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Xiaoyu Luo

Guizhou University

Fuhua Fan

fhfan1@gzu.edu.cn

Guizhou University

Article

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Stress memory triggered by combinatorial thermos-drought priming enhances cross-tolerance of *Pinus massoniana* seedlings: physio-biochemical evidence from multi-stress conditioning

Xiaoyu Luo^{1,2}, Fuhua Fan^{1,2*}

¹Institute for Forest Resources and Environment of Guizhou Province/Guizhou Key Laboratory of Forest Cultivation in Plateau Mountain, Guizhou University, Guiyang 550025, Guizhou, China

²College of Forestry, Guizhou University, Guiyang 550025, Guizhou, China

*Correspondence

FAN Fuhua

Institute for Forest Resources and Environment of Guizhou Province/Guizhou Key Laboratory of Forest Cultivation in Plateau Mountain, Guizhou University, Guiyang 550025, Guizhou, China

Tel: (+86)19971534309

Email: fhfan1@gzu.edu.cn

Abstract The intensification of climate change has rendered high-temperature and drought stress critical constraints on forest ecosystems. *Pinus massoniana* Lamb., an ecologically and economically significant conifer species renowned for its abiotic stress resilience, presents an ideal model for investigating stress memory formation in woody perennials. This study establishes a novel priming protocol involving cyclical stress exposure to systematically examine physiological memory formation and subsequent stress tolerance enhancement. Three-month-old seedlings underwent three distinct priming regimens: thermos priming (35/30°C, 14/10 photoperiod), drought priming (~25% field capacity), and combinatorial stress (thermos-drought), followed by a 15-day recovery phase before imposing identical combinatorial stress. Comprehensive analyses of growth dynamics, oxidative stress biomarkers, antioxidant systems, and osmoregulatory compounds were conducted through a multi-methodological approach encompassing morphometric measurements, spectrophotometric assays, and enzymatic activity quantification. Principal component analysis and membership function analysis were employed to evaluate the impact of different adversity training treatments on the tolerance of seedlings to combined stress, thereby clarifying the actual effectiveness of adversity training in enhancing the stress resistance. Results indicated that, compared with non-primed controls, those subjected to adversity priming exhibited a reduced inhibitory effect on stem elongation and root collar diameter growth under subsequent stress, along with decrease in lipid peroxidation (malondialdehyde content), reduced relative cell conductance, and enhanced antioxidant capacity. Notably, combinational priming induced synergistic osmoregulatory adaptation, elevating proline accumulation while reducing soluble sugar consumption compared to single-stress priming, suggested the most significant improvements in stress tolerance. The comprehensive evaluation index revealed a stress resilience hierarchy: combinational priming > thermos priming > drought priming > non-primed controls. This study provides the first empirical evidence of stress memory formation in *P. massoniana* through cross-tolerance mechanisms, demonstrating that cyclical sublethal stress exposure enhances subsequent stress tolerance. The superior efficacy of combinational priming highlights the necessity of multi-stress experimental designs to accurately simulate natural stress regimes. These findings advance our understanding of ecological memory in perennial woody plants and establish practical protocols for developing climate-resilient forestry through targeted stress hardening.

Keywords *Pinus massoniana*; High temperature; Drought; Combined stress; Stress memory

Introduction

The persistent rise in global temperatures in recent decades has been accompanied by intensifying heatwaves and altered precipitation patterns throughout this century^[1]. Crucially, terrestrial plants under natural conditions predominantly encounter concurrent abiotic stresses rather than isolated challenges - a critical distinction from controlled experimental paradigms^[2]. The thermo-drought nexus, in particular, induces membrane peroxidation through reactive oxygen species (ROS) overproduction while simultaneously disrupting photosynthetic electron transport and osmotic homeostasis, leading to metabolic dysfunction and inhibited plant growth^[3]. To mitigate these adverse effects, plants have evolved sophisticated adaptive strategies, including the regulation of stomatal conductance, synthesis of osmoprotectants, and activation of antioxidant defense systems. Furthermore, emerging evidence indicates that plants can develop a form of environmentally induced "stress memory" which primes them for enhanced resilience upon subsequent exposure to similar abiotic challenges, also known as priming^[4].

Emerging evidence reveals that plants possess molecular memory systems enabling enhanced response kinetics upon recurrent stress exposure - an ecological adaptation termed "somatic stress memory"^[5]. Research has demonstrated that upon initial exposure to stress, plants retain a form of memory that shapes their subsequent responses to analogous stressors, thereby enhancing their capacity to endure adverse conditions^[6]. Heat stress triggers a complex regulatory network associated with heat shock protein synthesis, redox homeostasis, phytohormone signaling, calcium-mediated signal transduction, and multiple metabolic pathways, which collectively enhance plant resilience to recurrent thermal stress^[7]. Researches have demonstrated that wheat (*Triticum aestivum* L.) acquires thermotolerance through two cycles of high-temperature preconditioning, significantly improving survival during subsequent heat stress exposure^[8]. Similarly, in barley (*Hordeum vulgare* L.), heat-induced stress memory has been shown to persist across generations. Furthermore, thermal acclimation contributes to cellular stress mitigation and homeostasis maintenance through the accumulation of osmoprotectants such as soluble sugars (SS) and proline (Pro)^[9]. Plant responses to drought stress have been widely investigated, with numerous species exhibiting the capacity to form stress memory under recurrent adverse conditions. This phenomenon has been documented in *Arabidopsis thaliana*^[10], olive tree (*Olea europaea* L.)^[11], rice (*Oryza sativa* L.)^[12], mulberry (*Morus multicaulis*)^[13] and citrus (*Citrus reticulata* Blanco)^[14]. Collectively, these studies underscore that controlled drought preconditioning can trigger the formation of stress memory in plants, ultimately improving their resilience upon subsequent exposure to similar drought conditions.

