

## RESEARCH ARTICLE

# Response of Root Growth and Root Border Cell Characteristics of *Hemerocallis fulva* to NaCl Stress

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**Abstract:** Taking three *Hemerocallis* cultivars ('Jingzhouhua', 'Qingchong', 'Changzuizihua') with different salt tolerance as materials, the effects of 0-200 mmol/L NaCl on root morphology and Root Border Cell (RBC) characteristics were investigated using hydroponic and root suspension culture methods and one-way analysis of variance (ANOVA). The results showed that the root morphology indicators and activity of the high-salt-tolerant 'Jingzhouhua' decreased less than those of the medium-salt-tolerant 'Qingchong' and salt-sensitive 'Changzuizihua'. As the salt concentration increased, the number and viability of RBCs in these three cultivars decreased in the order of 'Jingzhouhua' < 'Qingchong' < 'Changzuizihua'. NaCl stress increased the apoptotic rate of RBCs and the thickness of the mucilage layer; 'Jingzhouhua' had the lowest apoptosis rate and the thickest mucilage layer, followed by 'Qingchong' and 'Changzuizihua'. Under NaCl stress, maintaining a high number of RBCs, survival rate, and mucus layer thickness helps stabilize the root function and morphology of 'Jingzhouhua', which is the reason for its strong salt tolerance.

**Keywords:** *Hemerocallis fulva*, Root Morphology, Root Border Cells, NaCl Stress

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## Introduction

The global sustainability of agriculture is facing the threat of salinity, which is a major abiotic constraint on crop productivity and soil health [1]. Salinity-induced stress often triggers a rapid oxidative burst caused by overproduction of Reactive Oxygen Species (ROS) [2]. It can lead to membrane lipid peroxidation damage [3] and affect root morphology and function. Root Border Cells (RBCs) are a special cell population in the root cap of plants with certain functional activities. They can alleviate stress damage to root tips [4] and are the first line of defense for plant root tips against adversity [5]. Some studies have reported the protective effect of RBCs on root tips. For example, RBCs can alleviate the atrazine toxicity to the root tips of *Fagopyrum esculentum* [6]. Maize and soybean can alleviate the allelopathic stress of *Chenopodium ambrosioides* by upregulating the production and apoptosis of RBCs [7]. Under NaCl stress, RBCs have a strong capacity for signal transduction. The adaptive

response of RBCs to high salinity involves complex remodeling of cellular lipid profiles to maintain structural integrity. This metabolic modulation includes enriching membrane-stabilizing lipids, actively counteracting the destabilizing effects of Na<sup>+</sup> ions on the phospholipid bilayer. In addition to structural maintenance, the accumulation of specific signaling lipids also triggers a comprehensive defense response through the activation of phytohormone-mediated pathways. Specifically, the amplification of Jasmonic Acid (JA) and Salicylic Acid (SA) signal transduction cascades plays a critical role in orchestrating the expression of downstream stress-responsive genes [8]. These specialized cells may be key positive regulators in cereal models such as rice, significantly enhancing the overarching capacity of the organism to withstand NaCl-induced stress [9].

*Hemerocallis fulva* is a perennial herb of the genus *Hemerocallis* in the Asphodelaceae family. As a perennial herb of high ornamental and economic value, *Hemerocallis fulva* faces significant physiological constraints under salinity, which is the main abiotic barrier to its vegetative growth and overall biomass yield [10]. Studies on *Hemerocallis* under NaCl stress have mainly focused on growth, physiological traits, and transcriptome analysis. Some progress has been made in hybrid breeding and germplasm collection. However, research on its root system under NaCl stress remains limited. Although the importance of RBCs in alleviating stress has been widely recognized, empirical data on their response to salinity in the *Hemerocallis* genus is still limited. Specifically, only a small number of studies have systematically investigated the coordinated responses of root morphology and RBC characteristics in *Hemerocallis* cultivars with divergent salt tolerance, and few works have clarified the physiological mechanisms underlying differential salt tolerance from the perspectives of RBC mucilage layer thickness and cellular apoptosis. To explain the cytological mechanisms of differential salt tolerance, this study systematically evaluated the effects of salinity on three comprehensive dimensions of the physiology of *Hemerocallis fulva*: root system architecture (including total length, tip frequency, diameter, surface area, and volume), physiological vigor (root activity), and RBC characteristics (population count, viability, mucilage secretion, and apoptotic progression). Combined with the lack of relevant cytological evidence, this work fills the above research gap by integrating root morphological indicators and multi-dimensional RBC physiological traits to dissect genotype-specific salt-adaptive strategies in *Hemerocallis*. The synthesis of these data aims to establish a robust theoretical framework for improving the halotolerance of *Hemerocallis* germplasm.

