

## Evaluation of Salt Tolerance in Wheat Seedlings Based on Leaf Physiological Indicators (Post-print)

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### Abstract

Soil salinization severely affects wheat production, and improving wheat salt tolerance is one of the primary biological approaches to addressing this issue. The wheat germination stage is also relatively sensitive to salt, and the degree of salt tolerance during this stage is crucial for wheat cultivation in saline-alkali soils. To investigate the feasibility of evaluating wheat salt tolerance at the germination stage using leaf physiological indices, this study employed Cangmai 6005 and its 73 sodium azide mutagenized families as experimental materials. Under conditions of ultrapure water and 40% artificial seawater, we measured the contents of proline, soluble sugar, total protein, and soluble protein, as well as the activities of peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD) in leaves at the germination stage, and conducted a comprehensive evaluation of wheat salt tolerance at the germination stage using principal component analysis and cluster analysis. The results demonstrated that: (1) Under salt stress from 40% artificial seawater, the contents of proline and soluble sugar in wheat germination-stage leaves increased, while the contents of total protein and soluble protein decreased. Peroxidase (POD) activity was enhanced, whereas catalase (CAT) and superoxide dismutase (SOD) activities were reduced. (2) In the principal component analysis, the first and fourth principal components represented enzyme activity response factors for salt tolerance in wheat germination-stage leaves, while the second and third principal components represented osmotic adjustment factors. (3) In the salt tolerance evaluation, Cangmai 6005 was identified as having a germination-stage salt tolerance rating of level 2 (salt tolerant), and the families identified with a salt tolerance rating of level 1 (highly tolerant) were SAM1, SAM49, and SAM59, which aligns with field production experience. In conclusion, the activities of peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD), along with the contents of proline and soluble sugar, can serve as leaf physiological indices

for evaluating wheat salt tolerance at the germination stage. The findings of this study may provide a reference for the evaluation of wheat salt tolerance at the germination stage using leaf physiological indices.

## Full Text

### Preamble

#### Comprehensive Evaluation of Salt Tolerance in Wheat Based on Leaf Physiological Indices at the Germination Stage

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## Abstract

Soil salinization seriously impacts wheat production, and improving wheat salinity tolerance represents one of the primary biological approaches to addressing this challenge. The germination stage is particularly sensitive to salt stress, and the level of salt tolerance during this phase is crucial for wheat cultivation in saline-alkali soils. To explore the feasibility of evaluating wheat salt tolerance at the germination stage using leaf physiological indices, we used CM6005 and its 73 sodium azide mutagenesis families as experimental materials. Under ultrapure water and 40% artificial seawater conditions, we measured the contents of proline, soluble sugar, total protein, and soluble protein, as well as the activities of peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD) in germination-stage leaves. Principal component analysis and cluster analysis were employed for comprehensive evaluation of salt tolerance. The results showed: (1) Under 40% artificial seawater salt stress, proline and soluble sugar contents increased, while total protein and soluble protein contents decreased. POD activity increased, whereas CAT and SOD activities decreased. (2) In principal component analysis, the first and fourth principal components represented enzymatic response factors for salt tolerance in wheat germination-stage leaves, while the second and third components represented osmotic adjustment factors. (3) In salt tolerance evaluation, CM6005 was classified as grade 2 (salt-tolerant), and the families identified as grade 1 (highly salt-tolerant) were SAM1, SAM49, and SAM59, consistent with field production experience. In conclusion, the activities of POD, CAT, and SOD, along with proline and soluble sugar contents, can serve as leaf physiological indicators for evaluating wheat salt tolerance at the germination stage. These findings provide a reference for assessing wheat salt tolerance using leaf physiological indices at the germination stage.

