

## EFFECTS OF EXOGENOUS SALICYLIC ACID ON CELL WALL POLYSACCHARIDES AND ALUMINUM TOLERANCE OF *TRICHOSANTHES KIRILOWII* UNDER ALUMINUM TOXICITY

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### Abstract

A hydroponic experiment was conducted to study the effects of exogenous salicylic acid (SA) on root length, relative aluminum content in the apical cell wall, acid phosphatase (APA) and pectin methyl esterase (PME) activity, root pectin, hemicellulose 1 (HC1), and hemicellulose 2 (HC2) contents of Anguo *Trichosanthes kirilowii* (Al-tolerant genotype) and Pujiang *T. kirilowii* (Al-sensitive genotype) under 800  $\mu\text{mol/L}$  of aluminum stress. The results showed that the growth of Al-tolerant Anguo *T. kirilowii* and Al-sensitive Pujiang *T. kirilowii* was inhibited when exposed to 800  $\mu\text{mol/L}$  of aluminum solution. APA and PME activities were also enhanced for both genotypes. The contents of relative aluminum, pectin, HC1, and HC2, as well as Al accumulation in the root tips were increased under aluminum toxicity. Pujiang *T. kirilowii* showed higher enzyme activity and cell wall polysaccharide contents than Anguo *T. kirilowii*. In addition, the root cell wall pectin, HC1, and HC2 contents of Pujiang *T. kirilowii* were increased by a large margin, showing its greater sensitivity to aluminum toxicity. Root length is an important indicator of aluminum toxicity, and has an important relationship with cell wall polysaccharide content. Aluminum toxicity led to the accumulation of pectin and high PME activity, and also increased the number of free carboxyl groups, which have more aluminum binding sites. Membrane skin increased extensively with the increase in APA activity, damaging membrane structure and function. Different SA concentrations can decrease enzyme activity and cell wall polysaccharide content to some extent. With the addition of different SA concentrations, the root relative aluminum content, cell wall polysaccharide content, APA and PME activities decreased. Aluminum toxicity to both genotypes of *T. kirilowii* was relieved in different degrees as exogenous SA concentration increased. Inter-simple sequence repeat (ISSR) marker was used to examine the genetic distance, genetic identity, and phylogenetic relationships. A certain correlation was found between the differences in Al tolerance, genetic distance, genetic identity, and phylogenetic relationship of *T. kirilowii* genotypes.

**Key words:** Aluminum; Salicylic acid; Cell wall polysaccharide; PME activity; Gene distance.

### Introduction

Aluminum (Al) is the third most abundant element after oxygen and silicon, accounting for 8% of the earth's crust (Sverdlin, 2003). Aluminum normally has no harmful effects on plants in its typical forms, such as insoluble aluminosilicates or oxides. However, these insoluble Al forms release highly toxic soluble  $\text{Al}^{3+}$ , with the increasing soil acidification caused by the long-term use of acid fertilizers or frequent acid rain deposition. Under these conditions, these released Al ions are harmful to plants (Kochian, 1995). Al toxicity is one of the most important factors that determine the distribution of plant species and ecotypes, as well as the growth and yield of crops on acid mineral soil (Uexküll & Mutert, 1995).

Snake gourd (*Trichosanthes kirilowii* Maxim.), a perennial vine belonging to the genus *Trichosanthes* L., used as traditional Chinese medicines is distributed in East Asia, northern Australia, and China. *Trichosanthes* is an indigenous plant used as traditional Chinese medicine for preventing abortion for more than two thousand years. It is widely used as a folk medicine because its tuber can reduce mid-term abortion and its fruit rind can help cure heart and lung diseases (Anon., 1986). The seeds of *Trichosanthes kirilowii* are called snake gourd seed, which has high medicinal value and is rich in lipids and protein. The medical value of *T. kirilowii* seed has had a comprehensive development and application (Liang & Li,

2009). *Trichosanthin* can be presumed as a protective protein with ribosome-inactivating activity in the seeds of *T. kirilowii*, indicating its great potential role in the construction formation of fusion immunotoxin and anti-tumor therapy (Shu *et al.*, 2009).

Salicylic acid (SA) is a potent plant hormone (Raskin, 1992a) because of its diverse regulatory roles in plant metabolism (Popova *et al.*, 1997). SA is essential in the regulation of plant growth, development, interaction with other organisms, and in response to environmental stresses (Raskin, 1992a, 1992b; Yalpani *et al.*, 1994). Exogenous salicylic acid alleviates the toxic effects generated by mercury in *Medicago sativa* (Zhou *et al.*, 2009) and by cadmium (Cd) in barley (Metwally *et al.*, 2003) and maize plants (Pal *et al.*, 2002). Its effect on plants exposed to various biotic and abiotic stresses has also been discussed by Hayat (2010). A significant improvement in growth parameters was recorded, with a concomitant reduction in the rate of Cd-induced lipid peroxidation and electrolyte leakage in maize plants raised from the seeds soaked in SA (Krantev *et al.*, 2008). Shi & Zhu (2008) reported that exogenous SA alleviated the toxicity generated in *Cucumis sativus* by manganese exposure, and the response was mediated by the reduction in ROS level and lipid peroxidation. Antioxidant enzymes also showed varied response following SA treatment. However, there is still no study on the effect of exogenous SA on Al tolerance of *T. kirilowii* exposed to aluminum toxicity.