## Materials and Methods

### Test Materials and Cultivation Methods

The main experimental materials were three cultivars of *Hemerocallis fulva* with different salt tolerance: 'Jingzhouhua' (highly salt-tolerant), 'Qingchong' (moderately salt-tolerant), and 'Changzuizihua' (salt-sensitive). All germplasm was from the Suqian Institute of Agricultural Sciences at the Jiangsu Academy of Agricultural Sciences.

Healthy, uniformly sized, and disease-free *Hemerocallis fulva* plants were cultivated in a 24-hole hydroponic box (39×28×14 cm) with a hole diameter of 3.2 cm. The box with Hoagland nutrient solution was aerated using an aeration pump. After one month of normal culture (when new white roots grew on the root system), the plants were transferred to a 6-hole hydroponic box (18×12×10.5 cm) and divided into two groups for NaCl stress treatment using different methods, respectively. All plants were cultivated under standard laboratory conditions at 25°C.

### Determination of Root Morphological Indicators

The NaCl concentration gradients were set at 0, 50, 100, 150, and 200 mmol/L, respectively. NaCl stress was applied using hydroponic culture. The NaCl concentration was increased by 50 mmol/L per day until the predetermined concentration was achieved. All *Hemerocallis fulva* plants under each NaCl treatment were arranged in three biological replicates. One biological replicate consisted of three independent seedlings, resulting in a total of nine plants per treatment. Plants subjected to the same NaCl concentration were cultivated in one hydroponic container to guarantee the reliability of experimental data. After 10 d of NaCl treatment, significant phenotypic differences were observed, and root systems were gently collected to determine their root morphological traits.

### Determination of Root Border Cell Characteristics

Plants were fixed in the holes of hydroponic boxes with sponges, and a small amount of pure water was added to prevent roots from directly touching the box bottom. The root surface was sprayed with NaCl solution of corresponding concentrations (0, 50, 100, 150, 200 mmol/L) each hour until the roots were fully moist and water droplets formed at root tips. The setting of biological replicates is the same as the measurement of root morphological indicators. After 24 h of treatment, the number, viability, apoptosis rate, and mucilage layer thickness of RBCs, as well as root activity, were measured.

## Determination Items and Methods

### Determination of Root Morphological Indices

After rinsing with distilled water, the root systems of *Hemerocallis fulva* plants were laid flat on the transparent plate of a root scanner (Tuopu GXY-A) for scanning. The root images were saved and analyzed by a root analysis system (GXY-B).

### Determination of Root Activity

Root activity was determined using the TTC method [11]:

$$TTC\ reduction\ intensity = \frac{TTC\ reduction\ amount\ (g)}{root\ fresh\ weight\ (g) \times treatment\ time\ (h)} \quad (1)$$

### Acquisition of RBCs Suspension

The preparation procedure of suspensions used for detecting all Root Border Cell (RBCs) related indicators is described as follows: Three distinct root tips (15-25 mm in length) were collected from individual *Hemerocallis* plants and immersed in PCR tubes containing 100  $\mu$ L distilled water to obtain RBCs suspensions (Each treatment included three biological replicates with three plants per replicate, for a total of nine plants), followed by gentle shaking to fully release RBCs into the liquid and generate an RBCs suspension.

### Determination of RBC Number and Viability

To assess the viability and population density of blood cells, the RBC suspension was analyzed using a trypan blue dye exclusion assay. The collected suspension was combined with 0.5% trypan blue solution at a volume ratio of 10 to 1. This concentration was optimized to ensure that only non-viable cells with compromised membranes would internalize the dye. The mixture was carefully loaded onto a standard hemocytometer, and the cell number and viability in the four quadrants were observed and counted under a UY203i microscope. Only intact RBCs were counted, while cell debris and cell aggregates were excluded. The total cell count per microliter was calculated by averaging the counts from four quadrants, multiplied by the dilution factor and the hemocytometer constant. Dead cells were counted using the same procedure, and cell viability was finally calculated accordingly. This approach helps to quantify the total cells and the proportion of viable individuals, and remains clear under microscopic examination:

$$No.\ of\ RBCs = No.\ of\ viable\ RBCs + No.\ of\ dead\ RBCs \quad (2)$$

$$Viability\ of\ RBCs = (No.\ of\ viable\ \frac{RBCs}{Total}\ no.\ of\ RBCs) \times 100\% \quad (3)$$

