

Original Research

Aluminum Exposure Effect on Cell Wall Pectin Methyl Esterification in Alfalfa with Different Aluminum Tolerance

Na Jiang¹, Jian Ren¹, Yanqun Zu², Wenjun Sun¹, Xiangli Ma^{1*}, Yufen Bi¹

¹College of Animal Sciences and Technology, Yunnan Agricultural University, Kunming 650201, China

²College of Resources and Environment, Yunnan Agricultural University, Kunming 650201, China

Received: 11 January 2022

Accepted: 25 April 2022

Abstract

Aluminum (Al) toxicity is one of the factors limiting alfalfa production on acid soils. This study aims to investigate the different mechanisms in growth, Al content, pectin content, pectin methylesterase (PME) and the degree of pectin methyl-esterification (DM) in the two root segments of different ecotypes alfalfa during the time of Al stress. It was found interesting that lower root elongation and higher Al content were observed in the Al-sensitive ecotype compared to the Al-resistant ecotype of alfalfa. The Al accumulation increased with the increased of stress time. Meanwhile, the pectin and PME activity of cell wall significantly increased. While DM in Al-sensitive ecotype decreased, more free pectin acid residues on the cell wall of Al-sensitive ecotype. The Al content was higher in 0-5 mm root segments and the cell wall for both materials. The increase in pectin content and PME activity along with the decrease in DM in this segment could determine the alfalfa sensitivity to Al more effectively. The correlation analysis showed that there was a relationship between Al content, pectin content, PME activity of cell wall and DM. The different temporal dynamics and spatial differences caused the increase of Al content, pectin content and cell wall PME activity, changes of DM under Al toxicity cooperates to determine the difference of Al resistance that confers different ecotypes of alfalfa.

Keywords: alfalfa, aluminum, root, cell wall, pectin, pectin methyl-esterification

Introduction

In recent years, with the development of modern aluminum (Al) industry, Al alloys and their compounds have been widely used in all fields of social life, resulting in Al pollution. Moreover, sludge is more and

more used in farmland fertilization, and flocculants such as Al salt are widely used in sewage treatment, which increases the Al content in soil. Southwest China is mostly red soil, which intensifies the role of Al toxicity due to the acidic characteristics of red soil. Alfalfa (*Medicago sativa* L.), a nutrient rich forage legume grass, is also acknowledged as the “king of forage” worldwide due to its wide planting, strong adaptability, and high grass yield. It is also abundant in germplasm

*e-mail: xfmangli@126.com

resources. 92 alfalfa varieties, including 44 bred, 20 local, 5 wild cultivated, and 23 introduced varieties in 2017 [1] have been approved and registered in China. However, nowadays a very few varieties could adapt to the acidic soil as the Al poisoning of acid soil inhibits the alfalfa growth [2]. Notably, when the soil pH less than 5, the octahedral hexahydrate Al (H_2O)₆³⁺, referred as Al³⁺, becomes soluble in the soil solution and might interfere with a wide range of physical and cellular process, thereby inhibiting plant growth and functions [3].

Plant root growth inhibition is the first symptom of Al poisoning in plants, occurring within minutes to hours of Al exposure (micro molar concentration) [4]. The root elongation inhibition is usually employed for Al resistance screening in plant species and cultivars, which is positively correlated with Al accumulation [5].

Accumulating evidence have revealed that the cell wall polysaccharide composition is a novel Al resistance mechanism due to the correlation of cell wall binding capacity with Al accumulation capacity [6]. It has been reported that, more than 70% of Al could bind on the cell wall of roots in wheat [7-9]. Most of the Al absorbed is accumulated by the cell wall and the apoplast of root system plays a vital role in Al-resistance or sensitivity plants. Recent studies have proposed that Al binding to the cell wall is prerequisite for Al toxicity to plants [10]. Pectin is known as the essential anionic polysaccharide of cell wall polysaccharide composition and allow Al³⁺ to easily combine with pectin [11]. However, this phenomenon is still unexplored. Pectin accumulation in plants leads to cell wall hardening and cell growth inhibition, resulting in root growth inhibition. In most plant cells, the initial pectin synthesis is highly esterified, followed at demethylation under pectin methyl-esterase activity (PME) to meet the functional needs of the cell wall [12]. Al tolerance of varieties is closely related to pectin content and the degree of pectin methyl-esterification (DM) [5]. Studies have proved that about 79.60% to 87.33% of Al is accumulated by the cell wall, and the binding ability of the cell wall to Al mainly depends on the pectin content in the cell wall and DM [13, 14].

A few studies on the Al tolerance mechanism has been reported. Most of the studies focus on the secretion of organic acids, antioxidant enzymes, and growth performance [15, 16]. It should be noted that Al removal from the root tip through the exudation of root organic acids is the most important mechanism of Al resistance. The difference in pectin content and DM affected the Al resistance in different genotypes of *Brassica chinensis* L. (pakchoi) [17]. These studies were mostly focused on crops. Therefore, it is necessary to explore the difference of Al adsorption characteristics of root tip cell wall in different tolerant types and the regulatory mechanisms of cell wall DM against Al tolerance in alfalfa.

In our previous studies, 27 alfalfa resources were collected and identified in Yunnan, China [18, 19].

In this study, two Yunnan escaped alfalfa (Al-resistant ecotype NO.12 and Al-sensitive ecotype NO.21) were selected as study materials to determine the changes in pectin content, PME activity, and DM in the cell walls of root tips about 0-5 mm, 5-10 mm under different Al treatment times. To investigate the time and space of different ecotypes of alfalfa cell wall pectin methylation and its regulatory mechanism under Al stress.

Material and Methods

Two alfalfa ecotypes, No.12 with strong Al-tolerance and No.21 with Al-sensitivity, were used in this study. Seeds were collected from the Yunnan-Guizhou Plateau, southwestern China and the Al tolerance were evaluated in the early seedling stage [20].

Alfalfa seeds were sterilized for 20 min soaked in deionized water overnight and then germinated in the dark at 25°C. Petri dishes were used to raise seedlings in artificial greenhouse and hydroponically with Hogland nutrient solution., where the light and temperature was 14 h and 25°C, and the relative humidity was controlled at 70%. After reaching 1cm of height after germination, the seedlings were selected for uniformity and then treated with 10ml 50 mmol L⁻¹ AlCl₃ solution at pH 4.5 (n = 3). After 0, 6, 12, and 24 h of Al stress, the indexes of the root segments (0-5 mm and 5-10 mm root tip segments) were determined.