The impact of aluminum toxicity on the growth of *T. kirilowii* cannot be neglected in promoting the wide cultivation of *T. kirilowii* in acidic soils. Two gene types of *T. kirilowii*, which are different in Al resistance, were selected to solve the problem. A culture experiment was conducted to study the effects of different SA concentrations on cell wall polysaccharides, physiological and growth characteristics of *T. kirilowii* under Al stress, and correlation between the differences in aluminum tolerance of *T. kirilowii* genotypes and genetic distance.

## Materials and Methods

**Plant materials and growth condition:** Two *Trichosanthes kirilowii* genotypes with different Al resistance, Al-tolerance genotype of Anguo, and Al-sensitive genotype of Pujiang were used as research materials.

The constitution of the nutrient solution was according to Yoshida (1972). Whole seeds were selected. Seeds were surface-sterilized for 30 min in 0.1% H<sub>2</sub>O<sub>2</sub>, washed three times with sterile distilled water, and then planted in a sand pot. Individual plants of uniform size and vigor were transferred to a 3 L plastic bucket after developing the third leaf, and then cultured in 1/2 of the amount of the complete nutrient solution for one week. The culture pH was adjusted daily to 4.5, and nutrients were changed every three days.

Thereafter, the following treatment groups were formed: T<sub>0</sub> (complete nutrient solution); T<sub>1</sub> (AlCl<sub>3</sub>, 800 μmol/L); T<sub>2</sub> (AlCl<sub>3</sub>, 800 μmol/L; SA, 10 μmol/L); T<sub>3</sub> (AlCl<sub>3</sub>, 800 μmol/L; SA, 30 μmol/L); T<sub>4</sub> (AlCl<sub>3</sub>, 800 μmol/L; SA, 50 μmol/L), and cultured for 15 days. Al<sup>3+</sup> was supplied by AlCl<sub>3</sub>·6H<sub>2</sub>O. The concentrations used in the experiment were selected by pre-experiments.

$$(A) Z_{ij} = \frac{x_{ij} - x_{j\min}}{x_{j\max} - x_{j\min}}; (B) Z_{ij} = 1 - \frac{x_{ij} - x_{j\min}}{x_{j\max} - x_{j\min}}; (C) Z_i = \frac{1}{n} \sum_{j=1}^n Z_{ij}$$

A-type and B-type represents membership function value of the i genotype j indicator indicating its aluminum tolerance,  $x_{ij}$  means the determination ratio of the i genotype j indicator,  $x_{j\max}$ ,  $x_{j\min}$  represent the maximum and minimum values of the indicator. Calculation of each index coefficient of aluminum tolerance membership function value, Tolerance to aluminum toxicity coefficient of aluminum stress was positively correlated with the A-type; Tolerance to aluminum toxicity coefficient of aluminum stress was negatively correlated with B-type. C-type expressed tolerance to aluminum toxicity membership function of the average in i genotype, n is the number of indicators.

### The genetic diversity of nine genotypes of *T. kirilowii*:

DNA was extracted from young leaves of *T. kirilowii* by SDS method. The genetic distance, genetic identity, and phylogenetic relationships among nine genotypes of *T. kirilowii* were studied using ISSR marker.

**Statistical analysis:** Statistical analyses were performed via analysis of variance (ANOVA) using the Statistical Product and Service Solutions (SPSS) software.

**Aluminum measurement in the apical cell wall:** The apical part of the plant, *T. kirilowii*, (about 10 mm in length) was immersed in 5 mmol/L NH<sub>4</sub>OAC buffer (pH 5.0) for 5 min, stained in 100 μmol/L Morin (5 mmol/L NH<sub>4</sub>OAC, pH5.0) for 1 h, and immersed again in 5 mmol/L NH<sub>4</sub>OAC buffer (pH 5.0) for 10 min. The specimen was viewed and photographed under a fluorescence microscope (Nikon, Japan, 510 nm, blue light excitation).

**Relative content of aluminum in the root tip:** A fresh root tip (5 mm) was immersed in a solution of 0.2% hematoxylin and 0.02% KIO<sub>3</sub>, dyed for 15 min at room temperature, and immersed in distilled water for 15 min. After immersion, the 10 root tip was immersed in 200 μL of 1M HCl for 1 h, and a 490 nm full-wavelength on the microplate reader was then selected.