### Determination of RBCs Apoptosis Rate

The RBC suspension was mixed with Hoechst-33258 staining solution at a volume ratio of 1:1 and placed in the dark for 10 min. Then the apoptosis rate of RBCs was counted under a UY203i fluorescence microscope (nine fields were randomly selected to count the total number and apoptotic number of RBCs, respectively, and the average values were calculated):

$$Apoptosis\ rate = (No.\ of\ apoptotic\ \frac{RBCs}{Total}\ no.\ of\ RBCs) \times 100\% \quad (4)$$

### Determination of RBCs Mucilage Layer Thickness

The RBC suspension was mixed with India ink at a volume ratio of 1:1 and left to stand for 30 seconds. Micrographs were captured using a UY203i microscope equipped with a digital camera at 400 $\times$  magnification. ImageJ software (National Institutes of Health, USA) was used to measure the mucilage layer thickness at three positions (upper, middle, and lower parts) of each cell. The average of the three measurements was calculated as the mucilage thickness of the individual cell:

$$Mucilage\ layer\ thickness = (L1 + L2 + L3)/3 \quad (5)$$

Where L1, L2 and L3 represent the mucilage layer positions at the upper, middle and lower parts of RBCs, respectively.

### Data Analysis

This experiment used a one-way ANOVA and Duncan's multiple range test for multiple comparisons in different treatment groups. The significance level for all statistical tests was set at  $P < 0.05$ . Data processing and statistical analysis were performed using Excel 2016 and SPSS 26.0, and all figures were plotted with Origin 2025.

## Results and Analysis

### Effects of NaCl Stress on Root Morphology of *Hemerocallis fulva*

As shown in Table 1, 'Jingzhouhua' shows a non-monotonic response to salinity. The total root length first increased at 50 mmol/L NaCl, and then decreased at higher concentrations. In contrast, the total root length of 'Qingchong' and 'Changzuizihua' decreased linearly as the NaCl concentrations increased. The three *Hemerocallis fulva* cultivars had different physiological thresholds as NaCl concentrations increased. For the highly tolerant 'Jingzhouhua', root elongation was first stimulated by mild salinity, with the maximum total root length at 50 mmol/L. This indicates that low levels of Na<sup>+</sup> may trigger compensatory growth mechanisms or beneficial osmotic adjustments in this specific genotype. In sharp contrast, the salt-sensitive 'Changzuizihua' was proven to be highly sensitive. This is because its total root length was significantly affected even at a threshold of 50 mmol/L. The moderately resistant 'Qingchong' showed an intermediate response. Its structure remained stable until the concentration reached 100 mmol/L, at which point there was a significant decline in growth. As the salinity increased to 200 mmol/L, the root development of all cultivars was severely inhibited, resulting in their respective minimum growth values. However, the magnitude of this inhibition largely depends on the inherent salt tolerance of cultivars. Under extreme ionic stress, 'Jingzhouhua' exhibits a stronger capacity to sustain root elongation compared with more salt-sensitive cultivars.

