

Research Article

Differences in the Response of Diploid and Tetraploid *Anthurium andraeanum* Seedling Roots to Salt Stress

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Abstract: To investigate the effects of salt stress on the growth, development, and endogenous hormone content of roots in diploid and tetraploid seedlings of *Anthurium andraeanum*, the enhanced salt tolerance was elucidated. The seedlings of *A. andraeanum* 'Pink champion' were placed at NaCl concentrations of 0, 50, 100, 150, and 200 mmol·L⁻¹. The results indicated that NaCl stress can reduce leaf area, total root length, root volume, average root diameter, root tip number, root vitality, root hair length, root hair diameter, root hair density, and the growth rate of root hair. At the same time, it can also shorten the length of mature area, elongation area, meristem area, and root crown area. The reduction in diploids was greater than tetraploids. NaCl stress also increased mortality rate, Indoleacetic Acid (IAA) content, and Ethylene (ET) production rate, with a smaller increase in tetraploids. Overall, NaCl stress can elevate the IAA content and ET production in diploids and tetraploids, with tetraploids being less affected, indicating higher salt tolerance.

Keywords: *Anthurium andraeanum*, Salt Stress, Root System, Diploid, Tetraploid

Introduction

Salt stress has different degrees of inhibition on plant growth and development (Çatav *et al.*, 2022). It is mainly manifested in high concentrations of salt, leading to ion imbalance, ion toxicity, reduces osmotic regulation capacity, inhibits antioxidant enzyme activity and plant growth (Chauhan *et al.*, 2023; Ahmad *et al.*, 2022). In recent years, facility agriculture has flourished, and facility production has become an important mode of production in China. However, due to the factors such as the singularity of planting varieties, improper fertilization and irrigation, and continuous cropping obstacles, the salinization of soil in the facility greenhouse has intensified, severely affecting the growth and development of plants. Therefore, continuing to strengthen the research on plant breeding and cultivating more salt-tolerant plants is one of the main directions.

Polyploid plants tend to be more resistant than diploid plants (Lin *et al.*, 2023; Mulagund *et al.*, 2023). They are significantly superior to diploid plants in horticultural traits. For example, Polyploids are often more resistant to

pathogens than diploids; similarly, polyploid birch seedlings (2n=4x=56, 2n=3x=42) are more resistant to salinization than diploids (2n=28) (Mehlferber *et al.*, 2021; Mashkina *et al.*, 2021). *A. andraeanum* Linden is a perennial herbaceous plant belonging to the genus Typha in the Araceae family, originating from the tropical rainforests of Central and South America. It is a flower with great ornamental and cultivated value. It was introduced to China for cultivation in the 1970s and is currently widely cultivated in Southern and Southwestern China. *A. andraeanum* prefers warm, humid, well-ventilated, and semi-shaded environments, and it is mainly cultivated in facilities in China. The severe soil salinization in facility cultivation not only hinders the growth and development of *A. andraeanum*, but also reduces the yield and quality, restricting the development of *A. andraeanum* industry to some extent. Currently, the research on *A. andraeanum* mainly focuses on hybrid breeding, selective breeding, color variation mechanism and tissue culture (Tung *et al.*, 2021; Osorio-Guarín *et al.*, 2021; Bandyopadhyay *et al.*, 2022; Rittirat *et al.*, 2021). There has been no report on the effects of salt stress on *A. andraeanum* diploid and tetraploid plants.



Therefore, through exploring the impact of NaCl stress on *A. andraeanum* diploid and tetraploid plants, we aim to investigate why tetraploid seedlings have higher salt tolerance than diploid, providing a scientific basis for breeding new varieties with better salt and alkali resistance, and a reference for the resistance of polyploid plants.

Materials and Methods

Test Material

The *A. andraeanum* diploid and tetraploid seedlings were selected as test materials, which were provided by the Plant Cell Engineering Laboratory of Suqian University.