The root length of 30 replicates alfalfa was measured by ruler after treating with 0, 6, 12, and 24 h with and without Al, respectively. The root elongation was calculated using the following formula:

The cell wall of alfalfa was extracted, as described by Luo et al. [21]. Briefly, after treating for 6, 12, and 24 hours, the root segments at 0-5 mm and 5-10 mm of two materials were cut with sterilized blades and stored in a refrigerator at -80°C to extract the cell wall. Then, the roots were ground into powder by adding liquid nitrogen. After adding 70% ethanol into the powder, the solutions were ice-bathed for 20 min, centrifuged at 8000rpm for 10 min, and the supernatant was discarded. Later, acetone, methanol: chloroform (1:1 [v:v]), and methanol were added and the above steps were repeated to obtain the final precipitate of the cell wall samples. The cell wall was dried in an oven at 60°C (60°C can be used for the analysis of cell wall components, but not for the determination of PME activity) and stored at 4°C.

Al content was determined according to the procedure of Safari et al. [22]. After 6, 12, and 24 h of Al treatment, the samples at 0-5 mm and 5-10 mm of alfalfa root tips were cut with sterilized blade and placed in an oven. Later, these samples were dried at 60°C. Weigh 0.5g of sample into the digestion tank, add 10ml nitric acid, put it on the microwave digestion plate for digestion. After cooling, transfer it into a triangular flask, put it on the electric heating plate to drive out the acid, take it down and cool it when the solution is nearly

dry, dissolve the treated sample with an appropriate amount of deionized water, and then fix the volume to 10ml. The Al content was determined by inductively coupled with plasma atomic emission spectrometry (ICP-AES; Thermo Jarrel Ash, San Jose, CA, USA).

Cell wall pectin was extracted, as described by Luo et al. [21]. Pectin in cell wall powder was extracted by 4 mL of 0.5% ammonium oxalate buffer (containing 0.1% NaHB₄, pH = 4.0). The supernatant was obtained by centrifugation at 12000 rpm for 10 minutes and stored in a refrigerator at 4°C for further analysis.

The galacturonic acid (GalA) content was determined by hydroquinone spectrophotometry according to the methods of Wilson [23]. The pectin uronic acid content was determined by plotting a standard curve using galacturonic acid (GalA) as the standard substance.

The extraction and determination of PME activity was performed according to the methods of Richard et al. [5]. Different root segments (40 root segments for one replicate) were thoroughly ground by adding 150 µL of PME activity extract solution (containing 0.1 mol L⁻¹ citrate acid, 0.2 mol L⁻¹ Na₂HPO₄, 1.0 mol L⁻¹ NaCl, pH = 5.0) and a small amount of liquid nitrogen in the grinding bowl. Later, the sample was put into the centrifuge tube and placed in the ice bath for 1 h after full oscillation for 20 min. The supernatant was collected and extracted by centrifugation at 12000 rpm for 10 min at 4°C then stored at -20°C. The extracted PME activity samples were added to a 4 mL substrate solution (0.5% (w:v) citrus pectin, 0.2 mol L⁻¹ NaCl, 0.15% (w:v) methyl red, pH 6.8) and heat at 37°C for 2 hours. The absorbance was measured at a wavelength of 525 nm.

Spectrophotometric method was employed to determine DM [5, 21]. 50 µL of NaOH (1.5 mol L⁻¹) was added to the same pectin extract (100 µL) (described in 2.7 section) for saponification reaction, which lasted for 30 min at room temperature. Later, (the volume of HCl needs to be adjusted to ensure that the pH of the solution after the reaction is 7.5-8.0) 1.5 mol L⁻¹ of HCl was added to neutralize the excess alkali. The mixed solution was reacted for 20 min at 30°C by adding 100 µL of Tris-HCl, 40 µL of MBTH, and 10 µL of 0.01 units µL⁻¹ AO. Afterward, 200 µL of mixed solutions with 5 mg mL⁻¹ of ammonium ferric sulfate, and amino sulfonic acid were added to react at room temperature for 20 min. Finally, deionized water was added until the total volume was 2 mL, and A620 was determined. Methanol produced by saponification was determined by spectrophotometric method improved by Yang et al. [24]. The standard curve was prepared using methanol/formaldehyde as the standard substances. The calculation formula was as follows:

$$DM(\%) = \frac{\text{Methanol}}{C_{\text{Glucuronic acid}}} \times 100$$

DM: The degree of methylation, %, CMethanol: Determination of the concentration of methyl esterified

uronic acid, i.e., the amount of methanol produced after saponification, µmol L⁻¹, CGlucuronic acid: Pectin content, µmol L⁻¹.

Statistical analyses were performed using the statistical software package for social science (SPSS) version 20.0 analysis of variance (ANOVA), and correlation analysis followed by Duncan's multiple comparisons were performed to determine the Al stress effects. $P < 0.05$ was considered as significant difference.

Results and Discussion

Results

Root elongation was adopted to compare the Al sensitivity in different alfalfa ecotypes (Fig. 1). Root elongation was inhibited by Al toxicity in both the ecotypes, and root elongation slowed down after 12 hours of Al stress. Meanwhile, the root elongation in Al-resistant (No.12) was higher than Al-sensitive (No.21). For instance, under 0, 6, 12, 24h Al stress, the root elongation of No. 12 was 1.45, 1.81 and 1.54 times of that of No. 21, respectively.

The Al contents in different root segments and cell walls of two experimental ecotypes showed an accumulation tendency with the increase of Al stress time (Fig. 2). The Al content increased in most root segments and cell walls when exposed to Al stress, particularly at 0-5 mm root segments. The Al contents in different root segments and cell walls of Al-sensitive were significantly higher than those of Al-resistant. No significant difference was observed in Al content between 12 h and 24 h treated Al-sensitive, except for the 0-5 mm root segments cell wall ($P > 0.05$). Similarly, no significant difference was observed in Al-resistant between 6 h and 12 h for 0-5 mm root segments cell wall. Moreover, the Al contents in the root of the two alfalfa ecotypes were significantly lower than the cell

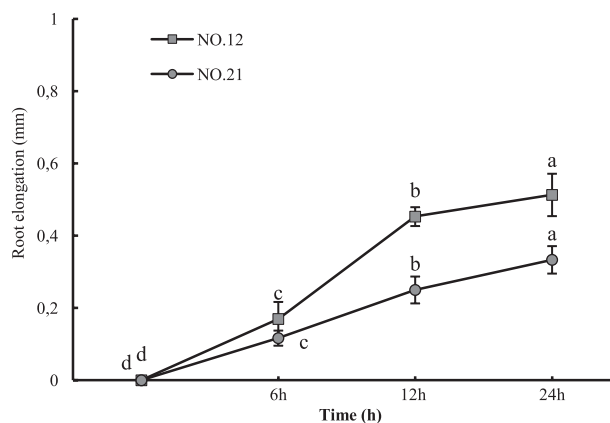


Fig. 1. Root elongation of two alfalfa ecotypes under different Al treatment time. Bars represent the mean±S.E (n = 30). Those bars that do not share the same letters are significantly different at $P < 0.05$.