**Keywords:** wheat; germination stage; physiological indices; salt tolerance; comprehensive evaluation

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Soil salinization is a major factor limiting grain production. Screening and utilizing new salt-alkali tolerant crop varieties represents an effective biological approach for improving saline-alkali soils (Zhang et al., 2013). As one of the world's primary food crops, wheat breeding for salt-alkali tolerance is of significant importance. Wheat growth and development begins with seed germination, and salt tolerance at the germination stage refers to the comprehensive ability of seeds to absorb water, swell, and initiate root growth under salt stress. Previous studies have shown that in severely saline-alkali soils, wheat emergence is delayed and emergence rates are significantly reduced (Zhao et al., 2005). Therefore, selecting wheat varieties with strong germination-stage salt tolerance is fundamental for wheat cultivation in saline-alkali lands and serves as an important basis for achieving "full emergence in one sowing" in production practice.

In wheat production on saline-alkali soils, cultivating and promoting salt-alkali tolerant wheat varieties is the most economical and effective measure for developing wheat production in these areas (Wang et al., 2012). Chemical mutagenesis breeding is an important method for wheat variety development and germplasm innovation (Cheng et al., 1990). Sodium azide ( $\text{NaN}_3$ ) is a highly efficient and low-toxicity mutagen widely applied in crops such as wheat, maize, and rice (Kiruki et al., 2006; Gao et al., 1992; Valentina et al., 2008; Cao et al., 1991; Jiang et al., 2006). Zhang et al. (2011) investigated the variation characteristics of agronomic traits such as plant height, awn type, and spike morphology in sodium azide-mutagenized wheat progeny, and demonstrated the mutagenic effect of sodium azide at the molecular level. However, few studies have reported on salt tolerance in wheat mutant libraries constructed through sodium azide mutagenesis based on physiological index detection.

The identification and evaluation of salt-tolerant wheat germplasm resources are important prerequisites and guarantees for breeding salt-alkali tolerant wheat varieties (Zhang et al., 2013). Morphological indicators such as emergence rate, plant height, spike length, spike number, and grain number per spike can be used to evaluate wheat salt-alkali tolerance (Mguis et al., 2008; Naruoka et al., 2011; Rathod & Anand, 2016). Regarding physiological and biochemical indices, Liu et al. (2013) found that salt-tolerant wheat varieties possess stronger osmotic adjustment capacity. Physiological and biochemical indices including malondialdehyde (MDA), POD activity, and chlorophyll content show significant increases under salt-alkali stress compared to controls (Shi et al., 2018; Ibrahim et al., 2020; Wu et al., 2019). Additionally, Shang et al. (2017) studied changes in physiological indices in different ploidy wheat at the three-leaf stage. Hormones are also closely related to plant salt-alkali tolerance, with abscisic acid and ethylene considered stress hormones (Kefu et al., 1991; Haihua et al., 2004; Waskiewicz et al., 2013). The germination stage is salt-sensitive in wheat,

and salt tolerance evaluation at this stage has primarily relied on morphological indicators such as germination rate, shoot length, and root length (Liu et al., 2001; Liu et al., 2014). This study used CM6005 and its 73 sodium azide-mutagenized families as research materials, applied 40% artificial seawater as salt stress treatment, and investigated salt tolerance during the germination stage based on wheat leaf physiological indices. Through comparative analysis with CM6005, we aimed to identify strongly salt-tolerant wheat germplasm resources suitable for actual agricultural production environments in coastal saline-alkali areas of Hebei Province, which is of great significance for breeding new salt-tolerant wheat varieties.

### 1.1 Experimental Materials

All test materials were developed by the Wheat Research Group of Cangzhou Academy of Agriculture and Forestry Sciences, including CM6005 and its 73 sodium azide-mutagenized families (designated SAM1-SAM76). These were planted at the Qianying Experimental Base of Cangzhou Academy of Agriculture and Forestry Sciences in 2019. After harvest, seeds were subjected to physiological index detection in wheat germination-stage leaves under indoor conditions.