**Table 1: Effects of NaCl stress on root morphology of *Hemerocallis fulva***

| Cultivar      | Treatment/mmol·L <sup>-1</sup> | Total root length/cm | Root tips          | Average root diameter/mm | Total root surface area/cm <sup>2</sup> | Total root volume/cm <sup>3</sup> |
|---------------|--------------------------------|----------------------|--------------------|--------------------------|-----------------------------------------|-----------------------------------|
| Jingzhouhua   | CK                             | 320.11±17.21a<br>b   | 237.67±8.3<br>3b   | 1.77±0.20a               | 333.98±6.18a                            | 97.72±7.31a                       |
|               | 50                             | 344.39±32.1a         | 286.67±8.7<br>4a   | 1.72±0.18a               | 312.19±6.98a                            | 96.67±11.58a                      |
|               | 100                            | 311.67±21.67a<br>b   | 254.33±21.<br>2ab  | 1.69±0.21a               | 311.74±17.97a                           | 89.09±5.49ab                      |
|               | 150                            | 294.32±45.84a<br>b   | 227.67±32.<br>35b  | 1.64±0.24a               | 262.81±34.1b                            | 76.08±5.11bc                      |
|               | 200                            | 277.72±30.42b        | 221.67±17.<br>79b  | 1.56±0.21a               | 251.94±39.49b                           | 68.52±9.33c                       |
| Qingchong     | CK                             | 587.89±41.95a        | 327.67±27.<br>32a  | 1.66±0.06a               | 344.04±28.68a                           | 63.94±5.10a                       |
|               | 50                             | 552.58±28.45a<br>b   | 325.33±8.1<br>8a   | 1.49±0.19ab              | 326.21±29.69ab                          | 57.56±9.21ab                      |
|               | 100                            | 417.18±36.32b<br>c   | 301.67±43.<br>5a   | 1.37±0.08b               | 260.15±13.59bc                          | 48.80±7.57b                       |
|               | 150                            | 412.12±19.69b<br>c   | 271.67±50.<br>14a  | 1.34±0.21b               | 217.69±27.19c                           | 34.11±6.97c                       |
|               | 200                            | 385.78±34.8c         | 245.67±32.<br>08a  | 1.28±0.09b               | 196.13±20.25c                           | 29.05±6.88c                       |
| Changzuizihua | CK                             | 540.34±44.01a        | 290.00±35.<br>68a  | 2.60±0.20a               | 330.09±14.51a                           | 70.16±8.09a                       |
|               | 50                             | 412.96±48.54b        | 216.33±23.<br>16b  | 2.02±0.12b               | 295.18±12.31b                           | 58.93±5.13b                       |
|               | 100                            | 319.81±15.95c        | 171.33±27.<br>15bc | 1.76±0.19bc              | 244.78±9.49c                            | 46.68±3.72c                       |
|               | 150                            | 280.17±55.2c         | 153.67±32.<br>15c  | 1.58±0.07c               | 189.79±17.37d                           | 32.31±3.00d                       |
|               | 200                            | 235.39±64.04c        | 150.33±7.0<br>9c   | 1.52±0.13c               | 150.00±30.70e                           | 16.94±1.63e                       |

Note: The lowercase letters in the same column indicate significant differences between NaCl treatments of specific Cultivars at a significance level of P<0.05. This statistical convention applies to all subsequent data

The root tips of 'Jingzhouhua' showed a non-monotonic trend. It reached its peak at 50 mmol/L NaCl and then declined at high concentrations. In contrast, the root tips of 'Qingchong' and 'Changzuizihua' decreased steadily as the salinity increased. The root tips of these three cultivars reached their minimum at a level of 200 mmol/L NaCl. Statistical analysis indicated that 'Jingzhouhua' and 'Qingchong' maintained the same number of root tips as the control, and 'Changzuizihua' was significantly reduced.

As NaCl concentrations increased, the average root diameter of all three *Hemerocallis fulva* cultivars generally declined, although the threshold for a significant response varies depending on cultivars. In the salt-sensitive 'Changzuizihua', the

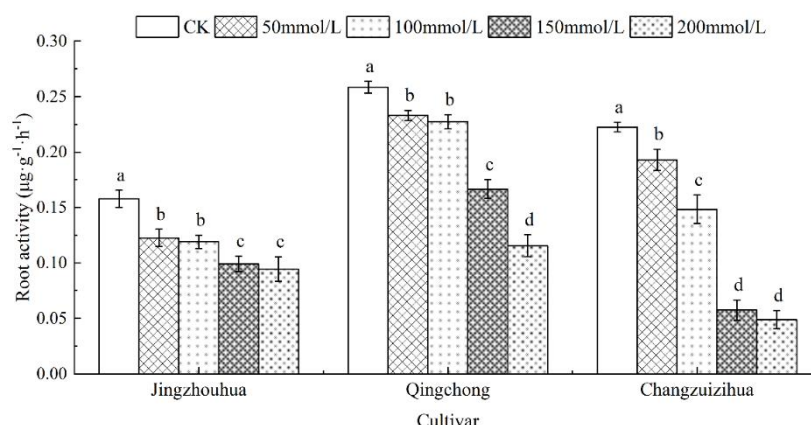
diameter decreased significantly at a concentration of 50 mmol/L NaCl. The moderately tolerant 'Qingchong' showed a similarly significant decrease when the concentration reached 100 mmol/L. In contrast, the highly salt-tolerant 'Jingzhouhua' maintained a stable average root diameter, and there were no significant changes even at a maximum concentration of 200 mmol/L.