Test Methods

Robust seedlings of *A. andraeanum*, diploid and tetraploid were selected. When they grew to 3 leaves and 1 bud, they were divided into two groups: Group 1 was used for plastic box substrate cultivation with NaCl solution irrigation treatment to measure leaf area, root morphology indicators, Indoleacetic Acid (IAA) content, Ethylene (ET) production rate, and root vitality; Group 2 was used for agar culture in petri dishes with different concentrations of NaCl solution to measure the growth of root tips and root hairs:

Group 1: Diploid and tetraploid seedlings of the *A. andraeanum* were transplanted into plastic boxes with a substrate mixture of peat: vermiculite: perlite = 2: 1: 1 (length × width × height = 15.5×11.5×7.5 cm), with 9 plants per box and a plant spacing of 4 cm. Each box contained 130g of substrate. They were then transferred to an artificial climate incubator for cultivation for 7 d, with cultivation conditions set at: 150 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity, 2 h light exposure, 12 h dark cultivation, light cultivation temperature, 28°C dark cultivation, 70% air relative humidity; after 7 d, treatment was carried out using a solution containing different concentrations of NaCl in Flower No.1 (1:1500), with final NaCl concentrations of 0, 50, 100, 150, 200 $\text{mmol}\cdot\text{L}^{-1}$. Each cultivation box was watered with 80 ml and treated once every 7 d for 4 consecutive times. After one month of the stress, samples were taken to measure leaf area, root morphology index and root vitality. Each cultivation box was filled with 80 ml of NaCl solution separately. After 7 days of stress, the roots were sampled and washed clean with deionized water to determine the content of dolo acetic acid and ethylene content.

Group 2: A certain amount of NaCl was added to the plant gel with a configured concentration of 0.45 $\text{g}\cdot\text{L}^{-1}$, so that the final concentration of NaCl was 0, 50, 100, 150 and 200 $\text{mmol}\cdot\text{L}^{-1}$, and then plant gel was injected into a petri dish with a diameter of 10cm and a depth of 1cm. The root systems of diploid and tetraploid seedlings of *A.*

andraeanum were cleaned with deionized water, and acclimated them in purified water for 1 d, and then placed the roots into plant gel for cultivation. The growth of root hairs was observed under a microscope for 15 consecutive hours.

Project Measurement

Measurement of Leaf Area and Root Morphology Index

The root morphology indicators of plant seedlings were determined by immediate measurement. The surface impurities of roots were washed thoroughly with clean water, and then rinsed them with pure water and spread them flat on the root scanner specimen plate, injecting 10-15mm deep pure water. It should be sure that these roots were not overlapping. A desktop scanner was used to scan the roots and save images. Root analysis system software was used to analyze the root images and obtain the parameters such as leaf area, total root length, total root volume, average root diameter and root tip number.

Measurement of Root Hair Growth Index

The fluorescence microscopy with a UY203i microscope was used to measure the root hair density 1-1.5 cm near the root tip under a 10x microscope, to determine the specific root segment and take the average. The lengths of root cap area, meristematic area, elongation area, and mature area were recorded under a 10x microscope. The diameters of root hairs were observed at 20x magnification and the growth rate of root hair was recorded every 3 hours. Subsequently, the analysis software UOP View was used to measure the length of root-cap area, meristem area, elongation area, and mature area of the root system, as well as the diameter, density, and growth rate of root hairs.

Determination of Auxin and Ethylene Content

Refer to the method of Tan *et al.* (2010), enzyme-linked immunosorbent assay was used to determine the content of indole acetic acid and ethylene. The supernatant was extracted and operated according to the instructions of the kit.

Determination of Root Vitality

Refer to Li (2000), the method was determined by Chlorinated Triphenyltetrazine (TTC). The formula is as follows: TTC reduction intensity = TTC reduction amount (g)/root weight (g) × time (h).

Data statistical Analysis

The data was processed by Microsoft Excel. ANOVA was performed by SPSS19.0, and multiple comparisons were performed by the Duncan method. The difference was defined as $P < 0.05$, and all data were the mean value of three repeated \pm standard error.