### 1.2 Material Culture and Treatment

Artificial seawater was prepared according to established formulations (Liu et al., 2001; Liu et al., 2014) and diluted to 40% concentration. Two layers of filter paper were placed in petri dishes, and 15 mL of either deionized water (control) or 40% artificial seawater (treatment) was added. Each treatment contained 30 seeds with three replicates. Seeds were germinated in an illuminated incubator at constant 22°C with 12 h light. After 7 days, physiological indices were measured in seedling leaves. The study detected contents of total protein, soluble protein, soluble sugar, and proline, as well as activities of POD, CAT, and SOD. All physiological indices were measured using ELISA kits following the operation manual of Jiangsu Kete Biotechnology Co., Ltd. Finally, relative values were calculated as:  $\text{Relative value (\%)} = (\text{Treatment index value} / \text{Control index value}) \times 100$  (Wu et al., 2014; Peng et al., 2017).

### 1.3 Measurement Methods

**1.3.1 Total Protein Content** A 0.5 g sample of wheat leaves was ground in 1.5 mL of pre-cooled buffer containing 0.1% PVP, 0.1 mol · L<sup>-1</sup> EDTA, and 1 mol · L<sup>-1</sup> ascorbic acid phosphate (pH=7.8) in an ice bath. The homogenate was centrifuged at 15,000 g for 30 min, and the supernatant was collected. Using Coomassie brilliant blue G-250 as the colorimetric agent, OD values were measured at 595 nm wavelength with a spectrophotometer. Protein content was calculated based on a standard curve using bovine serum albumin as the standard.

**1.3.2 Soluble Protein Content** Fifty mg of wheat leaves were ground thoroughly in liquid nitrogen, homogenized in  $0.01 \text{ mol} \cdot \text{L}^{-1}$  PBS (pH 7.4), and centrifuged at 5,000 g for 15 min. The supernatant was collected. Ten  $\mu\text{L}$  of supernatant was added to a coated enzyme-linked immunosorbent assay (ELISA) plate, incubated at  $37^\circ\text{C}$  for 30 min, washed, then enzyme-labeled secondary antibody was added and incubated at  $37^\circ\text{C}$  for 30 min. After washing, chromogenic solutions A and B were added, followed by stop solution. Absorbance was read at 450 nm wavelength using a microplate reader. Soluble protein concentration was calculated based on the standard curve using the soluble protein standard provided in the kit.

**1.3.3 Proline Content** Fifty mg of wheat leaves were ground thoroughly in liquid nitrogen, homogenized in  $0.01 \text{ mol} \cdot \text{L}^{-1}$  PBS (pH 7.4), and centrifuged at 5,000 g for 15 min. The supernatant was collected. Then 0.5 mL sample + 0.5 mL glacial acetic acid + 0.5 mL acidic ninhydrin were placed in a capped test tube and heated in boiling water for 30 min (tightly capped to prevent water loss), with shaking every 10 min. After cooling, 1 mL toluene was added to the test tube, shaken for 30 s, and allowed to stand for the pigment to transfer to the toluene layer. Approximately 0.8-1 mL of the upper layer was pipetted into a 1 mL glass cuvette, and absorbance was measured at 520 nm wavelength. Proline concentration in the sample was calculated using the appropriate formula.

**1.3.4 Soluble Sugar Content** Approximately 0.1-0.2 g of sample was weighed, ground into homogenate with 1 mL distilled water, and transferred to a capped centrifuge tube. The mixture was heated in boiling water for 10 min (tightly capped to prevent water loss). After cooling, it was centrifuged at 8,000 g at room temperature for 10 min. The supernatant was transferred to a 10 mL test tube, diluted to 10 mL with distilled water, and mixed thoroughly for later use. Two hundred  $\mu\text{L}$  of sample was added for measurement. The spectrophotometer was preheated for over 30 min, wavelength was adjusted to 620 nm, and zeroed with distilled water. Soluble sugar concentration was calculated based on a standard curve using soluble sugar as the standard.