Based on the average root diameter results, the total root surface area of all three cultivars decreased as NaCl concentrations increased. The salinity thresholds required for a significant reduction in surface area of 'Jingzhouhua', 'Qingchong', and 'Changzuizihua' were 150, 100, and 50 mmol/L, respectively. The reduction in root surface area of 'Jingzhouhua' was markedly lower than that of 'Qingchong' and 'Changzuizihua'.

Consistent with the changes in root surface area, the total root volume of all three cultivars decreased as the NaCl concentration increased. At 150, 100, and 50 mmol/L NaCl, the root volume of 'Jingzhouhua', 'Qingchong', and 'Changzuizihua' decreased significantly at 150, 100, and 50 mmol/L NaCl, respectively. At a NaCl level of 200 mmol/L, 'Jingzhouhua' maintained the maximum structural integrity, with a reduction of 29.88% compared to the control group. 'Qingchong' showed a moderate decline of 54.57%, and 'Changzuizihua' experienced the most severe loss at 75.86%. The decline of 'Qingchong' was moderate at 54.57%, and that of 'Changzuizihua' was the largest at 75.86%.

### Effects of NaCl Stress on Root Activity of *Hemerocallis fulva*

The root activity of all three *Hemerocallis* cultivars decreased as the NaCl concentration increased (Fig. 1). Compared with the control, treatments with 50-200 mmol/L NaCl solution significantly reduced the root activity of the three cultivars, with significant differences in the degree of reduction. For instance, under the treatment of 200 mmol/L NaCl, the root activity of 'Jingzhouhua', 'Qingchong' and 'Changzuizihua' decreased by 40.19%, 55.30% and 78.08% respectively compared to the control.



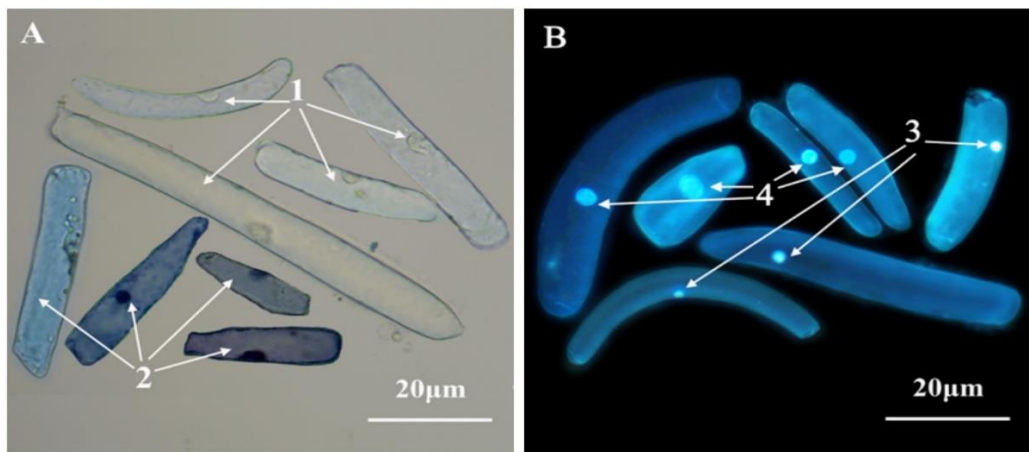
Note: The lowercase letters positioned above the data columns signify statistically significant differences among the various treatments for each individual cultivar at a significance level of  $P < 0.05$

**Fig. 1: Impact of NaCl stress on the root physiological vigor of three *Hemerocallis fulva* cultivars**

### Effects of NaCl Stress on RBC Number and Viability of *Hemerocallis fulva*

After staining with trypan blue solution, live RBCs appeared colorless and dead RBCs appeared blue (Fig. 2(A)). As shown in Table 2, the RBCs of all three *Hemerocallis fulva* cultivars decreased as the NaCl concentrations increased. The RBCs of 'Jingzhouhua' decreased significantly at 100 mmol/L NaCl, and 'Qingchong' and 'Changzuizihua' decreased at 50 mmol/L. At the maximum treatment level of 200 mmol/L NaCl, the magnitude of the reduction in RBC number was consistent with the salt tolerance ranking of the cultivars (reduction magnitude: 'Jingzhouhua' < 'Qingchong' < 'Changzuizihua').