**Cell wall polysaccharide content:** Cell wall polysaccharide content was measured based on the method by Zhong and Lauchi (1993). Each treatment group had 50 apical material (1cm), from which pectin, HC1, and HC2 were fractionated. Uronic acids, with galacturonic acid as a standard reference, were measured based on the method by Taylor and Buchanan-Smith (1992).

**PME and APA activities:** PME activity was determined as follows: (1) Each treatment group had 30 apical material (1cm), extraction of PME according to Bordenave and Goldberg (1994); (2) measurement of PME activity based on the method of Richard (1994); and (3) APA activity was measured according to Cao (2002).

Calculate the average of fuzzy membership function according to the method of Zhou (2008), as follows:

Differences between treatments were separated by the least significant difference test at a 0.05 probability level. The figures were mapped using Origin 8.0.

## Results

### Aluminum accumulation on the cell wall of *T. kirilowii*

**root tip:** Al accumulation in the apical cell wall is a prerequisite that Al is toxic to plant root tip, as shown in Figs. 1 and 2. The fluorescence level indicated that certain amounts of Al accumulated in cells at the root tip. Al accumulation occurred mainly in the root tip of *T. kirilowii*; however, more Al accumulation was observed near the apical regions, where showed brighter under the fluorescence microscope. The fluorescence intensity of the Al-sensitive Pujiang *T. kirilowii* is stronger than the Al-tolerant Anguo *T. kirilowii* in the T<sub>1</sub> group. An important feature of plant sensitivity to Al toxicity is the higher levels of Al accumulation in the apical cell wall. The fluorescence intensity of both Pujiang and Anguo *T. kirilowii* were significantly reduced with the addition of exogenous SA compared with the T<sub>1</sub> group. Fluorescence images indicated that the Al-tolerant genotype accumulated lower amounts of Al in the root tip.

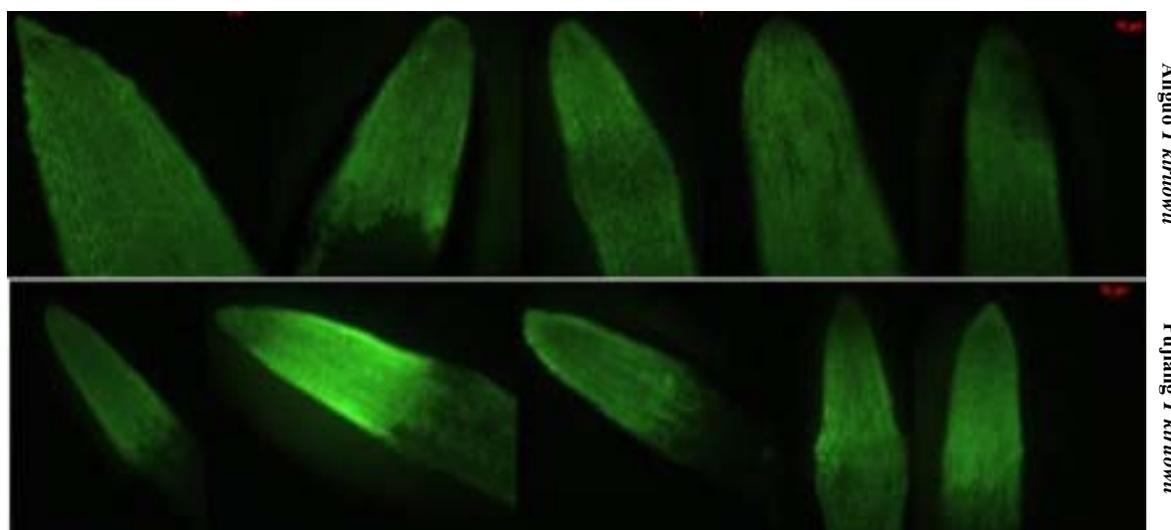


Fig. 1. Morin staining of *T. kirilowii* root (T<sub>0</sub> T<sub>1</sub> T<sub>2</sub> T<sub>3</sub> T<sub>4</sub> from left to right). T<sub>0</sub> (control), T<sub>1</sub> (AlCl<sub>3</sub> 800μmol/L), T<sub>2</sub> (AlCl<sub>3</sub> 800μmol/L, SA 10μmol/L), T<sub>3</sub> (AlCl<sub>3</sub> 800μmol/L, SA 30μmol/L), T<sub>4</sub> (AlCl<sub>3</sub> 800μmol/L, SA 50μmol/L). Each group has three independent experiments.

**Relative Al content in the root tip:** Fig. 2 showed that the Al content of *T. kirilowii* root tip of the T<sub>1</sub> group significantly increased compared with the T<sub>0</sub> group. Root relative Al content of the Al-sensitive Pujiang *T. kirilowii* was higher than that of the Al-tolerant Anguo *T. kirilowii*, indicating that the differences in Al resistance was correlate with differences in their potential to exclude Al from the root ape. The relative root aluminum content initially decreased, and then increased with the increase in exogenous SA concentration. The T<sub>3</sub> group exhibited the lowest relative aluminum content.