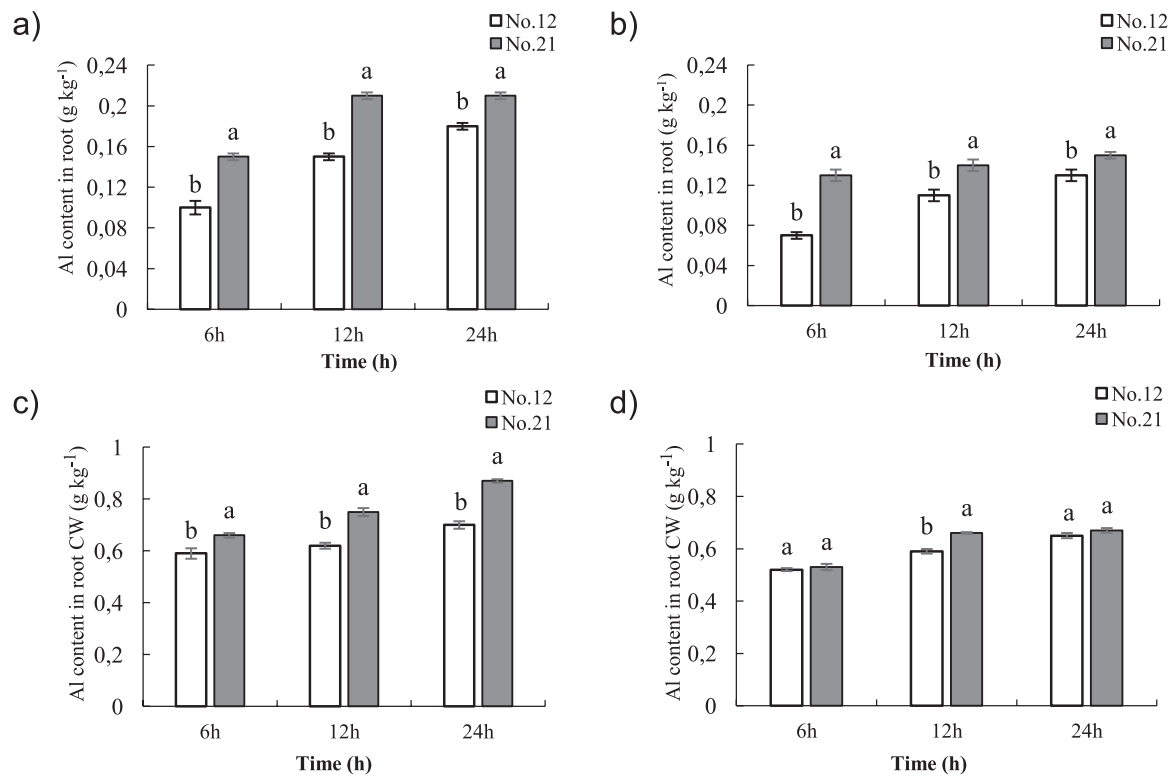


Fig. 2. Al contents in different root segments of two alfalfa ecotypes exposed to 6 h, 12 h and 24 h.

Al content in 0–5 mm roots (a), Al content in 5–10 mm roots (b), Al content in the cell wall (CW) of 0–5 mm roots (c); Al content in the CW of 5–10 mm roots (d). Bars represent the mean±S.E (n = 3). Different letters represent significant differences using the T-test at ($P < 0.05$).

wall, and reached the maximum level after 24 h stress, when the Al content of the cell wall was far more than 4 times than the root segments.

With the increase of Al stress, the pectin contents of the two ecotypes increased continuously, while the cell wall pectin contents in different root segments of Al-resistant were significantly lower than the Al-sensitive ($P > 0.05$) (Fig. 3). Furthermore, the accumulation of cell wall pectin in 0–5 mm root segment was higher than that in 5–10 mm root segment ($P < 0.05$), except for

the treatment in Al-resistant under Al stress for 12 h ($P > 0.05$). When exposed to Al stress for 24 h, the cell wall pectin contents in 0–5 mm of the two ecotypes were significantly higher than the initial stage of Al stress (6 h).

The PME activity of root segments decreased significantly with the increase of Al stress time ($P < 0.05$), and reached the highest within a short time (6 h) of Al treatment (Fig. 4a). The PME activity in root segment of Al-sensitive were higher than

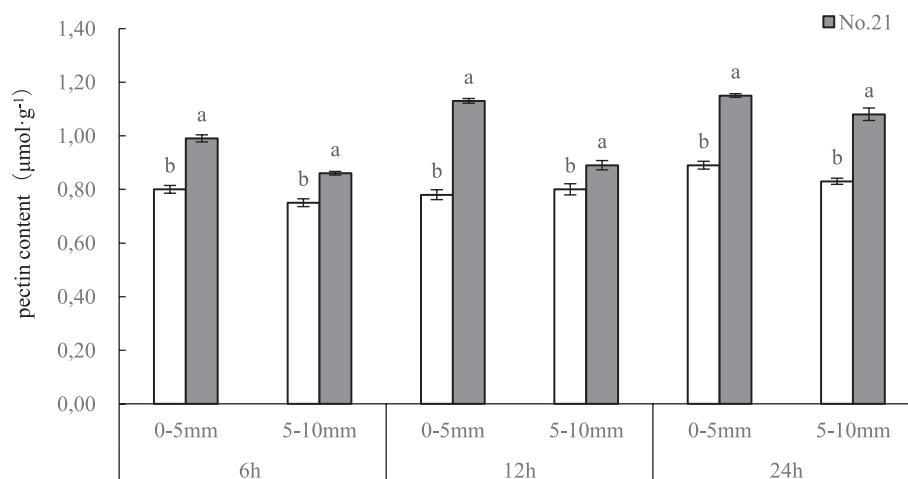


Fig. 3. Cell wall pectin content in different root segments (0–5 mm, 5–10 mm) of alfalfa treated with Al at 6 h, 12 h and 24 h.