Results

Effects of Salt Stress on the Growth of A. Andraeanum 'Pink Champion' Seedlings

As shown in Fig. 1, after one month of NaCl stress, as the salt concentration increased, the growth of diploid and tetraploid inhibited. The shoots were shorter and lower than the control group, and the leaves on the lower part of the plant gradually turn yellow and dry (Fig. 1). When the NaCl concentration reached 50 mmol·L⁻¹, the leaf edge of *A. andraeanum* seedlings began to turn yellow. As the NaCl concentration increased, the leaf atrophy of *A. andraeanum* seedlings became more severe. Especially, when the NaCl concentration reached 150 mmol·L⁻¹, the diploid plants were close to death, which was more serious than the tetraploids. When the NaCl concentration reached 200 mmol·L⁻¹, the plant died. The above results show that low NaCl concentration inhibited the growth of *A. andraeanum* seedlings, and the lower leaves became yellow and grew slowly. As NaCl concentration increased, the more obvious the inhibition effect, the greater the damage to the plant. A certain level of concentration will lead to the death of plant.

Effect of Salt Stress on Leaf Area of A. Andraeanum 'Pinkchampion' Seedlings

As the salt concentration increased (Table 1), compared to the control, the leaf areas of diploid and tetraploid *A. andraeanum* decreased. When treated with 50 mmol·L⁻¹ NaCl, the changes in the leaf area of diploid and tetraploid *A. andraeanum* were not significant ($P < 0.05$). However, the leaf area of diploid and tetraploid plants was significantly lower than that of the control at a concentration of 100 mmol·L⁻¹. As the NaCl concentration increased gradually, the growth of *A. andraeanum* was severely inhibited. When the NaCl concentration was 200 mmol·L⁻¹, the leaf area of diploid and tetraploid *A. andraeanum* was reduced by 93.41 and 92.96%, respectively. Compared with the control, the reduction in diploids was more pronounced. It can be seen that salt stress would affect the growth of *A. andraeanum* seedlings. The inhibitory effect of low concentration of salt stress is not significant, while high concentration of salt stress will inhibit the growth of *A. andraeanum* leaves. The diploid received a greater effect than the tetraploid.

Morphological changes are the most direct reflection of plants under stress (Ahmed, 2022). Leaves are important functional organs of plants and are one of the most responsive organs under stress (Zhang *et al.*, 2021). The results indicate that 50 mmol·L⁻¹ salt stress does not have a significant impact on the leaves of plants. However, as the salt concentration increased, diploid and tetraploid *A. andraeanum* leaves decreased. High

concentration of salt stress will inhibit the leaf growth, which may be related to ion toxicity and osmotic stress. Under salt stress, plants are difficult to absorb water, leading to a decrease in cell turgor pressure. The high turgor pressure maintained by plants under sufficient water is an important reason for plant cell extension. Xie *et al.* found that 100 mmol·L⁻¹ NaCl stress could significantly reduce the plant height, stem diameter, leaf area, and dry weight of melons. Cai Yubo *et al.* also found that under salt stress, the growth rate and leaf area of *Xanthocerasorbifolium* plants were significantly lower than those of the control, which is similar to the results of this study (Mao *et al.*, 2021).

Effects of Salt Stress on Root Morphology of A. Andraeanum 'Pink Champion' Seedlings

The total root length of diploid and tetraploid *A. andraeanum* decreased as the NaCl concentration (Table 2) increased. At the NaCl concentrations of 150 mmol·L⁻¹ and 200 mmol·L⁻¹, the total root lengths of diploid and tetraploid plants were significantly shorter than those of the control. In particular, at a NaCl concentration of 200 mmol·L⁻¹, the total root lengths of diploid and tetraploid decreased by 77.95 and 55.81% compared with the control. This shows that the effect of NaCl concentration on the root system of tetraploid was less than that of diploid.



Fig. 1: Effects of NaCl stress on phenotype of *A. andraeanum* seedlings

Note: 0, 50, 100, 150 and 200 respectively represent 0, 50, 100, 150 and 200 mmol·L⁻¹ NaCl, The same as below

Table 1: Effects of NaCl stress on the leaf area of *A. andraeanum* seedlings

NaCl concentration/mmol·L ⁻¹	Leaf area/mm ²	
	2n	4n
0	758.71±104.75a	565.69±114.66b
50	699.26±144.05a	558.65±62.33b
100	363.62±145.32c	86.63±1.94d
150	127.27±53.30d	56.51±0.83d
200	50.01±9.93d	39.83±2.82d

Note: The data are mean ± SD. Different lowercase letters indicate significant difference between all treatments ($P < 0.05$) and the same as below

As the salt concentration increased, the average diameter in the total root system of diploid and tetraploid *A. andraeanum* decreased. The decrease in average root diameter of diploid was significantly larger than that of tetraploid (Table 2). When the NaCl concentration was 200 mmol·L⁻¹, the average root diameter of diploid decreased by 82.48%, and that of tetraploid decreased by 49.77%. This indicates that the average diameter in the root system of tetraploid was less affected by NaCl than that of diploid.