Pinus massoniana Lamb., a coniferous species of the Pinaceae family, is a pioneer drought-resistant tree in southern China. It plays a crucial ecological and economic role in forest chemical industries, natural resource utilization, and ecosystem services^[15]. Drought stress markedly impairs the growth of *P. massoniana* seedlings. Under progressive drought conditions, these seedlings exhibit tissue-specific accumulation of non-structural carbohydrates (NSCs), concomitant with reduced chlorophyll content and a pronounced decline in photosynthetic capacity^[16, 17]. Exposure to elevated temperature and drought stress induces cellular membrane damage and reactive oxygen species (ROS) accumulation in *P. massoniana* seedlings, accompanied by significantly reduced stomatal conductance. Concurrently, increased superoxide dismutase (SOD) activity and elevated concentrations of malondialdehyde (MDA) and abscisic acid (ABA) were observed^[18]. Notably, combined high-temperature and drought stress exerted more severe effects on photosynthetic and transpiration rates compared to individual stress treatments. Transcriptomic analysis further revealed substantial alterations in carbon metabolic pathways under these co-stress condition^[19].

However, it remains unclear whether *P. massoniana* seedlings can establish stress memory following short-term exposure to heat, drought, or combined stress conditions, thereby enhancing their tolerance upon re-exposure to similar adverse environments. This study employs a multi-dimensional analytical approach to investigate the formation of stress memory and its physiological and biochemical regulatory mechanisms in *P. massoniana* seedlings under stress conditioning. The findings provide theoretical support and practical guidance for seedling cultivation and introduction strategies under climate change scenarios.

Results

Morphological Effects of High Temperature and Drought Hardening. Among the three acclimation treatments, *P. massoniana* seedlings in the HD group showed significant leaf wilting during stress exposure (Figure 1), but returned to normal after the stress was relieved. When stressed again, the leaves of CKS wilted the most severely, followed by DS.

During the initial stress period, the seedling heights of the priming groups (H, D, HD) were reduced by 14.37%, 12.98%, and 8.39% compared to CK. After the stress removal, their heights increased by 35.47%, 20.87%, and 25.08% respectively compared to the first period, all of which were higher than the growth rate of the CK group (16.88%). Upon re-stress, the differences in seedling height between the acclimated groups (HS, DS, HDS) and the CKS group gradually decreased. In the first sampling, the seedling heights of the HS, DS, and HDS groups were reduced by approximately 11.91%, 10.58%, and 10.08% respectively compared to the CKS group. In the second sampling, these differences were reduced to 4.47%, 7.21%, and 4.11% respectively (Figure 1). Regarding ground diameter, after the stress treatment, the stem diameters of all treatment groups were significantly lower than that of the CK group, with reductions of 30.86%, 35.11%, and 37.10% respectively. Post-stress removal, the stem diameters of these groups increased by 44.11%, 41.84%, and 64.46% respectively, surpassing CK's 12.31% growth rate. In the third phase, the H and HDS groups significantly increased. In the second sampling, the HDS group significantly outperformed all other groups, with increases of approximately 18.76%, 15.40%, and 30.57% compared to the CKS, HS, and DS groups respectively (Figure 1).

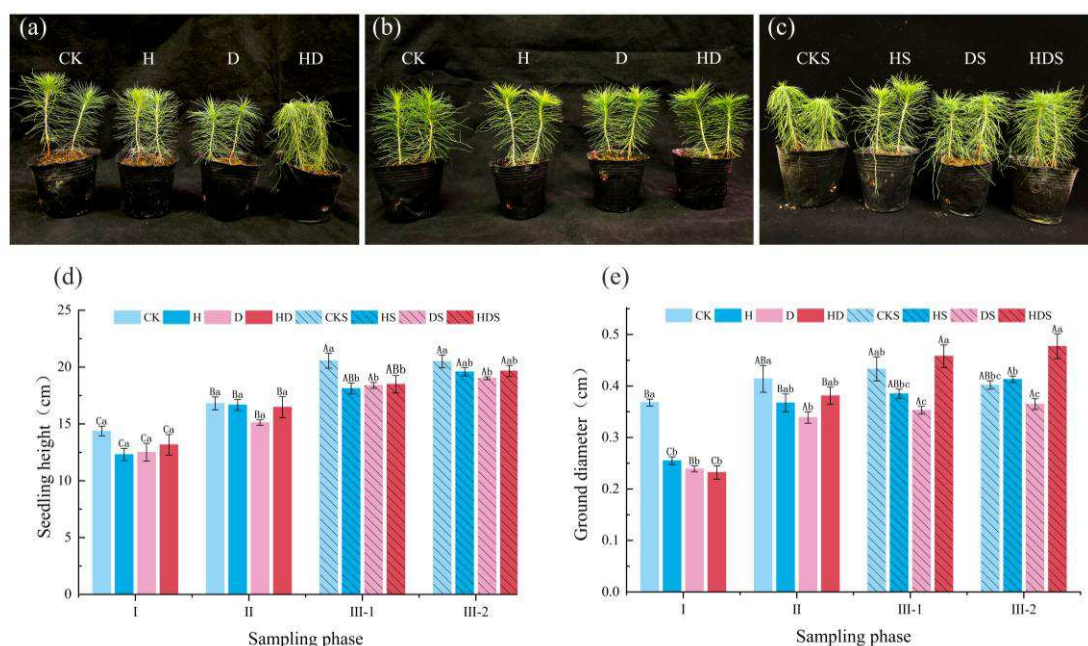


Figure 1. The Effects of High Temperature and Drought Training on Morphology. Figure a, b, and c are