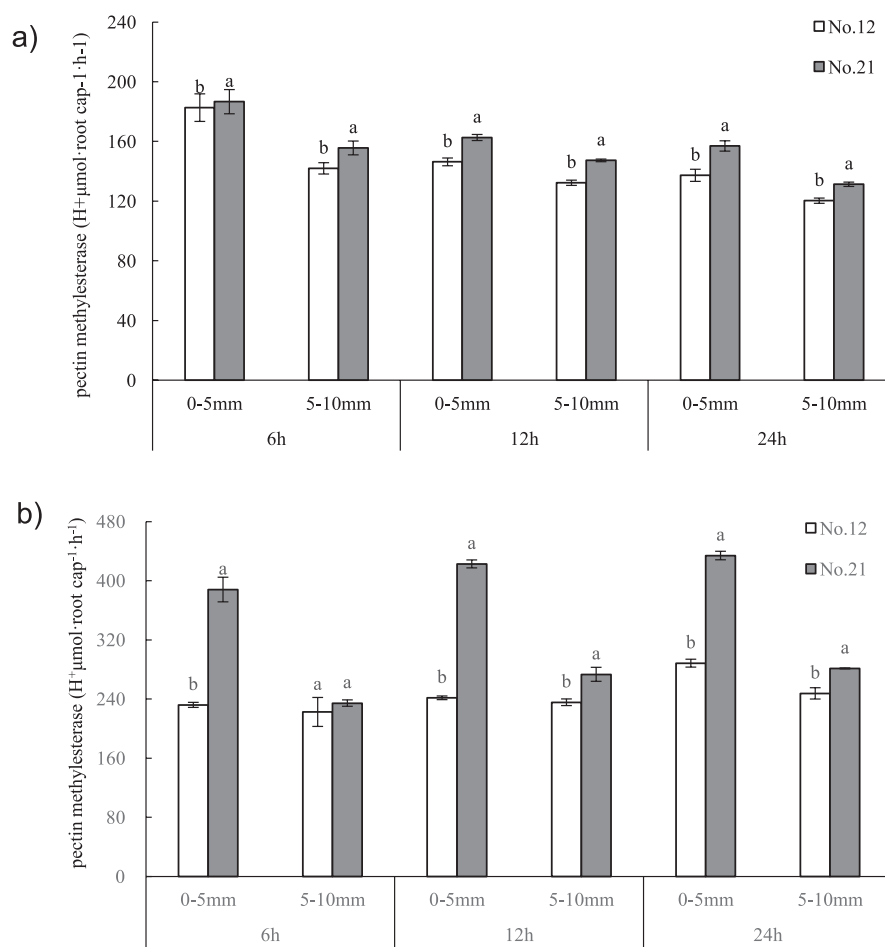


Fig. 4. Pectin methylesterase (PME) activity in different root segments (0-5 mm, 5-10 mm) of alfalfa treated with Al at 6 h, 12 h and 24 h.

the Al-resistant. Of them, the PME activity of 0-5 mm root segment was higher than 5-10 mm root segment.

The PME activity of cell wall exhibited an increasing trend, and the difference decreased by the increasing Al stress time (Fig. 4b). The difference of PME activity in the cell wall of 0-5 mm root segment between the two tested ecotypes was the highest at 12 h. The PME activity of Al-sensitive was 1.75 times

higher than the Al-resistant. After 12 h, the difference reduced significantly. The PME activity in the cell wall of the Al-sensitive was also higher than the Al-resistant, and the PME activity in the cell wall of 0-5 mm root segment was higher.

For both the tested ecotypes, the DM in root apex was prominently affected by the Al stress ranging from 27 to 46% in different segments (Fig. 5). However,

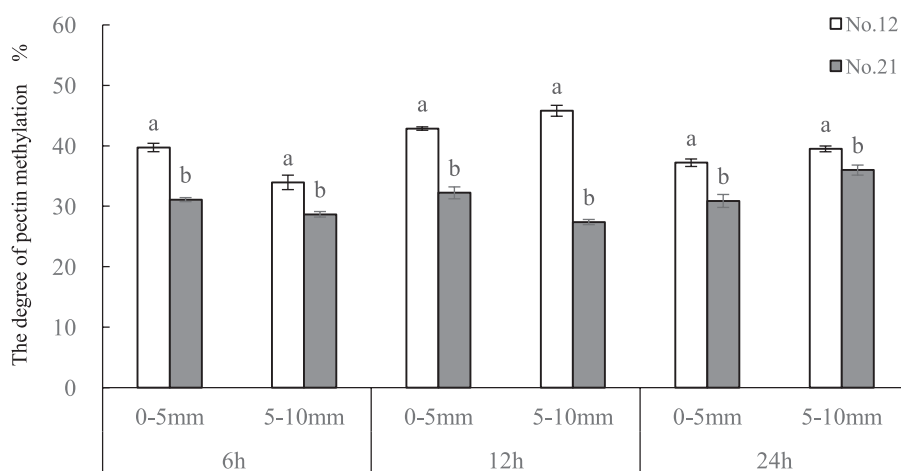


Fig. 5. The degree of pectin methylation in different root segments (0-5 mm, 5-10 mm) of alfalfa treated with Al at 6 h, 12 h and 24 h.

a significant difference was observed between two ecotypes. It was higher in Al-resistant ecotypes than the Al-sensitive ecotypes. With the increasing Al stress, the DM in the cell wall of most treatments increased at first and then decreased, whereas the DM in cell wall at 5-10 mm root segment of Al-resistant decreased at first and then increased. Moreover, a significant the difference between DM of different root segments at the same stress time was observed. At the initial stage of stress, the anterior root segment (0-5 mm) was higher than the posterior root segment (5-10 m). However, in the later stage of stress, the posterior root segment was higher than the anterior root segment.

The analysis of the correlation between Al stress time and various physiological indicators of alfalfa (Table 1) shows that stress time is extremely significantly correlated with root elongation, Al content, cell wall Al content and PME activity, and significantly correlated with pectin content.