**Effect of exogenous SA on the root length of *T. kirilowii* under Al toxicity:** The most conspicuous Al toxicity response is the reduction in root growth (Foy, 1992). Fig. 3 showed that Al reduced the root growth of *T. kirilowii* significantly, whereas Pujiang *T. kirilowii* was inhibited more extensively. On the contrary, root lengths in the T<sub>2</sub>, T<sub>3</sub>, and T<sub>4</sub> groups of Anguo *T. kirilowii* were improved by 16.59%, 35.05%, and 8.40%, respectively, compared with that of the T<sub>1</sub> group. Meanwhile, root lengths in the Pujiang *T. kirilowii* groups were improved by 38.47%, 72.99%, and 45.39%, respectively. These results suggested that 10 μmol/L, 30 μmol/L, and 50 μmol/L of SA can alleviate the inhibition of root elongation caused by Al.

**Effect of exogenous SA on cell wall polysaccharide content of *T. kirilowii* under Al toxicity:** Cell walls are essential in the defense response of plants to heavy metals (Krzyszowska, 2011). The cell wall is the first and major site of Al accumulation, thus the loss of viscoelastic extensibility in the cell wall of an elongating cell is one of the important mechanisms of Al-induced inhibition of root elongation (Ma *et al.*, 2004). To test the effect of exogenous SA on cell wall polysaccharide content of *T. kirilowii* under Al toxicity, we treated *T. kirilowii* with different concentration of SA addition with 800 μmol/L AlCl<sub>3</sub>. Table 1 showed that the T<sub>1</sub> group had a higher pectin content compared with the T<sub>0</sub> group. Moreover, pectin content in the Pujiang *T. kirilowii* groups was higher than that in the

Anguo *T. kirilowii* groups. Pectin content in the T<sub>2</sub>, T<sub>3</sub>, and T<sub>4</sub> groups decreased compared with the T<sub>1</sub> group. Pectin content in the Anguo *T. kirilowii* groups decreased by 43.15%, 16.36%, and 10.43%, respectively, whereas that in the Pujiang *T. kirilowii* groups decreased by 51.14%, 41.19%, and 23.13% respectively. The T<sub>2</sub> group had the lowest pectin content. These results indicated that SA can effectively reduce pectin content in the root cell walls.

The increase of hemicellulose and cellulose content in cell wall rapidly inhibits root growth, and primarily causes a decline in cell elongation (Van & Kuraishi, 1994). Al increases the amount of HC1 and HC2 significantly (Table 1). The HC1 and HC2 contents of the root cell wall were higher in Pujiang *T. kirilowii* compared with that of the Anguo *T. kirilowii*, indicating the difference in Al tolerance between them. Compared with the aluminum treatment group, the HC1 and HC2 content of the exogenous SA treatment groups (T<sub>2</sub>, T<sub>3</sub>, T<sub>4</sub>) decreased significantly with the increase in SA concentration. The HC1 and HC2 contents in the Pujiang *T. kirilowii* groups decreased successively, whereas those in the Anguo *T. kirilowii* groups initially decreased, and then increased.

**Effect of exogenous SA on PME activity of *T. kirilowii* under Al toxicity:** Pectin methylation degree is an important factor that affects the properties of the cell wall. Cell wall pectins are the main Al-binding component of the cell wall because the trivalent Al cation is attracted to the negatively charged carboxyl groups of unmethylated pectins (Horst, 1995; Chang *et al.*, 1999). The increase of PME activity reduces the amount of pectin methyl ester, thus, pectin contains much free carboxyl, thereby increasing the binding capacity of aluminum. To investigate whether exogenous SA plays a role on PME activity of *T. kirilowii* under Al toxicity, we detected the PME activity of *T. kirilowii* treated with SA under Al toxicity. As shown in Fig. 4, PME activity was significantly higher in the Al-sensitive Pujiang *T. kirilowii* groups in the absence of Al. Al treatment resulted in an increase in PME activity in both *T. kirilowii* genotypes. PME activity decreased significantly after the addition of

exogenous SA, indicating that SA can improve the degree of pectin methylation. The difference in cell wall polysaccharide composition (Table 1) and PME activity (Fig. 4) results in the different ability to bind Al between Anguo and Pujiang *T. kirilowii*. The above results indicate that SA has an effect to increase the degree of pectin methyl ester under Al stress.