the morphological diagrams of the seedlings sampled in the first, second, and third stages, respectively. Figures d and e show the height and diameter of the seedlings. I, II, and III represent the three sampling stages, with the third stage sampling twice. CK: non-primed controls, H: thermos priming, D: drought priming; HD: combined priming, CKS: CK + HD, HS: H + HD, DS: D + HD, HDS: HD + HD. Capital letters indicate significance within groups, while lowercase letters indicate significance between groups, the same below.

Effects of High Temperature and Drought Hardening on Cellular Membrane Damage. Under combined high-temperature and drought stress, the MDA content of the priming groups (H, D, HD) was significantly higher than that of the CK group, increasing by 46.14%, 80.13%, and 82.95% respectively. After stress removal, the MDA content of the H and D groups showed no significant difference from CK, but CK increased by approximately 67.0% compared to the first stage, while HD's MDA content was significantly lower than CK by about 27.73%. During the re-stress phase, in the first sampling, CKS had significantly higher MDA content than HS, DS, and HDS (10.61%, 9.40%, and 49.56% respectively), and in the second sampling, CKS was significantly higher than HS and HDS by about 32.40% and 34.95% respectively (Figure 2).

Similarly, leaf relative conductivity (REC) increased under stress (Figure 2), with H and HD groups significantly higher than CK by about 24.21% and 37.48% respectively. After stress removal, there were no significant differences in REC among the four groups. In the third phase, CKS had higher REC than the other three groups. In the first sampling, CKS was 60.10%, 41.65%, and 55.25% higher than HS, DS, and HDS respectively, and in the second sampling, CKS was significantly higher than HS and HDS by about 19.73% and 36.14% respectively (Figure 2). These results indicate that non-primed plants suffered more severe cellular damage compared to primed ones, with the combined primed group experiencing the least cellular damage.

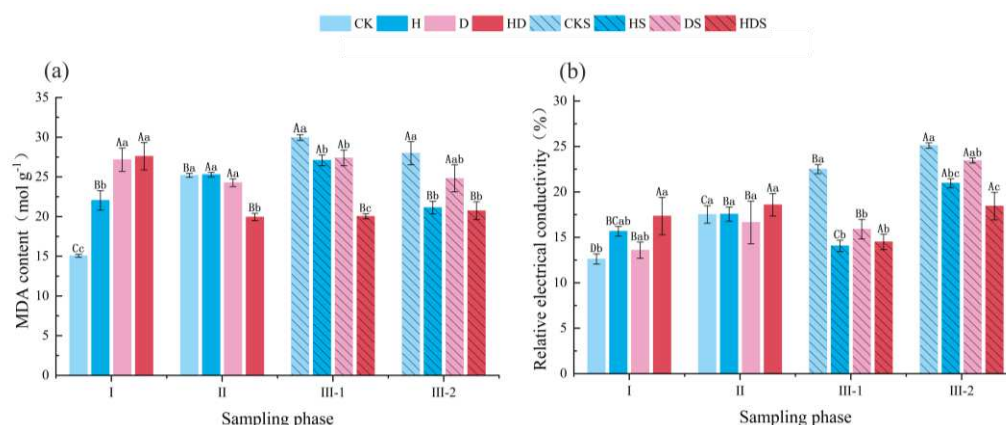


Figure 2. The impact of high-temperature and drought stress on cell membrane damage. Figure a and b respectively show the content of malondialdehyde and the relative conductivity of the leaves.

Effects of Acclimation to High Temperature and Drought on Chlorophyll Content. Under stress, the total chlorophyll (Chl), chlorophyll a (Chl a), and chlorophyll b (Chl b) contents of the plants decreased, except for the H group. The decreases in Chl, Chl a, and Chl b for the H, D, and HD groups were as follows: Chl decreased by approximately 5.22%, 12.85%, and 21.62%; Chl a decreased by 7.68%, 13.97%, and 24.18%; and Chl b decreased by 9.05% and 13.49% in the D and HD groups, respectively (Figure 3). Following stress removal, chlorophyll content increased in all groups but remained lower than

that of CK. Notably, the HD group exhibited significant increases in Chl and Chl a by about 39.67% and 44.13%, respectively. In the first sampling of the third phase, chlorophyll content significantly decreased in all groups: Chl decreased by 59.72%, 35.91%, 30.79%, and 34.19%; Chl a decreased by 56.94%, 33.57%, 37.47%, and 31.81%; and Chl b decreased by 68.14%, 42.73%, 39.92%, and 41.87%. The CKS group experienced the largest decline in chlorophyll content. In the second sampling, chlorophyll content increased in all groups, with the HDS group having the highest Chl and Chl a content in both samplings (Figure 3).