In the analysis of the correlation between different root segments of alfalfa and indicators (Table 2), the root segment and Al content, cell wall Al content, PME activity, and cell wall PME activity were extremely significantly correlated, but didn't reach a significant level with pectin PME.

The correlation analysis of different indexes under Al stress (Table 3) showed that Root elongation was significantly negatively correlated with PME, and significantly positively correlated with DM. Al content and cell wall Al content were positively correlated

with pectin and cell wall PME. However, Pectin was positively correlated with PME and negatively correlated with DM. PME was positively correlated with cell wall PME, while cell wall PME was negatively correlated with DM.

Discussion

The initial symptom of Al toxicity in plants is rapid inhibition of root elongation. Al-induced root elongation inhibition and Al accumulation were employed to determine Al-resistant and Al-sensitive of pea cultivars, as described by Li et al. [5]. The study results demonstrated that the root elongation of Al-resistant was higher than Al-sensitive and the exposure of root apex to Al could inhibit alfalfa root. It was confirmed that No.12 was Al-resistant ecotype and No. 21 was Al-sensitive ecotype. Since the root tip acts as a vital system for Al accumulation in higher plants, the Al tolerance is closely related to the Al content in root tip. In the study of two different Al-resistance watermelon cultivars, a higher Al content was identified in the root of tolerant cultivars than sensitive cultivars [25]. Our result was consistent with the above results. Plant roots produce different degrees of Al accumulation under Al stress at different times. 79.60% to 87.33% of Al is accumulated by the plant cell wall [13], which reduces the availability of cell wall relaxing, thereby increasing the cell wall rigidity and thickness, and inhibiting root elongation [26]. Yang et al. [24] further proved that cell

Table 1. Correlation analysis between Al stress time and physiological indexes of Alfalfa.

	Root elongation	Al	CW Al	Pectin	PME	CW PME	DM
Time	0.744**	0.482**	0.626**	0.417*	-0.610**	0.228	0.145

** indicate a significance level of $P < 0.01$, * indicate a significance level of $P < 0.05$. The same as.

Table 2. Correlation analysis of different root segments and physiological indexes of Alfalfa.

	Al	CW Al	Pectin	PME	CW PME	DM
Root segments	-0.594**	-0.514**	-0.328	-0.622**	-0.563**	-0.207

Table 3. Correlation analysis of physiological indexes of Alfalfa under Al Stress.

	Root relongation	Al	CW Al	Pectin	PME	CW PME	DM
Root relongation	1						
Al	0.215	1					
CW Al	0.278	0.869**	1				
pectin	-0.101	0.810**	0.792**	1			
PME	-0.732**	0.164	0.082	0.215	1		
CW PME	-0.136	0.804**	0.814**	0.858**	0.414*	1	
DM	0.640**	-0.206	-0.107	-0.552**	-0.254	-0.416*	1

wall Al content in rice accounted for more than 80% of the total content. The results showed that Al content of Al-sensitive alfalfa was higher than Al-resistant, indicating a negative correlation of alfalfa Al tolerance with the root tip or cell wall Al content. This result was consistent with the previous studies on wheat [27].

The composition and distribution of pectin can change the extensibility, hardness, porosity, and adhesion of plant cell walls, especially Al binds to the non-esterified pectin in the apoplast [28]. Silva et al. [29] found that Al could easily combine with the cell wall pectin to form pectin complex, thereby increasing the hardness of cell wall and hindering cell elongation. The present studies on cell wall pectin, PME, and DM are mostly focused on rice, barley, corn, and other food crops [2, 30, 31], but limited studies are available for alfalfa. Generally, Al toxicity in plant cells induces rapid pectin synthesis. Pectin is synthesized in the Golgi matrix and secreted into the cell wall in the form of high methyl esterification, while DM can greatly affect the fixation ability of plants to metal cations. The plant tolerance to Al is closely related to the cell wall pectin and DM. However, the non-methyl esterified cell wall pectin can only be completed by PME during the demethylation and esterification of pectin molecules. Non-methyl esterified pectin contains a large number of carboxyl groups (-COOH) and is the primary source of negative charge in the cell wall, while Al toxicity is mostly available in the form of trivalent Al^{3+} in the acidic soil. The degree of modification of cell wall DM can significantly affect the binding ability of pectin to Al. In this study, the root tip pectin content and PME activity of the two alfalfa ecotypes were significantly lower than Al-sensitive, while it was contradictory for DM. A study on DM in root tip cell wall revealed that the number of Al-resistant ecotypes were significantly higher than the Al-sensitive ecotypes. This was contradictory to the PME result. Li et al. [5] studied two pea varieties with different Al-resistance levels; the results showed that the DM in root tip cell wall of Al-resistant pea was higher than the Al-sensitive pea. The present experiment were consistent with those of Li et al. [5]. Collectively, the lower the DM, the higher will be the non-methyl esterified pectin content, pectin content, and free carboxyl (-COOH). Moreover, the enhanced ability of alfalfa cell wall to bind to Al^{3+} increased the Al^{3+} amount entering to the root system, and the sensitivity of the alfalfa ecotypes (NO.21) to Al, and reduced the alfalfa ecotypes tolerance. In contrast, the result was equivalent to Al extrusion from the root system, which indirectly improves the Al resistance of the alfalfa ecotypes (NO.12).

The plant root system is quite sensitive to Al toxicity, such as Al treatment for a few minutes or even seconds could mediate the toxic reaction [32]. In this study, alfalfa was exposed to Al stress at different times, and explored the differences caused by time dynamics of two ecotypes of alfalfa. Although the morphological characteristics of the aboveground part of the plant

was not significantly affected by a short term Al stress, the root morphology of the underground part changed obviously. With the increase of Al stress time, a significant inhibition of the main root elongation, root tip enlargement and shortening, and darker color of the root tip [33]. However, a prolonged Al stress inhibited the root elongation of the two ecotypes. This might attribute to the threshold effect of alfalfa ecotypes under Al stress. Alfalfa growth can be self-regulated and repaired in a certain period of time and the plant self-regulation system may collapse upon exceeding the threshold time. It affects the metabolic level of plants, slows down the growth and development of plants, and then affects the growth of plants and roots [34, 35]. In this study, the root system produced varying degrees of Al accumulation, which was significantly correlated with the stress time. Additionally, the cell wall pectin content increased with the increase of Al stress time, which was consistent with the previous reports. This result confirms that the cell wall is more responsive to Al. With the increase of stress time, the PME activity in the cell wall, the pectin content, and Al^{3+} combination with cell wall pectin increased, all reaching a higher level at 24h. However, the DM value showed a slight downward trend at 24 h as time prolonged, and the correlation with time were lower (Table 1). This might be related to the temporal heterogeneity of Al stress.