Treatments with 50-200 mmol/L NaCl significantly reduced the RBCs viability of the three cultivars. At a concentration of 200 mmol/L NaCl, the RBC viability of 'Jingzhouhua', 'Qingchong' and 'Changzuizihua' all decreased to the minimum, with 19.18%, 28.02% and 37.77% lower than that of the control group.



A. RBC viability of *Hemerocallis fulva*: 1. Viable RBCs; 2. Dead RBCs      B. RBC apoptosis rate of *Hemerocallis fulva*: 3. Normal RBCs; 4. Apoptotic RBCs

**Fig. 2: RBCs of *Hemerocallis fulva* stained with trypan blue (A) and Hoechst 33258 (B)**

**Effects of NaCl Stress on RBC Apoptosis Rate of *Hemerocallis Fulva***

After staining with Hoechst-33258, RBCs showed fluorescence: the nuclei of normal RBCs appeared as clear, bright blue spots, and the nuclei of apoptotic RBCs showed a diffuse fluorescence pattern (Fig. 2B). Compared with the control group, treatments with 50 to 200 mmol/L NaCl can significantly increase the RBC apoptosis rate of these three *Hemerocallis* cultivars. This apoptotic response increased linearly with NaCl concentration. At a maximum concentration of 200 mmol/L, the apoptosis rates for 'Jingzhouhua', 'Qingchong', and 'Changzuizihua' reached 117.33%, 121.33%, and 128.38% of their respective control values (Table 2). Under 200 mmol/L NaCl treatment, the RBC apoptosis rate of 'Jingzhouhua', 'Qingchong', and 'Changzuizihua' was 117.33%, 121.33%, and 128.38%, respectively, of that of their respective controls.

**Table 2: Effects of NaCl stress on RBCs of *Hemerocallis fulva***

| Cultivars     | Treatments/mm ol·L <sup>-1</sup> | RBCs number     | Viability of RBCs/% | Apoptosis rate of RBCs/% | Mucilage layer thickness of RBCs/µm |
|---------------|----------------------------------|-----------------|---------------------|--------------------------|-------------------------------------|
| Jingzhouhua   | CK                               | 4750.00±220.48a | 0.85±0.01a          | 0.75±0.01c               | 3.83±0.60e                          |
|               | 50                               | 4583.33±381.88a | 0.81±0.02b          | 0.79±0.01b               | 6.60±0.94d                          |
|               | 100                              | 4027.78±267.88b | 0.80±0.01b          | 0.81±0.02b               | 8.40±0.18c                          |
|               | 150                              | 3250.00±166.67c | 0.73±0.02c          | 0.85±0.02a               | 12.33±0.73b                         |
|               | 200                              | 2861.11±336.79c | 0.69±0.01d          | 0.88±0.02a               | 16.50±1.14a                         |
| Qingchong     | CK                               | 3916.67±166.67a | 0.86±0.03a          | 0.75±0.01d               | 3.81±0.56c                          |
|               | 50                               | 3361.11±336.79b | 0.82±0.01b          | 0.81±0.01c               | 5.29±0.53c                          |
|               | 100                              | 2777.78±267.88c | 0.79±0.02b          | 0.84±0.02b               | 8.10±0.71b                          |
|               | 150                              | 2027.78±346.94d | 0.67±0.02c          | 0.86±0.01b               | 9.43±0.48ab                         |
|               | 200                              | 1305.56±173.47e | 0.62±0.02d          | 0.91±0.01a               | 10.50±1.46a                         |
| Changzuizihua | CK                               | 3944.44±315.49a | 0.88±0.02a          | 0.74±0.01d               | 3.94±0.17c                          |
|               | 50                               | 3250.00±381.88b | 0.80±0.01b          | 0.83±0.01c               | 4.28±0.91c                          |
|               | 100                              | 2333.33±381.88c | 0.75±0.03c          | 0.88±0.03b               | 4.99±0.74c                          |
|               | 150                              | 1222.22±336.79d | 0.64±0.01d          | 0.92±0.02a               | 6.74±0.36b                          |
|               | 200                              | 805.56±127.29e  | 0.55±0.05e          | 0.95±0.03a               | 8.45±0.32a                          |

## Effects of NaCl Stress on RBCs Mucilage Layer Thickness of *Hemerocallis fulva*

According to the pattern in RBC apoptosis, treatments with 50-200 mmol/L NaCl significantly increased the mucilage layer of RBCs in these three cultivars (Table 2 and Fig. 3). The minimum NaCl concentrations significantly increased the thickness of the RBC mucilage layer of 'Jingzhouhua', 'Qingchong' and 'Changzuizihua', which were 50, 100 and 150 mmol/L respectively. At a concentration of 200 mmol/L NaCl, the RBC mucilage layer thickness of 'Jingzhouhua', 'Qingchong' and 'Changzuizihua' was 4.31, 2.75 and 2.14 times that of the control, respectively.