**Effect of exogenous SA concentration on APA activity of *T. kirilowii* under Al toxicity:** APA is a degreasing enzyme, and its activity is an indicator of skin reaction. Higher APA activity indicates more extensive degreasing of the skin membrane, leading to the damage of the

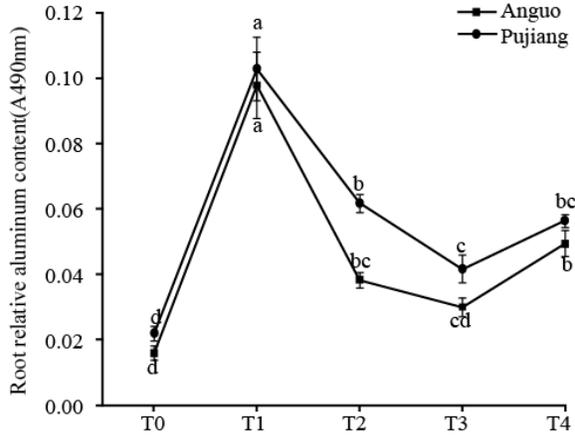


Fig. 2. Effect of exogenous SA concentration on root relative aluminum content of Anguo and Pujiang *T. kirilowii* under Al toxicity. T<sub>0</sub> (control), T<sub>1</sub> (AlCl<sub>3</sub> 800 μmol/L), T<sub>2</sub> (AlCl<sub>3</sub> 800 μmol/L, SA 10 μmol/L), T<sub>3</sub> (AlCl<sub>3</sub> 800 μmol/L, SA 30 μmol/L), T<sub>4</sub> (AlCl<sub>3</sub> 800 μmol/L, SA 50 μmol/L). Data are mean values ± SE of three in dependent experiments. Different lower case letters indicate statistically significant differences between different treatment groups ( $p < 0.05$ ).

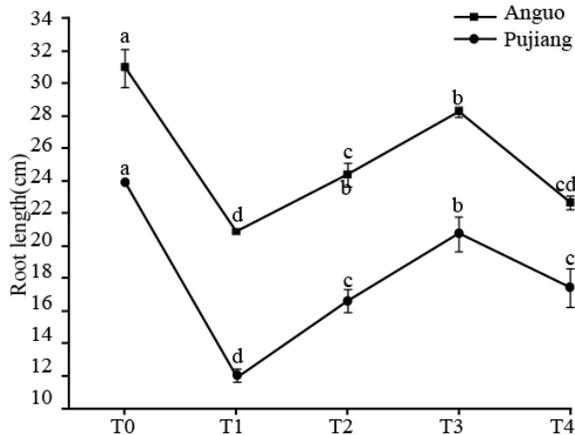


Fig. 3. Effect of exogenous SA concentration on root length of Anguo and Pujiang *T. kirilowii* Maxim under Al toxicity. T<sub>0</sub> (control), T<sub>1</sub> (AlCl<sub>3</sub> 800 μmol/L), T<sub>2</sub> (AlCl<sub>3</sub> 800 μmol/L, SA 10 μmol/L), T<sub>3</sub> (AlCl<sub>3</sub> 800 μmol/L, SA 30 μmol/L), T<sub>4</sub> (AlCl<sub>3</sub> 800 μmol/L, SA 50 μmol/L). Data are mean values ± SE of three in dependent experiments. Different lower case letters indicate statistically significant differences between different treatment groups ( $p < 0.05$ ).

membrane structure and function. To further explore the mechanism of how exogenous SA protect *T. kirilowii* from Al toxicity, APA activity was detected with treatment of different SA concentration under Al toxicity. As shown in Fig. 5, APA activity was significantly higher in the T<sub>1</sub> group compared with the T<sub>0</sub> group. APA activity in Anguo and Pujiang *T. kirilowii* groups improved by 64.39% and 106.06%, respectively, while the margin of increase was higher in the Al-sensitive Pujiang *T. kirilowii* groups. APA activities of T<sub>2</sub>, T<sub>3</sub>, and T<sub>4</sub> groups were lower than that of the T<sub>1</sub> group. APA activity was lowest at 30 μmol/L of SA in the Anguo *T. kirilowii* groups, and 50 μmol/L in the Pujiang *T. kirilowii* groups.

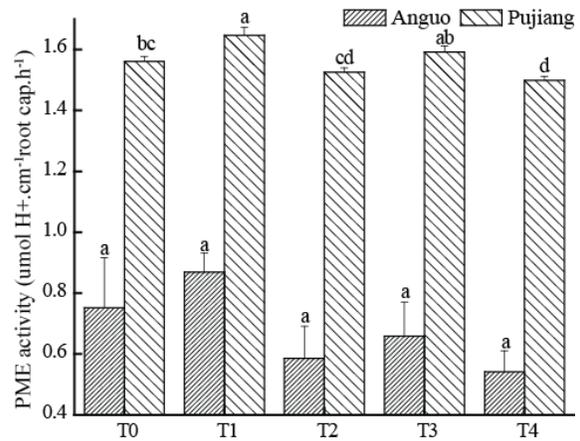


Fig. 4. Effect of exogenous SA on PME activity of Anguo and Pujiang *T. kirilowii* Maxim under Al toxicity. T<sub>0</sub> (control), T<sub>1</sub> (AlCl<sub>3</sub> 800 μmol/L), T<sub>2</sub> (AlCl<sub>3</sub> 800 μmol/L, SA 10 μmol/L), T<sub>3</sub> (AlCl<sub>3</sub> 800 μmol/L, SA 30 μmol/L), T<sub>4</sub> (AlCl<sub>3</sub> 800 μmol/L, SA 50 μmol/L). Data are mean values ± SE of three in dependent experiments. Different lower case letters indicate statistically significant differences between different treatment groups ( $p < 0.05$ ).