At the same time, as the salt concentration increased (Table 2), the total root volume and total root tip number of diploid and tetraploid *A. andraeanum* also decreased. When the NaCl concentration was 200 mmol·L⁻¹, compared with the control, the total root volume of diploid and tetraploid decreased by 89.52 and 59.24%, and the total root tip number decreased by 91.67 and 67.50%, respectively. This shows that the effect of NaCl on the total root volume and root tip number of tetraploids was less than that of diploids.

There is no report on the effect of salt stress on root morphology and structure of *A. andraeanum* (Tu *et al.*, 2014; Liu *et al.*, 2024). In this experiment, it was found that several morphological indexes such as total root length, average diameter and total root volume of diploid and tetraploid *A. andraeanum*, were significantly changed under NaCl stress. The inhibition in the growth of diploid root system was more significant. It was further confirmed that tetraploids were significantly stronger than diploids in resisting adversity. In addition, salt stress leads to an increase in root plasma membrane permeability, thereby reducing root water adsorption. Tetraploid *A. andraeanum* suffered less damage from salt stress than diploid, resulting in stronger root water absorption than that of diploid. This may also be the reason why tetraploid *A. andraeanum* is stronger than diploid *A. andraeanum* in resisting salt stress.

Effect of Salt Stress on Root Hair Growth of *A. Andraeanum* 'Pink Champion' Seedlings

As the NaCl concentration increased, the average diameter of root hairs, the length and density of root hairs of diploids and tetraploids of *A. andraeanum* decreased, and the decrease in diploids was more significant (Table 3). When the NaCl concentration reached 2 00 mmol·L⁻¹, compared with the control, the length of root hairs of diploid and tetraploid decreased by 51.88% and 24.52%, respectively, and the density of root hairs decreased by 46.53 and 41.46%, respectively. It can be seen that the inhibitory effect of NaCl on diploids was significantly stronger than that of tetraploids in the length and density of root hairs, indicating that tetraploid root hairs were more capable of resisting NaCl stress.

As shown in Table 3 and Fig. 2, the growth rates of root hairs of *A. andraeanum* diploids and tetraploids also decreased as the NaCl concentration increased. The growth of root hairs stopped at high concentrations. When the NaCl concentration was 0-50 mmol·L⁻¹, the growth rate of diploid root hairs was faster than that of tetraploid when the NaCl concentration was 150 mmol·L⁻¹. Compared with that of control, the growth rate of root hairs of diploid and tetraploid decreased by 99.69 and 84.98%, respectively. At this time, the growth rate of diploid was slower than that of tetraploid. When the NaCl concentration reached 200 mmol·L⁻¹, both root hairs stopped growing. This indicates that higher NaCl concentration will inhibit the growth of diploid root hairs to a greater extent than that of tetraploid. This also proved that the ability of tetraploid root hairs to resist NaCl stress was superior to that of diploid.

Table 2: Effects of NaCl stress on the roots of *A. andraeanum* seedlings

NaCl concentration n/mmol·L ⁻¹	Total root elongation/cm		Average diameters of total roots/mm		Root volume of total roots/mm ³		Total root tips	
	2n	4n	2n	4n	2n	4n	2n	4n
0	20.36±3.14a	18.60±1.36ab	1.37±0.22d	2.13±0.20 a	401.29±28.60c	592.36±135.40 a	12.00±0.82 a	10.00±0.82b
50	18.07±3.54ab	17.46±3.68ab	1.13±0.02d	1.88±0.06 b	314.80±25.40de	505.85±64.19 b	8.00±1.83c	9.00±0.82bc
100	11.37±2.35de	15.51±0.83bc	0.90±0.09e	1.63±0.09 c	252.48±1.67e	419.13±34.59 c	5.25±0.50d	6.00±0.83d
150	8.39±0.17e	12.40±1.74cd	0.61±0.03f	1.33±0.13 d	148.37±6.71f	328.15±31.82d	3.50±1.29e	5.00±0.81d
200	4.49±0.87f	8.22±0.47e	0.24±0.09g	1.07±0.36e	42.06±4.12g	241.44±8.65e	1.00±0.00f	3.25±1.26e