'Jingzhouhua' has a more stable RBC population and a thick mucilage protective layer. It is more conducive to maintaining normal root growth and stable physiological metabolism under high-salinity conditions.



A~E. 'Jingzhouhua', A1~E1. 'Qingchong', and A2~E2. 'Changzuizihua' treated with 0, 50, 100, 150, and 200 mmol/L NaCl, respectively

**Fig. 3: Mucilage layer thickness of *Hemerocallis fulva* under NaCl stress**

## Discussion

The well-developed root morphology and high root activity facilitate the effective absorption of water and nutrients by plant roots, and enhance their adaptability to different stresses [12]. Salinity typically limits root elongation and reduces cumulative surface area and volume [13]. Some studies suggest that controlling the reduction of root surface area may be a defensive strategy to limit the passive influx of phytotoxic ions [14]. In this investigation, the highly salt-tolerant cultivar 'Jingzhouhua' showed a unique growth-stimulating effect at 50 mmol/L NaCl. The characteristics increased in total root length and tip frequency. In contrast, the moderately salt-tolerant cultivar 'Qingchong' only exhibited slight compensatory root growth with marginal growth improvement, and the salt-sensitive cultivar 'Changzuizihua' had mild root growth inhibition. These three cultivars exhibited significant phenotypic differences under low-concentration NaCl stress. This localized proliferation indicates a compensatory mechanism. Low-level salinity triggers the expansion of the absorptive surface area, thereby optimizing nutrient acquisition to counteract initial osmotic pressure. This adaptive expansion is consistent with the similar growth responses in tomato [15]. 100-200 mmol/L NaCl treatments decreased consistently in all measured parameters (including total root length, root tip number, average diameter, surface area, and volume, as well as overall root activity of the three *Hemerocallis* cultivars). 'Jingzhouhua' showed the smallest reduction in root indices and the most stable growth, and 'Qingchong' showed moderate damage. 'Changzuizihua' showed the greatest inhibition and the most severe root degradation. This may be due to the massive influx of Na<sup>+</sup> and Cl<sup>-</sup> into root cells, causing ionic toxicity and disrupting the structure and

function of cell membranes. In addition, the imbalance in ROS metabolism caused by this may damage the structural and functional stability of the root systems [16], ultimately leading to the inhibition of root growth in *Hemerocallis fulva*. The results are similar to the findings of cowpea [17]. Under NaCl stress, various root morphology indicators of the high salt-tolerant cultivar 'Jingzhouhua' were less inhibited (Table 1). Compared with 'Qingchong' and 'Changzuizihua', 'Jingzhouhua' maintained a more intact root spatial structure, larger effective absorption area, and more stable root metabolic activity under moderate and high NaCl stress. In contrast, 'Qingchong' exhibited impaired root structural integrity, and 'Changzuizihua' had disordered root architecture, drastically declined root activity, and severely damaged root absorption function (Fig. 1). This may be because the root systems of salt-tolerant cultivars have higher root oxidizing capacity, a larger absorption area, and a stronger ability to absorb water and nutrients, resisting NaCl stress damage to a certain extent [18].

Under different environmental conditions, plants activate various defense strategies, including regulating ROS production and modulating the number and activity of RBCs [19]. To cope with different environmental conditions, RBCs and the mucus they secrete play a crucial role. Root cap cells accumulate abundant glycerophospholipids, unsaturated fatty acids, and betaines. These compounds are crucial for maintaining cell membrane stability and regulating permeability, thereby effectively improving the salt tolerance of plant roots [8].

This study demonstrates that as NaCl concentrations increased, the population density and viability of RBCs in the three *Hemerocallis* cultivars decreased. The apoptosis rate and mucilage layer thickness of RBCs increased (Table 2). Under NaCl stress, the number and survival rate of RBCs are higher; the apoptosis rate is lower, and the mucus layer of root tips is thicker, which can effectively protect root tip tissues to a certain extent [6, 20]. This indicates that these characteristics are conducive to alleviating the inhibition of root activity. In contrast, the moderately salt-tolerant variety 'Qingchong' has significantly lower numbers of RBCs, cell viability, and mucus layer thickness than 'Jingzhouhua', resulting in weaker overall protection ability of root tips. The salt-sensitive cultivar 'Changzuizihua' had RBC apoptosis and rapid loss of cell activity, with no significant increase in the thickness of the mucilage layer. It failed to form effective physical and biochemical barriers at root tips, resulting in the continuous invasion of toxic ions into root tissues and causing sustained damage to the root's structural and physiological functions.