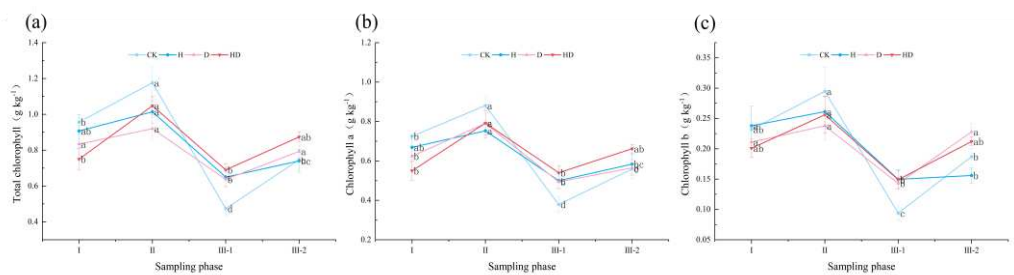


Figure 3. The effect of high-temperature and drought stress on chlorophyll. Figures a, b, and c respectively represent the total chlorophyll, chlorophyll a, and chlorophyll b contents. CK, H, D, and HD respectively represent different adaptation methods in the early stage.

Effects of Acclimation to High Temperature and Drought on the Activities of Related Enzymes.

During the stress acclimation period, the SOD activity of the three groups of seedlings increased (Figure 4), with H and HD being significantly higher than CK by approximately 41.76% and 51.54%, respectively. After the stress was alleviated, SOD activity decreased in all treatment groups, but the primed seedlings still had significantly higher activity than CK (Figure 4), by about 34.82%, 40.03%, and 72.07%. In the first sampling of the third phase, SOD activity significantly increased in all four groups (Figure 4), by 124.98%, 75.04%, 68.09%, and 52.62%, respectively, with HDS being significantly higher than CKS by about 16.73%. In the second sampling, SOD activity significantly decreased (Figure 4), by 44.77%, 31.93%, 42.36%, and 43.67%, respectively, while HS and HDS remained significantly higher than CKS by 15.10% and 17.62%, respectively.

Similarly, during the acclimation period, PAL activity in the stressed groups was significantly higher than CK (Figure 4), by approximately 73.63%, 82.53%, and 477.55%. After stress removal, HD's activity significantly decreased by 9.47%, while H and D groups' activities significantly increased by 84.78% and 101.19%, respectively. CK's activity remained unchanged and was still significantly lower than those primed groups (Figure 4). In the first sampling of the third phase, CKS, HS, and HDS activities significantly increased by 298.01%, 53.59%, and 35.64%, respectively, with CKS having the lowest activity (Figure 4), being approximately 32.56%, 15.23%, and 53.14% lower than the primed groups. In the second sampling, CKS and DS activities significantly increased and were higher than HS and HDS (Figure 4), while DS and HDS activities significantly decreased, with HDS having the lowest activity.

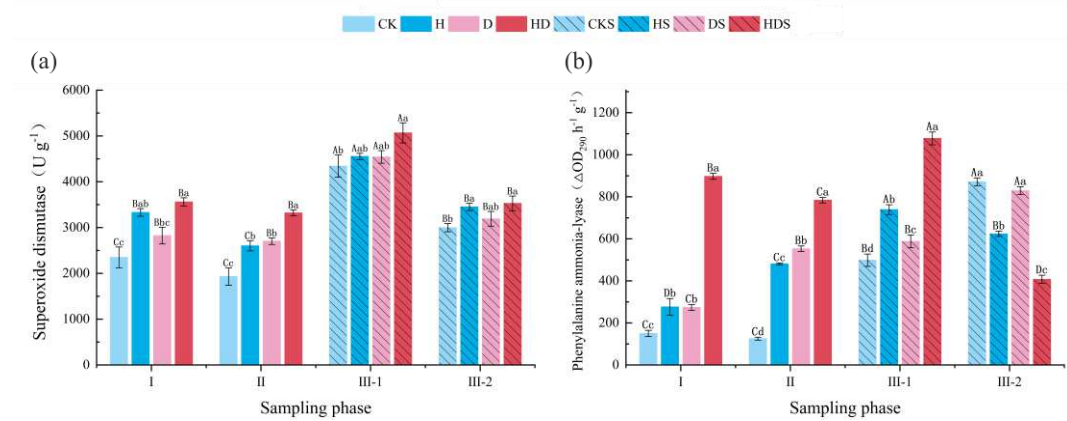


Figure 4. The influence of high-temperature and drought stress on the activities of related enzymes. Figure a and b respectively show the activities of superoxide dismutase and phenylalanine ammonia-lyase.

Effects of High Temperature and Drought Stress Conditioning on Osmoregulatory Substances.

During the acclimation period, the Pro content in the stressed groups increased (Figure 5), with D and HD significantly higher than CK by approximately 33.09% and 152.69%, respectively. After the stress removal, the Pro content in the H and D groups showed no significant difference from before stress removal (Figure 5), while HD's Pro content significantly decreased by about 35.26%. In the third phase, Pro content continued to rise in all groups. By the second sampling, Pro content had increased by 110.30%, 125.58%, 66.21%, and 61.74% compared to pre-stress levels, with plants acclimated to heat-related treatments (HS, HDS) having significantly higher content than those non-primed (Figure 5).

Similarly, under the same acclimation conditions, the SS content was significantly higher than CK (Figure 5), by approximately 44.07%, 64.31%, and 108.94%. After the stress was alleviated, SS content in the H and D groups showed no significant difference from CK, while HD's SS content was significantly lower than CK (Figure 5), by about 30.36%. During the third phase, SS content continued to increase in all groups. In the later stages of combined stress, plants acclimated to drought-related treatments (DS, HDS) having significantly lower content than those non-primed, by 11.25% and 13.51%, respectively.