Table 3: Effects of NaCl stress on the root hairs of *A. andraeanum* seedlings

NaCl concentration /mmol·L ⁻¹	Root hair average length/μm		Root hair average diameter/μm		The density of root hairs/mm ²		Root hair growth rate/(μm·h ⁻¹)	
	2n	4n	2n	4n	2n	4n	2n	4n
0	1118.69±48.59a	767.28±17.58c	20.26±1.85ab	20.63±0.88a	25.25±0.96a	20.50±1.29b	3.20±0.22a	2.13±0.20b
50	1039.94±12.99b	683.33±13.22d	18.19±2.39bc	18.89±2.23abc	21.50±1.29b	20.25±2.50b	1.57±0.11c	0.75±0.09d
100	780.59±93.77c	633.98±12.17d	17.69±1.62c	18.04±1.90bc	14.75±1.71c	15.00±0.82c	0.01±0.04f	0.32±0.02e
150	752.09±25.78c	639.94±24.13d	13.91±0.87de	15.16±0.95d	14.00±1.29cd	14.75±1.50c	-	-
200	538.26±16.32e	579.18±11.67e	12.48±0.46d	14.77±0.39de	13.50±0.82cd	12.00±1.41d	-	-

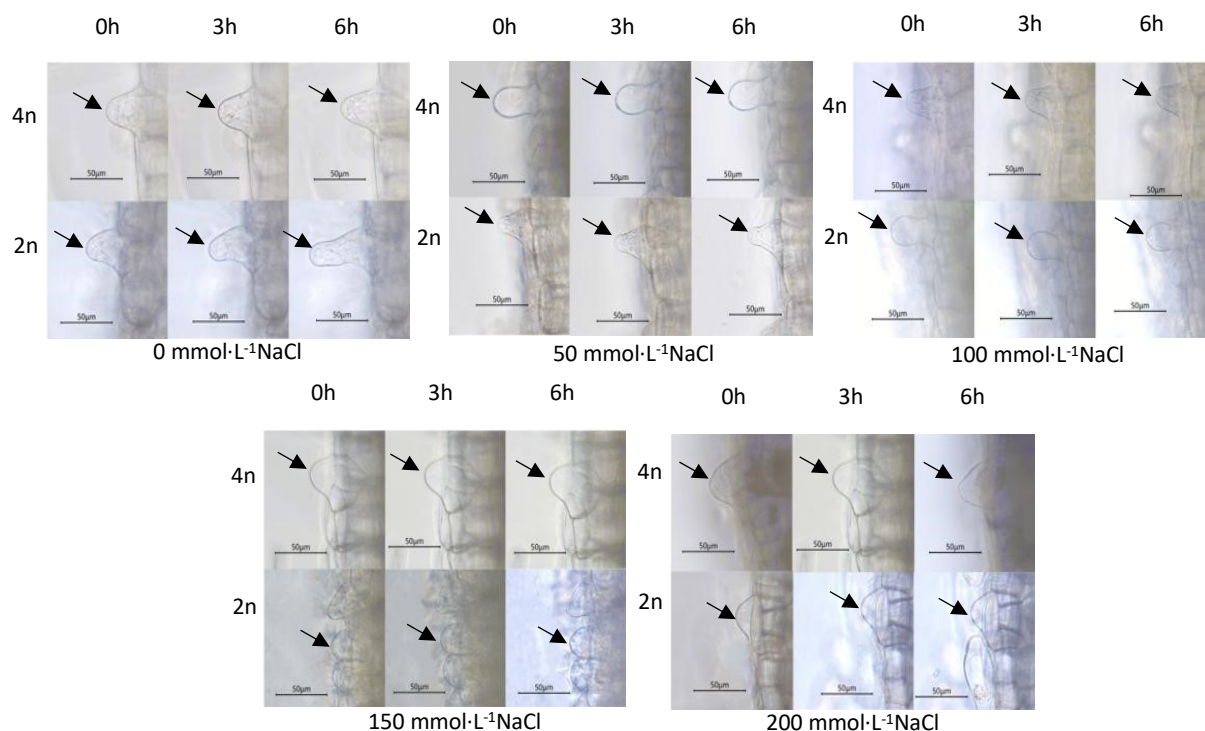


Fig. 2: Impact of NaCl on the maturation of root hairs of *A. andraeanum* seedlings