The two ecotypes of Alfalfa had spatial heterogeneity and uncertainty of stress effects under Al stress. According to the analysis of Al content, the cell wall accumulated more Al in spatial distribution, and the adsorption capacity about Al is significantly higher than the root tip. the analysis suggests that a number of carboxyl groups and negative charges exist in the cell wall, allowing the trivalent Al^{3+} to bind to these negatively charged groups easily. Hence, the cell wall has a good fixation effect on Al. The study on the Al content of root tip and cell wall revealed that the Al content at 0-5 mm root segment of two alfalfa ecotypes was higher than that at 5-10 mm root segment. An increased Al accumulation by the 0-5 mm root segment increases the degree of toxicity and sensitivity of the alfalfa. Moreover, the position adjacent to the apical front of root is more sensitive. In this study, a comparative study between the root tip PME activity and cell wall PME activity of the two alfalfa ecotypes revealed that the PME activity of the root tip cell wall of the two alfalfa ecotypes was significantly higher than the root tip PME activity. Furthermore, the comparison of different root segments revealed that the PME activity of two alfalfa ecotypes in anterior root segment (0-5 mm) was significantly higher than that in the posterior root segment (5-10 mm), which was consistent with the comparison of Al content of the two alfalfa ecotypes. The DM in the anterior root segment of the two alfalfa ecotypes was higher than in the posterior root segment during initial period of Al stress. However, this was different from the above research results on the esterification degree of pectin methyl

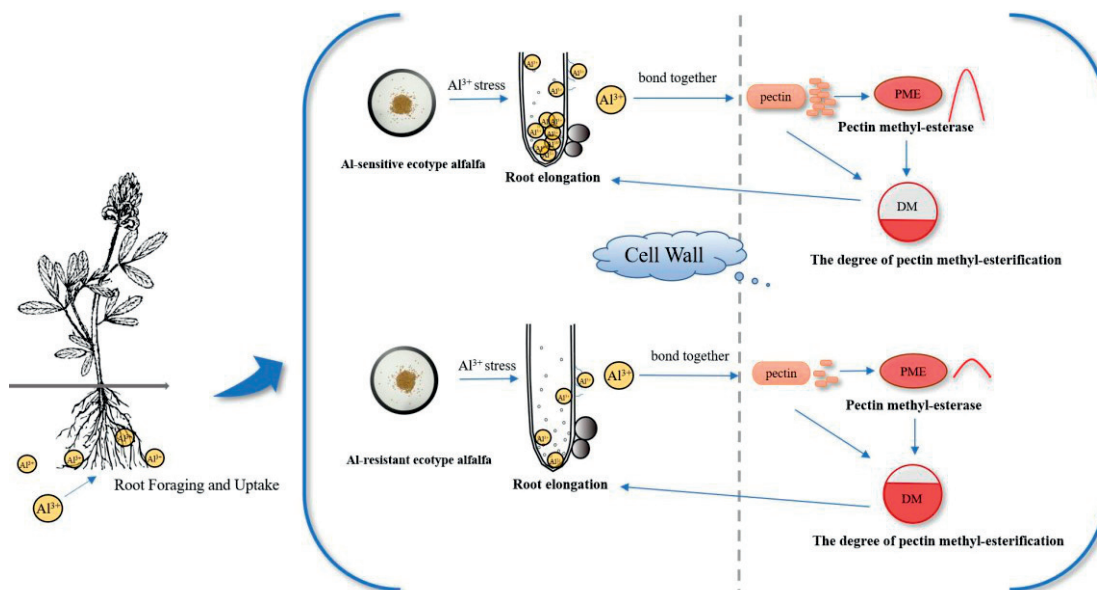


Fig. 6. Differences between the two ecotypes of alfalfa roots under Al Stress.

in different root segments at 12 h and 24 h. It is suggested that the spatial heterogeneity of the root segments of the two ecotypes of alfalfa is more obvious than the temporal heterogeneity.

In recent years, some studies have shown that Al toxicity induces cell damage through cell wall, plasma membrane and cytoskeleton [29, 36]. Therefore, it could be speculated that cell wall plays a vital role in Al resistance. The cell wall pectin content has a significant effect on Al accumulation. However, the amount of pectin is not the only factor determining its binding ability to Al as DM affects the number of free carboxyl groups in pectin. Generally, low-methyl esterified pectin has a higher affinity for metal ions than high-methyl esterified pectin, and the former contains more carboxyl groups that directly bind to the metal ions [37]. PME catalyzed demethylation of pectin can lead to an increase in the amount of low-methyl esterified pectin, thereby increasing the number of metal ion binding sites in the cell wall [38]. The differences in pectin content, PME and DM in root tip cell wall of two ecotypes of alfalfa revealed that Al and pectin accumulation increased with the extension of treatment time, forming a vicious circle. Besides, the special distribution of pectin in the cell wall to express the cell response to Al, including the number and distribution of methyl esterified pectin need further exploration. Al content, cell wall Al content, cell wall PME, DM and pectin content have significant correlation in this experiment, which can be used as a reference standard for screening different ecotypes of alfalfa. Temporal and spatial heterogeneity of different ecological alfalfa showed significant differences. 0-5mm was the most sensitive location for root of alfalfa under Al stress compared with 5-10 mm. And the cell wall is more

sensitive. Moreover, the values of indexes tended to be more stable with increasing time of Al stress in both ecotypes of alfalfa. Integrating the above indicators, we found that the magnitude of change was overall small after 24 h. But to a certain extent, it has spatiotemporal heterogeneity, and the spatial heterogeneity is more obvious than the temporal heterogeneity. Additionally, the special distribution of pectin in the cell wall to express the cell response to Al, including the number and distribution of methyl esterified pectin need further exploration.