**1.3.5 Activities of Peroxidase, Catalase, and Superoxide Dismutase** Fifty mg of wheat leaves were ground thoroughly in liquid nitrogen, homogenized in  $0.01 \text{ mol} \cdot \text{L}^{-1}$  PBS (pH 7.4), and centrifuged at 5,000 g for 15 min. The supernatant was collected. Ten  $\mu\text{L}$  of supernatant was added to a coated ELISA plate, incubated at  $37^\circ\text{C}$  for 30 min, washed, then enzyme-labeled secondary antibody was added and incubated at  $37^\circ\text{C}$  for 30 min. After washing, chromogenic solutions A and B were added, followed by stop solution. Absorbance was read at 450 nm wavelength using a microplate reader. POD, CAT, and SOD activities were calculated based on respective standard curves using POD, CAT, and SOD standards provided in the kit (Becana et al., 1986; Li et al., 2015).

#### 1.4 Statistical Analysis

Data were organized and analyzed using Excel 2010 and SPSS 21.0. Means and relative values of each trait were calculated for descriptive statistical analysis, simple correlation analysis, and partial correlation analysis. For relative values of each index, principal component analysis was first performed to obtain principal component factor scores for each test material. Then, membership function values and weight values of each factor were calculated using the membership function method to obtain comprehensive evaluation D values for salt tolerance of each material. Finally, cluster analysis of the comprehensive evaluation D values was conducted to classify salt tolerance levels of each material (Peng et al., 2017).

#### 2.1 Changes in Physiological Indices of CM6005 Sodium Azide-Mutagenized Population at Germination Stage Under Artificial Seawater Stress

Table 1 shows that under 40% artificial seawater stress, proline and soluble sugar contents in wheat germination-stage leaves increased by 75.17% and 47.20%, respectively, while soluble protein and total protein concentrations decreased by 46.31% and 33.82%, respectively. Meanwhile, the effect of 40% artificial seawater salt stress on the enzyme activity system in wheat germination-stage leaves manifested as enhanced POD activity and reduced CAT and SOD activities, with changes of 82.40%, 28.47%, and 2.83%, respectively. These results indicate that increased contents of the osmotic adjustment substances proline and soluble sugar in wheat germination-stage leaves help protect cellular water potential and maintain normal physiological characteristics. SOD, POD, and CAT are the major antioxidant enzymes that scavenge reactive oxygen species in plant cells. The differential effects of artificial seawater stress on these antioxidant enzyme activities suggest that their coordinated action enables wheat plants to mitigate or defend against salt stress to a certain extent.

#### 2.2 Correlation Analysis of Salt Tolerance Physiological Indices in CM6005 Sodium Azide-Mutagenized Population at Germination Stage Under Artificial Seawater Stress

Table 2 shows that relative proline content was significantly negatively correlated with relative total protein content, while relative SOD activity was extremely significantly positively correlated with relative CAT activity. Other pairwise simple correlations among relative indices were not significant.

Table 3 shows that the partial correlation between relative SOD activity and relative CAT activity was extremely significant, while all other pairwise partial correlations were not significant. These correlation and partial correlation analysis results indicate that when identifying salt tolerance based on wheat germination-stage leaf physiological data, special attention should be paid to

relative SOD activity and relative CAT activity, while the seven physiological indices should be simplified. Therefore, this study further employed principal component analysis to transform multiple indices into a few independent comprehensive indices for accurate evaluation of wheat salt tolerance.

### **2.3 Principal Component Analysis of Salt Tolerance Physiological Indices in CM6005 Sodium Azide-Mutagenized Population at Germination Stage Under Artificial Seawater Stress**

Principal component analysis was performed on seven salt tolerance physiological indices of CM6005 and its 73 mutagenized families at the germination stage. shows that four independent principal components could be selected as comprehensive physiological indices for salt tolerance identification at the germination stage, explaining 70.66% of total variation and essentially representing most information from the seven original indices.