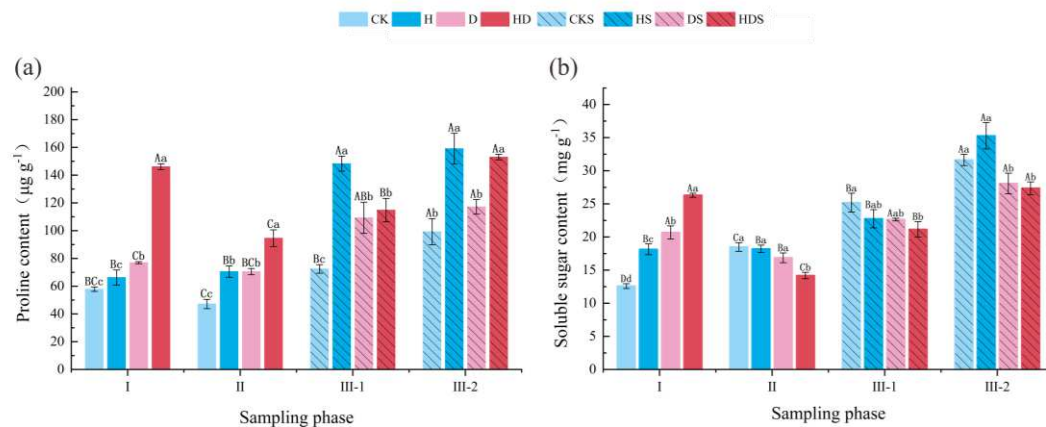


Figure 5. The influence of high-temperature and drought exercise on osmotic regulatory substances. Figure a and b represent the contents of proline and soluble sugar respectively

Comprehensive Evaluation of Seedling Responses to Combined Stress after Adversity Conditioning Using Principal Component Analysis and Membership Function.

In order to avoid the one-sidedness of evaluation by a single or a few indicators and to overcome the interference of information overlap among indicators, principal component analysis and membership function were used to comprehensively evaluate the impact of high-temperature and drought exercise on the growth of *P. massoniana* seedlings under combined stress conditions. To eliminate the influence of differences in variable scales and orders of magnitude, the deviations of the 11 measured original values were standardized. To avoid the cancellation of positive and negative indicator information, the negative correlation MDA and REC were transformed by inverse deviation standardization. The standardized values were analyzed by principal component analysis using SPSS 26.0 software. The results are shown in Table 1. The eigenvalues of the first 4 principal components were 4.946, 1.830, 1.650, and 1.053, with contribution rates were 44.97%, 16.64%, 15.00% and 9.57% respectively, and the cumulative contribution rate reached 86.18%. Therefore, all the information of the original 11 single indicators can be replaced by these 4 principal components, and their eigenvalues are all greater than 1.0.

The comprehensive values D of the membership functions for each treatment calculated from the 4 principal components are shown in Table 2: After the stress was relieved, there was no significant difference in the D value between the acclimation group and the control group; after the re-stress, the D value of the primed seedlings was significantly greater than that of the non-primed ones. At the first sampling, the D values of all groups significantly decreased, and the D value of the combinational primed seedlings was significantly higher than that of the single acclimation group; at the second sampling, the D value of the combinational primed seedlings was still significantly higher than the other 3 groups (CKs, HS、DS); the D value of thermos primed was greater than drought primed. As the duration of compound stress extended, the D values of all groups increased gradually, indicating that the adaptability of the seedlings enhanced over time.

PC	1	2	3	4	5	6	7	8	9	10	11
EV	4.946	1.830	1.650	1.053	0.628	0.356	0.197	0.150	0.095	0.084	0.00
Var. Explained%	44.97	16.64	15.00	9.57	5.71	3.24	1.79	1.36	0.86	0.76	0.00
Cumul. Var.%	44.97	61.61	76.60	86.18	91.89	95.13	96.92	98.28	99.14	99.90	100

Table 1. Principal component analysis of characteristic values, contribution rates and cumulative contribution rates of each index of *P. massoniana* seedlings. Principal component (PC), eigenvalue (EV), contribution rate (Var. Explained), cumulative contribution rate (Cumul. Var.)

AM	II	III-1	III-2
CK	0.573±0.01Aa	0.101±0.04Cc	0.261±0.02Bc
H	0.477±0.02Aa	0.301±0.01Bb	0.406±0.03Ab
D	0.480±0.04Aa	0.245±0.02Bb	0.349±0.04Bbc
HD	0.578±0.05Aa	0.429±0.03Ba	0.523±0.02ABa

Table 2. Stress on the comprehensive evaluation value D of the hybrid poplar seedlings under combined stress conditions. AM: Acclimation methods, including normal growth (CK), thermos priming (H), drought priming (D) and combinational priming (HD).

Correlation among Traits during Combined Stress. To further understand the correlations among various indicators, Origin 2024 was used to conduct a correlation analysis on the growth and final physiological indicators of *P. massoniana* seedlings under combined stress (Figure 6). The results

showed that during subsequent stress, primed plants (HS, DS, HDS) show positive correlations between growth traits (Sht, GD) and chlorophyll synthesis (Chl, Chl a, Chl b), while non-primed ones with negative correlations. Single-stress-acclimated seedlings link chlorophyll synthesis positively to plant height; combined-stress ones correlate it with height and ground diameter. Unacclimated plants have positive ties between membrane damage (REC, MDA) and osmotic regulation (Pro, SS), weakened by acclimation, with combined-acclimated showing negative correlations. In addition, thermos and drought priming strengthens SS-SOD correlation, significant in combined-acclimated seedlings.