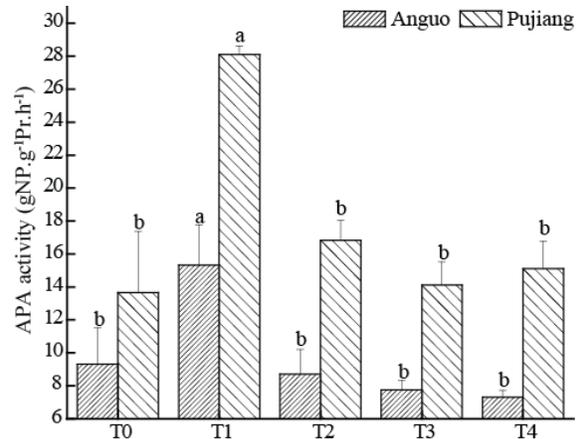


Fig. 5. Effect of exogenous SA on APA activity of Anguo and Pujiang *T. kirilowii* Maxim under Al toxicity. T<sub>0</sub> (control), T<sub>1</sub> (AlCl<sub>3</sub> 800 μmol/L), T<sub>2</sub> (AlCl<sub>3</sub> 800 μmol/L, SA 10 μmol/L), T<sub>3</sub> (AlCl<sub>3</sub> 800 μmol/L, SA 30 μmol/L), T<sub>4</sub> (AlCl<sub>3</sub> 800 μmol/L, SA 50 μmol/L). Data are mean values ± SE of three in dependent experiments. Different lower case letters indicate statistically significant differences between different treatment groups ( $p < 0.05$ ).

**Table 1. Effect of exogenous SA on cell wall polysaccharides of Anguo and Pujiang *kirilowii* under Al toxicity.**

Genotype	Treatment	Pectin content (mg.g <sup>-1</sup> )	Hemicellulose 1 content (mg.g <sup>-1</sup> )	Hemicellulose 2 content (mg.g <sup>-1</sup> )
Anguo	T <sub>0</sub>	0.511 ± 0.10a	1.333 ± 0.01b	1.180 ± 0.11ab
	T <sub>1</sub>	0.722 ± 0.08a	1.460 ± 0.01a	1.418 ± 0.10a
	T <sub>2</sub>	0.410 ± 0.10a	1.054 ± 0.02c	1.237 ± 0.02ab
	T <sub>3</sub>	0.604 ± 0.04a	0.881 ± 0.06d	1.045 ± 0.03b
	T <sub>4</sub>	0.646 ± 0.13a	1.043 ± 0.06c	1.260 ± 0.02ab
Pujiang	T <sub>0</sub>	0.968 ± 0.06b	1.464 ± 0.06c	1.900 ± 0.12b
	T <sub>1</sub>	1.282 ± 0.08a	2.719 ± 0.07a	2.421 ± 0.12a
	T <sub>2</sub>	0.627 ± 0.01c	2.432 ± 0.10ab	2.230 ± 0.17ab
	T <sub>3</sub>	0.754 ± 0.16c	2.198 ± 0.08b	2.124 ± 0.06ab
	T <sub>4</sub>	0.986 ± 0.09b	1.741 ± 0.131c	1.953 ± 0.06b

Note: T<sub>0</sub> (control), T<sub>1</sub> (AlCl<sub>3</sub> 800μmol/L), T<sub>2</sub> (AlCl<sub>3</sub> 800μmol/L, SA 10μmol/L), T<sub>3</sub> (AlCl<sub>3</sub> 800μmol/L, SA 30μmol/L), T<sub>4</sub> (AlCl<sub>3</sub> 800μmol/L, SA 50μmol/L). Data are mean values ± SE of three independent experiments.