The larger the absolute value of the eigenvector for a single index, the greater its role in the principal component. The eigenvectors of each leaf physiological index are shown in Table 5. The main indices in the first principal component were relative CAT and relative SOD, representing enzymatic factor I for salt stress. The main index in the second principal component was relative proline content, representing osmotic adjustment factor I for salt stress. The main index in the third principal component was relative soluble sugar content, representing osmotic adjustment factor II for salt stress. The main index in the fourth principal component was relative POD activity, representing enzymatic factor II for salt stress. Principal components 1 and 4 primarily represented two factors of the enzymatic system response to salt tolerance in wheat germination-stage leaves, both related to the antioxidant enzyme system response to salt stress, mainly reflecting enzymatic performance. Principal components 2 and 3 primarily represented two factors of osmotic adjustment for salt tolerance in wheat germination-stage leaves.

### **2.4 Comprehensive Evaluation of Salt Tolerance in CM6005 Sodium Azide-Mutagenized Population at Germination Stage Under Artificial Seawater Stress**

Factor scores of each principal component obtained from principal component analysis were used as comprehensive indicators for identifying salt tolerance physiology in wheat germination-stage leaves. Membership function values and weight values of each comprehensive indicator were calculated to obtain comprehensive evaluation D values for salt tolerance of each material (Table 6). The D value is a pure number ranging from 0 to 1, where larger D values indicate stronger salt tolerance. Then, K-means clustering was used to classify test materials into five salt tolerance levels: highly tolerant, tolerant, moderately tolerant, sensitive, and highly sensitive (Table 7).

Table 6 shows that CM6005 was classified as grade 2 (salt-tolerant) at the germination stage, while families SAM1, SAM49, and SAM59 were identified as grade 1 (highly salt-tolerant). Moreover, the comprehensive salt tolerance performance of SAM1, SAM49, and SAM59 in the field was also superior to CM6005. This demonstrates that using comprehensive evaluation D values based on wheat germination-stage leaf physiological index detection is a feasible method for representing wheat salt tolerance strength, with results consistent with practical experience.

Table 7 shows that the numbers of wheat materials at the five germination-stage salt tolerance levels were 3, 17, 22, 22, and 10, accounting for 4.05%, 22.97%, 29.73%, 29.73%, and 13.51% of test materials, respectively. Based on salt tolerance identification using wheat germination-stage leaf physiological indices, 20 wheat materials (approximately one-third of all test materials) showed high tolerance or tolerance (grades 1 and 2) at the germination stage.

Wheat salt tolerance is influenced by multiple factors, involving many physiological and biochemical responses to salt stress. Therefore, to more accurately evaluate wheat salt tolerance, the combined effects of multiple physiological indices must be considered (Wang et al., 2014). This study primarily investigated changes in wheat leaf physiological indices under artificial seawater salt stress and their impact on salt tolerance identification and evaluation at the germination stage. Salt tolerance identification at the germination stage allows rapid evaluation of numerous varieties (lines) in a short time, enabling preliminary assessment of salt tolerance in large batches of wheat materials and facilitating the breeding of salt-tolerant wheat varieties.

### 3.1 Effects of Artificial Seawater Stress on Wheat Germination-Stage Leaf Physiological Indices

Proline and soluble sugar are important osmotic adjustment substances in plant cytoplasm. Plants adapt to different saline-alkali environments by continuously accumulating organic compounds such as proline and soluble sugar. The accumulation of proline and soluble sugar represents a stress-protective response for salt resistance and tolerance (Wang et al., 2014). In this study, under 40% artificial seawater salt stress, proline and soluble sugar contents in wheat germination-stage leaves increased compared to the control to adapt to saline conditions and maintain osmotic adjustment system stability, consistent with previous research findings (Yang et al., 2010).

Antioxidant enzymes in plants play important roles in eliminating superoxide free radicals (Zhao & Dou, 1998). CAT, POD, and SOD work coordinately to maintain reactive oxygen metabolism balance. Some studies have shown that enzyme activities such as SOD, CAT, and POD decrease under salt stress (Zeng, 2017). In this study, POD activity increased while CAT and SOD activities decreased under artificial seawater salt stress. This may be because wheat salt tolerance is not only a complex quantitative trait affected by multiple factors,

but also because different wheat varieties have different salt tolerance mechanisms, leading to inconsistent responses of specific physiological indices to salt stress across different materials.