Previous studies have indicated that the imbalance in ROS metabolism caused by NaCl stress may be one of the reasons for the decrease in RBC number and viability, and the increase in apoptosis rate of *Hemerocallis fulva* under NaCl stress [21]. An increase in mucilage layer thickness may be associated with NaCl stress stimulating cells to secrete a series of bioactive chemicals into the extracellular space [22]. The elevated apoptosis rate of RBCs might be due to the initiation of programmed cell death in RBCs after absorption of NaCl, promoting cellular apoptotic processes [23]. In addition to direct ionic toxicity, the deceleration in root development is fundamentally linked to the population density of RBCs and the physical thickness of the protective mucilage layer [9]. In this study, the mucilage layer thickness of the high salt-tolerant cultivar 'Jingzhouhua' was significantly greater than that of the other two cultivars. This fully indicates that 'Jingzhouhua' has stronger adaptability under NaCl stress and can rapidly secrete various active substances to cope with environmental stimuli and alleviate stress-induced damage. Consistent with previous studies on grape under 4-HBA stress, grape RBCs synergistically resist 4-HBA stress through cell proliferation, mucilage secretion, salicylic acid accumulation, and enhanced secondary metabolism [24]. Therefore, the root morphology of the high salt-tolerant cultivar 'Jingzhouhua' was less affected by NaCl stress than that of 'Qingchong' and 'Changzuizihua', making it better able to resist root damage caused by NaCl stress.

These different cellular responses reveal the essential differences between the two genotype defense strategies. 'Jingzhouhua' can maintain intact RBCs and a stable mucilage barrier, effectively capture toxic ions, and maintain the physiological integrity of root tips. In contrast, 'Changzuizihua' fails to activate such protective mechanisms, which explains its high sensitivity to salt environments. NLP7 is a core transcription factor released by boundary-like cells and regulates disease resistance by mediating the release of RBCs [25]. The regulatory mechanism of plant salt tolerance may be similar. The divergent root responses to NaCl stress in different *Hemerocallis* cultivars are probably associated with genes regulating RBCs, which will be further verified in the future.

## Conclusion

This study reveals the associations between RBC quantity, viability, apoptosis rate, mucilage layer thickness, and salt tolerance of *Hemerocallis fulva* under NaCl stress. NaCl stress inhibits root growth and alters RBC characteristics of *Hemerocallis fulva*, with significant differences among cultivars with contrasting salt tolerance. The key findings include:

- 1) The salt-tolerant cultivar 'Jingzhouhua' maintains better root architecture and higher root activity under salt stress

- 2) 'Jingzhouhua' retains a larger population of viable RBCs with lower apoptosis rates
- 3) The mucilage layer thickness of RBCs increases under NaCl stress, with 'Jingzhouhua' showing the most substantial thickening

These results provide new insights into the cytological mechanisms of salt tolerance in ornamental plants, demonstrating that RBC mucilage secretion is an important component of the root defense system against NaCl stress. The strong correlation between RBC characteristics and salt tolerance suggests that RBC parameters, particularly mucilage layer thickness, can serve as reliable cytological markers for screening salt-tolerant *Hemerocallis* germplasm. These findings also have important implications for the cultivation and breeding of *Hemerocallis fulva* in saline soils. Future studies should further investigate the molecular mechanisms underlying RBCs' mucilage secretion and its regulation under NaCl stress, and explore the potential of exogenous regulators to enhance RBCs-mediated salt tolerance in *Hemerocallis fulva*.

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## Author's Contributions

Jinxu He and Yongxu Qiao: Experimental design, experimental execution, data analysis, and manuscript preparation.

Fen Chen, Yongping Zhang and Weihua Zhang: Material collection and experimental support.

Jiayi Zhang, Yuanyang You and Hongchao Li: Experimental material cultivation and maintenance and critical revision of the manuscript.

## Ethics

Authors should address any ethical issues that may arise after the publication of this manuscript.

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