Conclusions

In conclusion, the cell wall is an important location affected by Al toxicity. Different ecotypes of Alfalfa have different responses to spatio-temporal heterogeneity. With the increase in Al stress time, Al accumulation in the root and cell wall of alfalfa increased significantly, thereby increasing the pectin content and PME activity in alfalfa cell wall. The contents of Al, pectin, and the PME activity in the root of Al-sensitive alfalfa were higher than Al-resistant alfalfa ecotypes, especially at 0–5 mm root tip, where life activities were most active, indicating more sensitivity of anterior root tip to Al. However, the comparison of DM between the two ecotypes demonstrated that the DM of tolerant alfalfa was higher. Therefore we have reasons to believe that the temporal and spatial heterogeneity promotes the differential resistance among ecotype alfalfa. Further studies are required to reveal the quantity and distribution of methyl esterified pectin and Al redistribution in root cells of Al-resistant and Al-sensitive ecotypes.

Acknowledgments

The current research was supported by the General Project of Yunnan Fundamental Research (No.2019FB044), Yunnan Eco-friendly Food International Cooperation Research Center Project of Yunnan provincial key programs (No.2019ZG009).

Conflict of Interest

The authors declare no conflict of interest.

References

- BI Y.F., JIANG H., MA X.L. Research and utilization of alfalfa in Yunnan. Yunnan Science and Technology Press, Kunming, China. **2019**.
- JASKOWIAK J., KWASNIEWSKA J., MILEWSKA-HENDEL A., KURCZYNSKA E.U., SZURMAN-ZUBRZYCKA M., SZAREJKO I. Aluminum alters the histology and pectin cell wall composition of barley roots. *Int J Mol Sci.* **20**, 3039, **2019**.
- MA X.L., REN J., DAI W.R., YANG W., BI Y.F. Effects of aluminium on the root activity, organic acids and free proline accumulation of alfalfa grown in nutrient solution. *New Zeal J Agr Res.* **63**, 341, **2020**.
- RIAZ M., YAN L., WU X.W., HUSSAIN S., AZIZ O., JIANG C.C. Boron increases root elongation by reducing aluminum induced disorganized distribution of HG epitopes and alterations in subcellular cell wall structure of trifoliolate orange roots. *Ecotoxicol. Environ. Saf.* **165**, 202, **2018**.
- LI X.W., LI Y.L., QU M., XIAO H., FENG Y.M., LIU J.Y., WU L., YU M. Cell wall pectin and its methylesterification in transition zone determine Al resistance in cultivars of pea (*Pisum sativum*). *Front. Plant Sci.* doi: <https://doi.org/10.3389/fpls.2016.00039>. **01** (2), **2016**.
- YANG J.L., ZHU X.F., PENG Y.X., ZHENG C., LI G.X., LIU Y., SHI Y.Z., ZHENG S.J. Cell wall hemicellulose contributes significantly to aluminum adsorption and root growth in Arabidopsis. *Plant Physiol.* **155**, 1885, **2011**.
- SUN C.L., LV T., HUANG L., LIU X.X., JIN C.W., LIN X.Y. Melatonin ameliorates aluminum toxicity through enhancing aluminum exclusion and reestablishing redox homeostasis in roots of wheat. *J Pineal Res.* Article ID: **68:e12642**, **2020**.
- SUN C.L., LU L.L., YU Y., LIU L.J., HU Y., YE Y.Q., JIN C.W., LIN X.Y. Decreasing methylation of pectin caused by nitric oxide leads to higher aluminum binding in cell walls and greater aluminum sensitivity of wheat roots. *J Exp Bot.* **67**, 979, **2016**.
- YU Y., JIN C.W., SUN C.L., WANG J.H., YE Y.Q., LU L.L., LIN X.Y. Elevation of arginine decarboxylase-dependent putrescine production enhances aluminum tolerance by decreasing aluminum retention in root cell walls of wheat. *J Hazard Mater.* **299**, 280, **2015**.
- KOPITTKÉ P.M., MOORE K.L., LOMBI E., GIANONCELLI A., FERGUSON B.J., BLAMEY F.P.C., MENZIES N.W., NICHOLSON T.M., MCKENNA B.A., WANG P. Identification of the primary lesion of toxic aluminum in plant roots. *Plant Physiology.* **167**, 1402, **2015**.
- WEHR J.B., BLAMEY F.P., HANNA J.V., KOPITTKÉ P.M., KERVEN G.L., MENZIES N.W. Hydrolysis and speciation of Al bound to pectin and plant cell wall material and its reaction with the dye chrome azurol S. *J. Agric. Food Chem.* **12**, 58 (9), **2010**.
- GENG X., HORST W.J., GOLZ J.F., LEE J.E., DING Z., YANG Z.B. LEUNIG-HOMOLOG Transcriptional Co-repressor Mediates Aluminum Sensitivity through PECTIN METHYLESTERASE46-Modulated Root Cell Wall Pectin Methylesterification in Arabidopsis. *The Plant Journal.* **90** (3), 491, **2017**.
- YAN L., RIAZ M., LIU J., LIU Y., ZENG Y., JIANG C. Boron reduces aluminum deposition in alkali-soluble pectin and cytoplasm to release aluminum toxicity. *Journal of Hazardous Materials.* **266**, 123388, **2020**.
- ZHU C.Q., CAO X.C., BAI Z.G., ZHU L.F., HU W.J., HU A.Y., ABLIZ B., ZHONG C., LIANG Q.D., HUANG J., ZHANG J.H., JIN Q.Y. Putrescine alleviates aluminum toxicity in rice (*Oryza sativa*) by reducing cell wall Al contents in an ethylene-dependent manner. *Physiol Plant.* **167** (4), 471, **2019**.
- ZHU C., CAO X., ZHU L., HU W., HU A., ABLIZ B., BAI Z., HUANG J., LIANG Q., SAJID H., LI Y., WANG L., JIN Q., ZHANG J. Boron reduces cell wall aluminum content in rice (*Oryza sativa*) roots by decreasing H₂O₂ accumulation. *Plant Physiology and Biochemistry.* **138**, 80, **2019**.
- CHEN S., LI X., LIU X., WANG N., AN Q., YE X.M., ZHAO Z.T., ZHAO M., HAN Y., OUYANG K.H., WANG W.J. Investigation of Chemical Composition, Antioxidant Activity, and the Effects of Alfalfa Flavonoids on Growth Performance. *Oxid Med Cell Longev.* **3**, 1, **2020**.
- WANG L., LI R., YAN X.X., LIANG X.F., SUN Y.B., XU Y.M. Pivotal role for root cell wall polysaccharides in cultivar-dependent cadmium accumulation in Brassica chinensis L. *Ecotoxicol. Environ. Saf.* **194**, 110369, **2020**.
- MA X.L., BI Y.F., CHE W.G., REN J., SUN Y.D., DU J. Ecological adapt ability of wild and escaped alfalfa germplasm resources in Yunnan. *Acta Agrestia Sinica.* **21** (02), 265, **2013**.
- SUN W.J., TANG M., REN J., XU Z., JIANG N., MA X.L., BI Y.F. The physiological tolerance response of 8 alfalfa species in Yunnan to aluminum stress. *Acta Agrestia Sinica.* **26** (05), 1190, **2018**.
- JIANG N., TANG M., HAN B., REN J., XU W.H., MA X.L. Effects of Al stress on different germplasm materials of alfalfa and its comprehensive evaluation. *Acta Agrestia Sinica.* **27** (03), 620, **2019**.
- LUO J., NI D., LI C., DU Y., CHEN Y. The relationship between fluoride accumulation in tea plant and changes in leaf cell wall structure and composition under different fluoride conditions. *Environmental Pollution.* **270**, 116283, **2021**.
- SAFARI M., GHANATI F., SAFARNEJAD M.R., CHASHMI N.A. The contribution of cell wall composition in the expansion of Camellia sinensis seedlings roots in response to aluminum. *Planta.* **247** (2), 381, **2017**.
- WILSON L.A., DELIGEY F., WANG T., COSGROVE D.J. Saccharide analysis of onion outer epidermal walls. *Biotechnology for Biofuels.* **14**, 66, **2021**.
- YANG J.L., LI Y.Y., ZHANG Y.J., ZHANG S.S., WU Y.R., WU P. Cell wall polysaccharides are specifically involved in the exclusion of aluminum from the rice root apex. *American Society of Plant Biologists.* **146** (2), 602, **2008**.