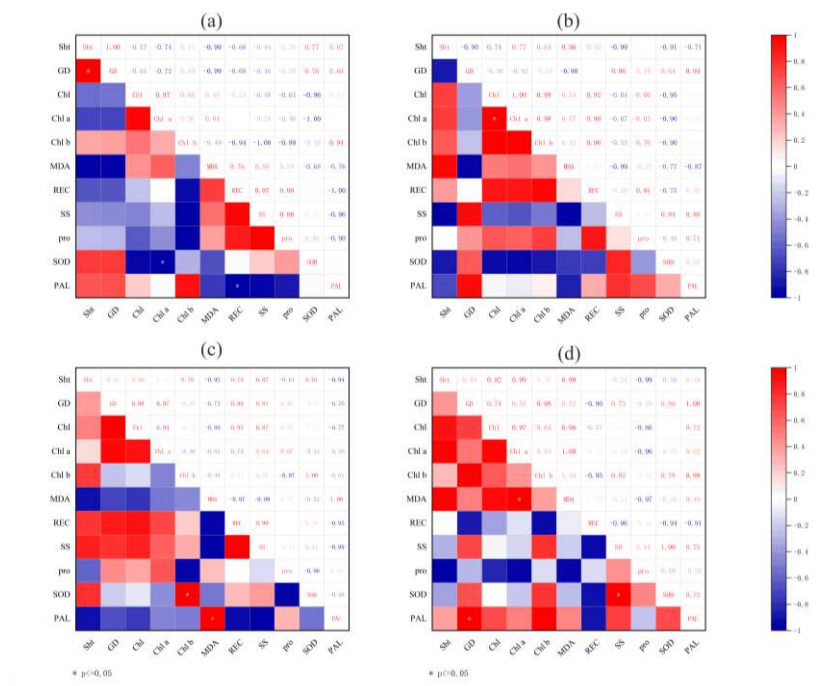


Figure 6. Correlation diagram under combined stress. Figures a, b, c, and d represent the control group, the thermos primed group, the drought primed group, and the combinational primed group respectively. * means significant correlation, $P < 0.05$.

Discussion

Plants will form "stress memory" for the first time when exposed to environmental stress. This memory enables them to show greater and faster activation potential in subsequent stress situations^[20]. We subjected the *P. massoniana* seedlings to high-temperature and drought pre-adaptation treatments to investigate whether they could form stress memory under stress conditions and enhance their tolerance to subsequent similar environmental stress.

Plant growth inhibition under abiotic stress conditions constitutes an evolutionarily conserved survival strategy. Our findings align with observations in *Populus × euramericana* 'Nanlin-895', where combined thermo-drought stress induced significantly greater growth suppression than singular stressors^[21]. Similar studies have also demonstrated that plants pre-exposed to drought stress have higher above-ground biomass compared to those not subjected to drought stress^[22]. This indicates that stress, especially combined stress, acclimation can mitigate the inhibitory effects of subsequent stress on height and ground diameter.

Under abiotic stress, ROS overproduction triggers membrane lipid peroxidation, generating MDA^[23, 24]. This oxidative cascade disrupts membrane integrity through increased permeability and electrolyte leakage, quantified as elevated REC-dual biomarkers for oxidative damage assessment. Our data

demonstrate synergistic effects of combined high-temperature and drought stress, showing higher MDA accumulation and greater REC elevation compared to isolated stressors, aligning with previous reports^[25, 26]. Post-stress recovery reduced MDA levels, though transient increases in control and high-temperature groups suggest developmental ROS signaling^[27]. Notably, combinatorial priming conferred superior protection during re-exposure, reducing more MDA and REC through dual mechanisms: peroxidation mitigation and cellular repair potentiation.

Chlorophyll, essential for light absorption and photosynthesis, directly influences plant growth and stress adaptation^[28]. While high temperature, drought, and their combined stress typically reduce chlorophyll content and photosynthetic efficiency^[29], our study revealed nuanced responses: initial stress elevated Chl b content but decreased total chlorophyll, with drought showing greater impact on Chl and Chl a than heat, and combined stress causing most severe reduction—aligning with existing reports^[25, 30]. Post-stress recovery of chlorophyll levels demonstrated *P. massoniana*'s adaptive resilience^[31]. In this research, primed plants maintained higher chlorophyll during re-stress, particularly the combined-stress group, confirming priming-enhanced adaptability^[32]. These findings demonstrate that stress priming modulates chlorophyll metabolism to enhance photosynthetic resilience under recurrent stress conditions.

SOD, a key antioxidant enzyme, regulates ROS balance by converting superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2)^[33, 34]. Lukić^[35] posits that the antioxidant system, by modulating the activity patterns of enzymes like SOD, plays a significant role in plant stress and drought memory. Our results align with reports of elevated SOD activity under drought/heat stress, peaking under combined stress^[25]. After stress relief, the reduction in ROS leads to a decrease in SOD activity across all groups^[36, 37]. When being stressed again, compared to non-primed plants, those pre-exposed to heat and drought stress exhibit higher SOD activity^[8, 12], which is consistent with our experimental findings. This suggests that prior stress conditioning enables seedlings to have higher SOD enzyme activity, which can eliminate ROS and alleviate oxidative stress damage.