Root hairs are tubular extensions of epidermal cells on the surface of plant roots, playing a crucial role in anchoring plants to soil, absorbing water and inorganic salts, and facilitating information exchange between the plants roots and the external environment. Their development is highly plastic influenced by various plant hormones and environmental factors (Jin *et al.*, 2023; Şekerci *et al.*, 2023). This study found that root hairs growth in diploid and tetraploid *A. andraeanum* was significantly inhibited, with the inhibition in diploid *A. andraeanum* being more pronounced. Therefore, when applying fertilizers in the future, the concentration of fertilizers should not be too high to prevent the root hairs of *A. andraeanum* from being damaged and affecting the growth of plants.

Effects of Salt Stress on Root Tip Structure of A. Andraeanum 'Pink Champion' Seedlings

As shown in Table 4, as the NaCl concentration increased, the lengths of maturation area, elongation area, meristematic area, and root crown area of root tips of *A. andraeanum* diploid and tetraploid roots were gradually shortened. When the NaCl concentration reached 200 mmol·L⁻¹, compared with the control, the length of each structure of the root system decreased to the lowest level. At this time, the length of mature area of diploid and

tetraploid decreased by 54.31 and 44.67%, respectively; the length of elongation area decreased by 64.04% and 53.56%, respectively, and the length of meristematic area decreased by 78.32 and 66.38%, respectively. There was little difference in the reduction of the length of the root crown area between them. It can be seen that the reduction in the length of major structures of diploid root system was significantly higher than that of tetraploid under high NaCl stress. This indicates that the tetraploid root system is more resistant to NaCl stress than the diploid.

Effects of Salt Stress on Endogenous IAA and ET in the Root System of A. Andraeanum 'Pink Champion'

Under 200 mmol·L⁻¹NaCl stress, compared with the control, the content of IAA in the roots of diploid and tetraploid *A. andraeanum* increased rapidly, with 33.81% and 19.29% respectively. The content of IAA in the roots of diploid plants was higher than that of tetraploids, both in control and treatment. In addition, 200 mmol·L⁻¹NaCl also promoted the production rate of ET in the roots of diploid and tetraploid plants. The ET production rate in the roots of both increased by 28.73 and 24.00% respectively compared to the control (Table 5). No matter IAA or ET, at 200 mmol·L⁻¹NaCl stress, the content in diploid roots of *A. andraeanum* was higher than that in tetraploids.

Table 4: Effects of NaCl stress on the root tip structure of *A. andraeanum* seedlings

NaCl concentration/ mmol·L ⁻¹	Mature area length of root tip/μm		Elongation area length of root tip/μm		Meristematic area length of root tip/μm		Root cap meristem area length/μm	
	2n	4n	2n	4n	2n	4n	2n	4n
0	1497.74±269.59a	1506.57±195.85a	588.67±82.41b	622.16±80.88a	547.67±58.20a	508.45±96.61a	161.62±24.24a	159.26±28.67a
50	1315.62±114.15b	1231.25±209.31c	336.11±47.06cd	358.28±64.49c	432.36±60.53b	445.52±75.74b	130.15±18.87b	125.86±20.14bc
100	1309.16±170.19b	1221.24±195.40cd	281.35±70.51f	322.52±73.15de	316.53±61.09d	381.16±60.99c	119.66±23.93c	86.72±19.23d
150	1175.04±199.76d	1070.34±132.19e	230.98±30.03g	318.94±54.22de	284.62±34.15e	377.58±56.64c	85.81±9.44d	78.66±14.95de
200	684.39±95.81g	833.51±133.36f	211.68±23.28g	288.91±40.45df	118.71±21.37g	170.92±18.80f	70.11±11.92ef	68.65±9.61f