Different letters indicate significant differences with the varieties of the same column data ( $p < 0.05$ )

**Table 2. Membership function mean of aluminum tolerance indicators and comprehensive evaluation.**

Treated	Anguo				Pujiang			
	CK(T <sub>0</sub> )	SA	SA	SA	CK(T <sub>0</sub> )	SA	SA	SA
		10μmol/L	30μmol/L	50μmol/L		10μmol/L	30μmol/L	50μmol/L
Mean	0.4910	0.4928	0.7115	0.3774	0.7687	0.2449	0.4471	0.4776
Sequence	3	2	1	4	1	4	3	2

**Table 3. Genetic identity and genetic distances among the nine genotypes of *T. kirilowii* Maxim.**

popID	shuyang	Sanmenxia	Wuyi	Linyi	Quzhou	Changxing	Pujiang	Anguo	Anqing
shuyang	1.000	0.7273	0.6608	0.6701	0.6624	0.6646	0.6746	0.7237	0.7037
Sanmenxia	0.3184	1.000	0.7574	0.6487	0.7241	0.7363	0.7583	0.6185	0.5877
Wuyi	0.4143	0.2779	1.000	0.6923	0.7521	0.7225	0.7634	0.5965	0.5950
Linyi	0.4003	0.4327	0.3677	1.000	0.6914	0.6234	0.6220	0.6555	0.6133
Quzhou	0.4119	0.3228	0.2849	0.3690	1.000	0.8791	0.7761	0.6220	0.6104
Changxing	0.4085	0.3061	0.3250	0.4726	0.1288	1.000	0.8652	0.6161	0.6153
Pujiang	0.3936	0.2766	0.2700	0.4728	0.2535	0.1448	1.000	0.5951	0.5831
Anguo	0.3234	0.4804	0.5166	0.4224	0.4749	0.4843	0.5191	1.000	0.8400
Anqing	0.3514	0.5316	0.5193	0.4890	0.4937	0.4857	0.5394	0.1743	1.000

Note: Genetic similarities are listed above the diagonal, and genetic distances are listed below the diagonal

**Aluminum tolerance analysis:** This experiment used fuzzy membership function method to analyze the seven measured indicators, and comprehensive evaluated the ability of three salicylic acid concentrations to alleviate aluminum toxicity. As shown in Table 2, to Anguo *T. kirilowii*, the capacity of SA to alleviate Al toxicity is 30 μmol/L > 10 μmol/L > 50 μmol/L, and Pujiang *T. kirilowii* is 50 μmol/L > 30 μmol/L > 10 μmol/L. In conclusion, low concentrations have a better effect to Al tolerance genotype, and high concentrations have a better effect to Al-sensitive genotype. And 30 μmol/L is an optimal concentration to alleviate the aluminum toxicity of Al-tolerant and Al-sensitive genotype.

**Genetic relationship among genotypes:** We use ISSR marker to study the genetic distance and genetic identity between Anguo and Pujiang *T. kirilowii*. As shown in Table 3, the genetic distance among genotypes of *T.*

*kirilowii* ranged from 0.1288 to 0.5394. The highest genetic distance was between the genotypes of Anguo and Anqing (0.5394), whereas the smallest was between the genotypes of Quzhou and Changxing (0.1288). The genetic distance between Anguo and Pujiang was 0.5191. Genetic identity ranged from 0.5831 to 0.8791, whereas between Anguo and Pujiang was 0.5951. The genetic distance between the genotypes of Anguo and Pujiang was high, but the genetic identity was lower.

Unweighted pair group method using arithmetic averages (UPGMA) was performed to estimate the phylogenetic relationships among the nine genotypes (Fig. 6). The nine genotypes were divided into two clusters, showing their geographical distribution. Al-tolerant Anguo *T. kirilowii* and Al-sensitive Pujiang *T. kirilowii* belonged to different clusters, and the phylogenetic relationship between them was far.

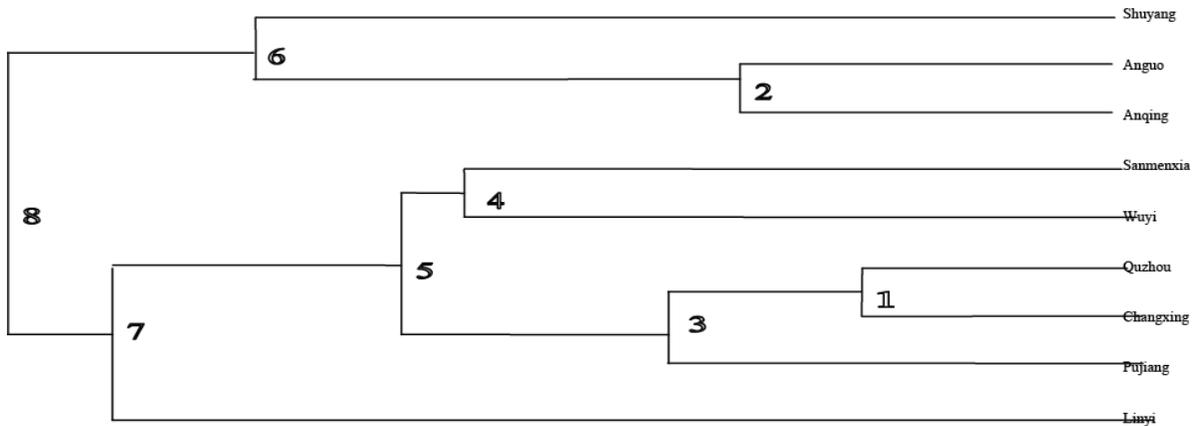


Fig. 6. UPGMA dendrogram for the genotypes of *T. kirilowii* based on Nei's genetic distance.