PAL, a key enzyme in the phenylpropanoid pathway, catalyzes phenylalanine deamination to produce trans-cinnamic acid, providing precursors for secondary metabolites^[38, 39]. Plants enhance PAL activity to synthesize lignin, flavonoids, etc., for adapting to adverse environments^[39, 40]. Our study found stress conditioning elevated seedling PAL activity, indicating stress coping via secondary metabolites like lignin. Upon stress relief, PAL activity decreased in the HD group but increased in H and D groups, possibly due to resource reallocation: severe combined stress prioritized primary recovery over secondary metabolism, whereas milder single stresses allowed faster recovery and enhanced PAL activity for secondary metabolism. In early re-stress, primed seedlings had higher PAL activity. In subsequent sampling, seedlings primed with heat stress or combined stress showed lower PAL activity than those primed with drought stress or unprimed seedlings. This suggests the subjected to heat-related priming had sufficient metabolites, whereas the other two groups require sustained high PAL activity for resistance.

Osmoregulation is one of the key mechanisms by which plants adapt to adverse environments. Plants accumulate osmotic regulatory substances (SS, Pro) to cope with stress. The more osmotic regulatory substances they accumulate, the stronger their resistance will be^[41]. Under emergency stress, SS and Pro levels rise, with combined stress showing the most significant effect. Upon stress relief, SS levels normalize, and Pro is rapidly broken down and utilized, participating in the synthesis of chlorophyll and other substances to help plants resume growth^[42, 43]. In our study, changes in osmotic substances mirrored previous findings. After stress relief, SS levels in stressed seedlings returned to normal, while Pro in the HD group decreased significantly, with no change in the H and HD groups. This

could be due to Pro breakdown for chlorophyll synthesis post-combined stress. In contrast, single stress did not deplete as many osmotic substances. Primed plants can swiftly adjust SS and Pro levels upon re-exposure to stress. Chakraborty's^[44] study indicates that the young seedlings of heat-acclimated chickpea (*Cicer arietinum* L.) exhibit a significant increase in Pro content under high-temperature stress. Similarly, when water stress was applied to rice seeds before germination, the Pro content of the seedlings increased and the SS content decreased under subsequent drought stress^[12]. In our investigation, re-exposure revealed that non-primed plants exhibited lower Pro and higher SS levels compared to conditioned plants. This indicates that primed plants have accumulated Pro to prepare for subsequent similar stresses, thereby effectively adapting to environmental pressures. Non-primed plants do not have a similar mechanism for forming such a memory and cannot obtain sufficient Pro to quickly adapt to adverse conditions, therefore, they need to synthesize more SS to adapt to the adverse environment.

This study analyzed 11 indicators of *P. massoniana* seedlings under combined heat and drought stress, but individual indicators alone could not fully reflect plant tolerance. PCA transforms multiple correlated, overlapping indicators into a smaller set of independent composite indicators with minimal information loss^[45]. Using membership function analysis to calculate values for each composite indicator, a weighted sum yields a comprehensive stress resistance score, enabling assessment of relative tolerance across treatments. PCA extracted 4 independent composite indicators (eigenvalues>1.0) accounting for 86.18% cumulative variance, effectively replacing the original 11 overlapping variables. Weighted membership function analysis of these indicators produced a comprehensive evaluation value D. Results showed that the comprehensive stress resistance of the primed seedlings is significantly better than non-primed ones, with the order: combinational priming > thermos priming > drought priming > non-primed controls. Correlation analysis revealed pre-acclimation optimized synergies between osmotic regulators and antioxidant enzymes, aiding growth: drought and heat acclimation respectively depend on the osmotic regulation of soluble sugar and proline, while compound acclimation takes precedence over the photosynthetic system and antioxidant defense, with osmotic substance accumulation mitigating membrane damage. These findings confirm that heat, drought, and combined acclimation enhance *P. massoniana* seedlings' physiological responses and tolerance to subsequent combined stress.

Conclusion

Under high temperature, drought and combined stress conditions, the cell structure of the leaves of *P. massoniana* seedlings is damaged, photosynthesis is restricted, and the growth of the plants is inhibited. Appropriate stress priming can reduce the degree of cell damage during subsequent stress and maintain a high chlorophyll content, thereby reducing the inhibitory effect on growth. Under adverse stress conditions, *P. massoniana* seedlings will form stress memory in the antioxidant defense and osmotic regulation. When facing adverse stress again, the seedlings will maintain higher SOD activity to effectively remove ROS, reduce MDA production; can have higher PAL activity, enhance the antioxidant defense system and improve the mechanical strength of the plant, and enhance the adaptability to adverse conditions. During the subsequent stress, the primed plants can accumulate more Pro to regulate the intracellular osmotic pressure. However, under the same stress level, the SS content of the primed plants is lower than non-primed plants. Principal component analysis and membership function comprehensive evaluation indicate that high-temperature and drought stress priming can enhance the tolerance of *P. massoniana* seedlings in a combined stress environment.

Materials and Methods

Experimental Material. The experimental seeds were obtained from the 1.5-generation seed orchard of the *P. massoniana* National Forest Tree Breeding Base located in Duyun, Guizhou Province, China. Prior to germination, seeds were surface-sterilized with 0.5% potassium permanganate solution. Following disinfection, the seeds were subjected to germination under controlled conditions, and the seedlings were transplanted into 72-cell plug trays filled with a sterilized (carbendazim-treated) substrate composed of yellow soil and nutrient soil at a volume ratio of 6:4. On September 10, 2024, healthy and uniform 3-month-old *P. massoniana* seedlings were selected for the experiment. The measured soil parameters showed a field capacity (FC) of 59.9% and a pH range between 5.5 and 6.0.