25. MALANGISHA G.K., YANG Y.B., MOUSTAFA M.F., FU Q., SHAO W.Q., WANG J., SHEN L., HUAI Y., LV X.L., SHI P.B., ALI A., KHAN J., REN Y.Y., YANG J.H., HU Z.Y., ZHANG M.F. Subcellular distribution of aluminum associated with differential cell ultra-structure, mineral uptake, and antioxidant enzymes in root of two different Al-resistance watermelon cultivars. *Plant physiology and biochemistry*. **155**, 613, **2020**. doi:10.1016/j.plaphy.2020.06.045.
26. WEHR J.B., MENZIES N.W., BLAMEY F.P.C. Inhibition of cell-wall autolysis and pectin degradation by cations. *Plant Physiol Biochem*. **42**, 485, **2004**.
27. KOPITTKE, P.M., MCKENNA B.A., KARUNAKARAN C., DYNES J.J., ARTHUR Z., GIANONCELLI A., et al. Aluminum complexation with malate within the root apoplast differs between aluminum resistant and sensitive wheat lines. *Front. Plant Sci*. **8**, 1377, **2017**. PMID: 28824696.
28. GENG X., HORST W.J., GOLZ J.F., LEE J.E., DING Z., YANG Z.B. Leunighomolog transcriptional co-repressor mediates aluminium sensitivity through PECTIN METHYLESTERASE46-modulated root cell wall pectin methylesterification in Arabidopsis. *Plant J*. **90**, 491, **2017**.
29. SILVA T.F., FERREIRA B.G., DOS SANTOS ISAIAS R.M., ZHENG S.J. Immunocytochemistry and Density Functional Theory evidence the competition of aluminum and calcium for pectin binding in *Urochloa decumbens* roots. *Plant Physiol. Biochem*. **153**, 64, **2020**.
30. ZHU C.Q., CAO X.C., ZHU L.F., HU W.J., ABLIZ B., ZG B.A.I., HUANG J., LIANG Q.D., SAJID H. Boron reduces cell wall aluminum content in rice (*Oryza sativa*) roots by decreasing HO accumulation. *Plant Physiol. Biochem*. **138** (5), **2019**.
31. HORST W.J., KOLLMEIER M., SCHMOHL N., SIVAGURU M., WANG Y., FELLE H.H., HEDRICH R., SCHRÖDER W., STAß A. Significance of the root apoplast for aluminium toxicity and resistance of maize. *The Apoplast of Higher Plants: Compartment of Storage, Transport and Reactions*. **49**, **2007**.
32. ZHANG X., LI L., YANG C., CHENG Y.B., HAN Z.Z., CAI Z.D. GsMAS1 Encoding a MADS-box Transcription Factor Enhances the Tolerance to Aluminum Stress in. *International Journal of Molecular Sciences*. **21** (6), **2004**, **2020**.
33. SILVA R.G.D., ROSA-SANTOS T.M., FRANCA S.D.C., KOTTAPALLI P., KOTTAPALLI K.R., ZINGARETTI S.M. Microtranscriptome analysis of sugarcane cultivars in response to aluminum stress. *PLoS ONE*. doi:10.1371/GUO P., QI Y.P., CAI Y.T., YANG T.Y., YANG L.T., HUANG Z.R., CHEN L.S. Aluminum effects on photosynthesis, reactive oxygen species and methylglyoxal detoxification in two citrus species differing in aluminum tolerance. *Tree Physiol*. **2018**.
34. GUO P., QI Y.P., HUANG W.L., YANG L.T., HUANG Z.R., LAI N.W., CHEN L.S. Aluminum-responsive genes revealed by RNA-seq and related physiological responses in leaves of two Citrus species with contrasting aluminum-tolerance. *Ecotoxicol. Environ. Saf*. **158**, 213, **2018**.
35. LI H., YANG L.T., QI Y.P., GUO P., LU Y.B., CHEN L.S., KOMATSU S. Aluminum toxicity-induced alterations of leaf proteome in two citrus species differing in aluminum tolerance. *International Journal of Molecular Sciences*. **17** (7), 1180, **2016**.
36. LOIX C., HUYBRECHTS M., VANGRONSVELD J., GIELEN M., KEUNEN E., CUYPERS A.N.N. Reciprocal interactions between cadmium-induced cell wall responses and oxidative stress in plants. *Front. Plant Sci*. **8**, 1867, **2017**.
37. JIA H., WANG X., WEI T., ZHOU R., DING Y.Z. Accumulation and fixation of Cd by tomato cell wall pectin under Cd stress. *Environ. Exp. Bot*. **167**, 103829, **2019**.