## Discussions

Al-dependent changes in cell wall viscosity and elasticity are involved in the inhibition of root growth (Ma & Shen, 2004). Extensibility of the cell wall is an important factor in the regulation of cell elongation in plant tissues (Sakurai, 1991). Cell wall synthesis is associated with the mechanism of Al tolerance (Taylor, 1991). The study of Yang (2008) suggests that cell wall polysaccharides are important in excluding Al, specifically from the rice root apex. This study confirmed that different concentrations of SA and aluminum affect the pectin, HC1, and HC2 contents of the root, thereby affecting the extensibility of the cell wall and root growth. Exogenous SA can alleviate aluminum toxicity to a certain extent, which is consistent with the results of a previous study (Liu, 2011).

Cell wall pectins are the main Al-binding component of the cell wall, suggesting that binding of Al to pectin matrix is an important step in Al toxicity expression. Al binding on pectin carboxyl groups will make cell wall hardening and extension process distorted. Pectins are secreted into the wall as highly methylesterified forms. Subsequently, they can then be modified by pectinases, such as PME (Micheli, 2001). Root cell wall binding capacity for Al largely depends on the amount of negatively charged free carboxyl of pectin. It is not only related to the cell wall pectin content, but also depends on pectin methylation degree. Colzi (2012) found that the degree of pectin methylation was higher in the tolerant genotype compared with the sensitive ones. The current study showed that Al-sensitive genotypes of Pujiang *T. kirilowii* have higher pectin content and PME activity in the T<sub>1</sub> group, indicating that Al stimulates the cell secretion of pectin and PME. The improvement of PME activity promotes pectin demethylation, such that pectin have more free carboxyl sites, more aluminum binding sites, and subsequently with enhanced ability to combine with Al<sup>3+</sup>. Al causes accumulation of cell-wall polysaccharides, especially hemicellulosic ones in the growing region (Tabuchi & Matsumoto, 2001). Cell wall composition of the root apex was measured in this study. Results showed that cell wall polysaccharides, including pectin, HC1, and HC2 were significantly higher in Al-sensitive Pujiang *T.*

*kirilowii*. The root cell wall extension was blocked, and the root length was decreased in Pujiang *T. kirilowii*. The Al-sensitive Pujiang *T. kirilowii* root length was inhibited greater than the Al-tolerant Anguo *T. kirilowii*.

APA plays an important role in terms of phosphoric acid secretion and plant aluminum tolerance. APA catalyzes phosphate monoester decomposition and facilitates the release of phosphate (Pi) that can form insoluble complexes with Al, thereby reducing Al toxicity. Several studies have shown that APA activity mainly increased in fragile organization. In the T<sub>1</sub> group, APA activity was significantly increased, indicating more phosphate monoester was decomposed. The combination of Al with the cell membrane results in cell membrane extensive degreasing, affecting its structure and function. Obviously, this is also one of the reasons for the reduced root length of the two *T. kirilowii* genotypes.

The above result suggests that Al mainly binds to the root cell wall, and Al content in the cell wall may be significantly related to different Al resistance. In the present study, inter-simple sequence repeat (ISSR) marker was used to examine the genetic distance, genetic identity, and phylogenetic relationships. Genetic distance between the genotypes of Anguo and Pujiang was higher, and genetic identity was lower. Clustering results of the phylogenetic relationship showed that Al-tolerant Anguo *T. kirilowii* and Al-sensitive Pujiang *T. kirilowii* belonged to different clusters. The phylogenetic relationship between them was far, indicating a certain correlation between differences in Al tolerance, genetic distance, genetic identity, and phylogenetic relationship of *T. kirilowii* genotypes.

Cell wall polysaccharide content and PME activity were reduced significantly after the addition of 10 μmol/L, 30 μmol/L, and 50 μmol/L of exogenous SA in the corresponding groups (T<sub>2</sub>, T<sub>3</sub>, T<sub>4</sub>), such that the free carboxyl group (cation adsorption sites) on the pectin, binding capacity with aluminum, and root relative aluminum content were reduced, as revealed by Morin staining. Thus, exogenous SA prevented cell wall structure and function changes and played a role in alleviating Al toxicity at different degrees. APA activity decreased after the addition of SA, indicating that SA weakens the effect of Al on the cell skin membrane and eases the inhibition of root growth.

Fluorescence detection also proved that fluorescence intensity became weaker after the addition of SA. Macro performance was the recovery of root growth. According to the analysis of fuzzy membership function, we can conclude that low concentrations have a better effect to alleviate aluminum toxicity of Al-tolerant genotype, and high concentrations to Al sensitive genotype. 30 mol/L is an optimal concentration in this experiment.

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