Additionally, salt stress disrupts the balance of protein metabolism in plants, causing protein degradation rates to far exceed synthesis rates. Generally, excessive salt conditions inhibit protein synthesis while promoting decomposition, ultimately reducing soluble protein content in cytoplasm with increasing salt stress intensity (Liu & Zhu, 2014). In this study, total protein and soluble protein contents in wheat germination-stage leaves decreased under artificial seawater salt stress, consistent with the findings of Liu & Zhu (2014).

### **3.2 Salt Tolerance Identification Based on Germination-Stage Leaf Physiological Indices**

Based on seven leaf physiological indices, this study first reduced the dimensionality of these indices to four independent principal component factors through principal component analysis: two enzymatic response factors and two osmotic adjustment factors. Then, using the membership function method and weight values, a comprehensive evaluation D value was obtained for each material as a standard for measuring germination-stage salt tolerance strength, which can exclude environmental influences. Finally, cluster analysis of D values was performed to classify salt tolerance levels, providing a relatively objective evaluation of wheat germination-stage salt tolerance. Regarding selection of physiological indices for salt tolerance identification, previous studies have shown that POD is a universally present and highly active enzyme in plants that can serve as an indicator for plant salt tolerance evaluation (Peng et al., 2014). Thus, the activities of antioxidant enzymes (POD, CAT, SOD) and the contents of osmotic adjustment substances (proline and soluble sugar) can be used as leaf physiological indices for salt tolerance identification at the germination stage. However, whether total protein and soluble protein contents in leaves can serve as physiological indices for wheat salt tolerance identification requires further investigation.

### **3.3 Evaluation of Wheat Germination-Stage Salt Tolerance Under Artificial Seawater Stress**

Salt tolerance identification in wheat has primarily focused on germination and seedling stages, mainly using morphological indicators such as shoot length and root length (Liu et al., 2001; Liu et al., 2014). This study primarily used physiological indices from wheat germination-stage leaves for salt tolerance identification. In the evaluation results, CM6005 was classified as grade 2 (salt-tolerant). CM6005 is a wheat variety with outstanding salt-alkali tolerance, consistent with production experience. Additionally, new lines with stronger salt tolerance than CM6005 were identified as SAM1, SAM49, and SAM59, which also aligns with their field salt tolerance performance. The comprehensive results demonstrate that using 40% artificial seawater stress combined with detection

of wheat germination-stage leaf physiological indices and appropriate statistical analysis methods can achieve salt tolerance identification and evaluation at the germination stage. This method is highly operable, short-cycle, and efficient, suitable for preliminary screening of large batches of wheat materials, representing an efficient, rapid, and feasible indoor method for wheat germination-stage salt tolerance identification.

## Conclusions

- (1) Under 40% artificial seawater salt stress, proline and soluble sugar contents increased, total protein and soluble protein contents decreased, POD activity increased, and CAT and SOD activities decreased in wheat germination-stage leaves.
- (2) Relative SOD activity and relative CAT activity mutually promoted each other, jointly representing a coordinated response of wheat germination-stage leaves to artificial seawater salt stress.
- (3) Principal component analysis integrated seven individual indices into four independent comprehensive indices. The first and fourth principal components primarily represented two enzymatic system response factors for salt tolerance in wheat germination-stage leaves, while the second and third components represented two osmotic adjustment factors. The activities of POD, CAT, and SOD, along with proline and soluble sugar contents, can serve as leaf physiological indices for screening salt tolerance at the germination stage.
- (4) Cluster analysis classified CM6005 and its 73 families into five categories. CM6005 was identified as grade 2 (salt-tolerant), while families SAM1, SAM49, and SAM59 were identified as grade 1 (highly salt-tolerant).

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