Table 5: The influence of different NaCl concentration treatments on IAA content and ET production in roots of *A. andraeanum* seedlings

NaCl concentration/mmol·L ⁻¹	Indole acetic acid content/(ng·g ⁻¹ ·FW)		Ethylene/(p mol·g ⁻¹ ·FW·h ⁻¹)	
	2n	4n	2n	4n
0	6.24±0.63a	5.43±0.66a	14.34±2.08a	12.25±1.86a
200	8.35±0.75b	6.37±0.61a	18.46±1.86b	15.19±2.11a

Both IAA and ET can positively regulate the growth of plant root hairs (Qiu *et al.*, 2023; Chen *et al.*, 2024). There is a complex signal exchange in root hair regulation (Jiang *et al.*, 2024). On the one hand, IAA is involved in root hair growth and regulates the development of root hair (da Silva *et al.*, 2022). On the other hand, ET has a decisive effect on root structure and root growth, root pith formation, elongation and cluster root formation. In addition, high concentrations of phytohormones can significantly inhibit the growth rate of diploid root hairs (Zhang *et al.*, 2016), while had less effect on the growth of tetraploid root hairs (Table 5). The mechanism of the difference between the two needs to be further studied.

Effects of Different Concentrations of NaCl Treatment on the Root Vitality of *A. Andraeanum* Diploid and Tetraploid Seedlings

As shown in Fig. 3, as the NaCl concentration increased, the root vitality of diploid and tetraploid seedlings of *A. andraeanum* decreased, with the diploid seedlings experiencing a more pronounced decline than the tetraploid seedlings. The inhibition of root vitality in diploid seedlings at low NaCl concentrations was significantly higher than that in tetraploid seedlings. Lower concentrations of NaCl (0, 50, 100 mmol·L⁻¹) had little effect on the reduction of root vitality in tetraploid seedlings, but significantly reduced the vitality of diploid seedling roots. High NaCl concentrations (150,200 mmol·L⁻¹) will significantly inhibit the root vitality of diploid and tetraploid seedlings. Under 200 mmol·L⁻¹NaCl stress, the root vitality of diploid and tetraploid seedlings reached the lowest level, decreasing by 68.78% and 51.88% respectively compared to the control. This indicates that the root vitality of diploid seedlings is more severely affected by NaCl stress.

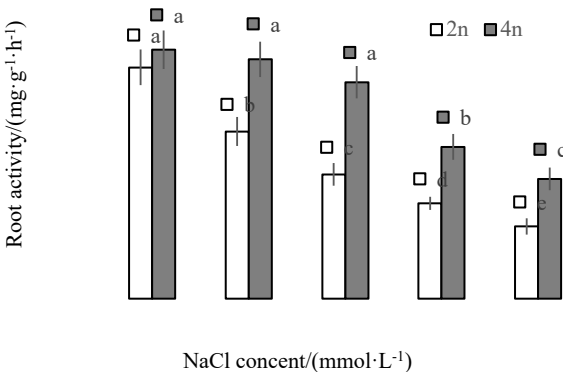


Fig. 3: Effects of NaCl on root activity of *A. andraeanum* seedlings

Conclusion

This study investigated the effects of salt stress on root systems and root hairs of diploid and tetraploid *A. andraeanum* seedlings. NaCl stress can reduce leaf area, root morphology indicators, root vitality, and root hair morphology of diploids and tetraploids, with more pronounced effects on diploids. NaCl can increase the IAA content and ET production rate of diploids and tetraploids, and tetraploids were less affected. It is inferred that NaCl-induced IAA accumulation is a significant factor in inhibiting root growth, and the growth in ET production may be a coping mechanism. More pronounced morphological and structural damage was observed in diploid plants, highlighting the higher salt tolerance of tetraploids.

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Authors Contributions

Yun Zhang, Yongping Zhang: Study design, experimental execution, data analysis, and manuscript preparation.

Yongxu Qiao and Weihua Zhang: Material collection and experimental support.

Jinxu He, Jiayi Zhang and Lihong Gao: Experimental design and critical revision of the manuscript.

Declarations

The authors declare that they have no conflict of interest and the corresponding author affirms that all authors have read and approved this manuscript.

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