Experimental Design. The experiment was conducted in an artificial climate chamber and consisted of three sequential phases:

Phase I (Acclimation Period): The control group (CK) was maintained at a temperature of 25/20°C(day/night) with soil moisture content held at 55~60% FC. The high-temperature group (H) experienced 35/30°C(day/night) while maintaining the same soil moisture content of 55~60% FC. The drought group (D) was subjected to 25/20°C with a reduced soil moisture content of 25~30% FC. The combined high-temperature and drought group (HD) was exposed to 35/30°C and a soil moisture content of 25~30% FC. All groups were kept under consistent environmental conditions, with 65% relative humidity and a photoperiod of 14/10 h (day/night). This acclimation phase lasted for 17 days.

Phase II (Recovery Period): Following the acclimation phase, all four groups of seedlings were transferred to a controlled environment with a temperature of 25/20°C and soil moisture content of 55~60% FC. Other environmental parameters, including 65% relative humidity and a 14/10 h photoperiod, were kept constant. The recovery period lasted for 15 days.

Phase III (Re-stress Period): In the final phase, all four groups of *P. massoniana* seedlings were simultaneously subjected to combined high-temperature and drought stress. The temperature was set at 35/30°C, and the soil moisture content was maintained at 20~25% FC. Other environmental parameters, including 65% relative humidity and a 14/10 h photoperiod, were kept constant. The re-stress treatment lasted for 20 days.

Index Measurement and Methods. Soil moisture was maintained using the gravimetric method, with daily irrigation performed at 9:30 AM. Sampling was conducted at the end of Phase I and Phase II, and during Phase III at 10-day intervals with two sampling events total. All measurements were carried out with triplicate biological replicates. Seedling height was measured with a steel tape, and root collar diameter was recorded using digital calipers. Leaf relative electrical conductivity (REC) was quantified following Zhang's protocol^[46]. MDA content, SOD and phenylalanine ammonia-lyase (PAL) activities, as well as Pro and SS content were determined using commercial assay kits (Suzhou Comin Biotechnology Co., Ltd.) according to the manufacturer's protocols. Chlorophyll extraction was performed with 95% ethanol according to Zhu's method^[47] with absorbance measured at 665 nm and 649 nm for subsequent chlorophyll concentration calculation using the specified formula:

$$C_a(\text{mg/L}) = 113.95 \times A_{665} - 6.88 \times A_{649} \quad (1)$$

$$C_b(\text{mg/L}) = 24.96 \times A_{649} - 7.32 \times A_{665} \quad (2)$$

$$C_T(\text{mg/L}) = C_a + C_b \quad (3)$$

In this equation, C_a , C_b and C_T represent the concentrations of chlorophyll a, chlorophyll b, and total chlorophyll, respectively; A_{665} , A_{649} represent the absorbance values of the chloroplast pigment extract at 665 nm and 649 nm, respectively.

After obtaining the chlorophyll concentration, the content of each pigment in the tissue is calculated using the following formula:

$$\text{Chlorophyll content (mg/g)} = (C \times V \times N) / (m \times 1000) \quad (4)$$

In this equation, C is the pigment concentration(mg /g); V is the volume of the extraction solution(mL); N is the dilution factor; m is the sample mass(g); and the factor of 1000 converts liters to milliliters.

Statistical analysis. All experimental data were analyzed using one-way analysis of variance (ANOVA) in SPSS 26.0 (IBM Corp.), with graphical visualization performed using Origin 2024. Results are presented as mean $\pm$ standard error (SE) from triplicate biological replicates. Significant differences among treatments were determined using Duncan's multiple range test at the $P < 0.05$ significance level. Notably, logarithmic transformation (base-10) was applied to chlorophyll b (Chl b) data from the control group (CK) prior to statistical analysis to address heterogeneity of variance.

To holistically assess the impact of different conditioning regimens on *P. massoniana* seedlings under combined heat-drought stress, the original data of 11 morphological and physiological indicators from the second and third phases were subjected to deviation standardization using Equation (5), following the methodology established by Liu et al^[48]. Principal component analysis (PCA) extracted four principal components, whose score coefficients were multiplied by corresponding standardized variables and summed to derive composite indices for each treatment. Membership function values $U(X_j)$ were then computed via Equation (6), with component weights ($W_j = 0.529, 0.197, 0.155, 0.119$) determined by Equation (7). The final comprehensive evaluation metric D was calculated using Equation (8). Finally, the D values of each group were analyzed using SPSS 26.0.

$$\text{Positive Correlation: } X_j = \frac{y_j - \bar{y}_j}{S_j}; \text{ Negative Correlation: } X_j = 1 - \frac{y_j - \bar{y}_j}{S_j} \quad (5)$$

In the formula, $\bar{y}_j$ and S_j represent the mean and standard deviation of the j-th indicator's data y_j respectively.

$$U(X_j) = \frac{X_j - X_{\min}}{X_{\max} - X_{\min}} \quad (6)$$

In the formula, X_j represents the j-th comprehensive indicator; $X_{\min}$ represents its minimum value; $X_{\max}$ represents its maximum value.

$$W_j = \frac{P_j}{\sum S_j} \quad (7)$$

In the formula, W_j represents the weight of the j-th composite indicator; P_j is the contribution rate of the j-th composite indicator for each treatment group.

$$D = \sum [U(X)_j \times W_j] \quad (8)$$

In the formula, the larger the D value, the stronger the plant's ability to tolerate stress.

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Author contributions

L.X. and F.F. conceived the project and designed the study. L.X. conducted these experiments. L.X. analyzed the data and wrote the manuscript. F.F. provided the resources for the study, and revised the manuscript. All authors read and approved the final manuscript.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reason able request.

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Declaration of Interest Statement

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