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Effects of Acid Precipitation and Aluminum on Carbohydrate Metabolism in Mycorrhizae of *Pinus massioniana*

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Aluminum (Al) is one of the most important growth-inhibiting factors for plants in acid precipitation environment suffering from acid stress. It can exist in various forms such as mononuclear Al (Al^{3+}), polynuclear Al (Al_{13}) and complex compounds（Al-F、$Al-SO_4$） in soils, where Al is involved in decreasing the carbohydrate synthesis and inhibiting the energy supply in plant to resist environmental stress (Brown et al. 1985; Parker et al. 1992; Kong 1995). Recent research showed that under acid precipitation, plants can induce water potential of cytoplasm by accumulation of carbohydrate, formation of mycorrhizae and synthesis of trehalose and mannitol with glucose and fructose in mycorrhizal roots to enhance the resistance to adverse stress (Thompson et al. 1984; Willenborg et al. 1990; Kong et al. 2000). Christian and Paul (1991) found that the content of trehalose was much higher in healthy mycorrhizal roots than that in the injured, and the healthy plants grew much better than the injured ones. Considerable attention to the toxicity of acid and Al stress has been paid in acid precipitation studies, however, few studies has been reported in the sugar metabolism and transportation in mycorrhizal plants in response to acid precipitation and Al stress.

The objective of the study was to determine the effects of acid precipitation and Al stress on carbohydrate metabolism in *P. massoniana* inoculated with *Pisolithus tinctorius*. After exposure to different Al concentrations and pH levels, chlorophyll content, dry weight and sugar (glucose, fructose, trehalose) allocation in roots, stems and leaves of mycorrhizal and non-mycorrhizal plants were investigated. Glucose-6-phosphate dehydrogenase (G6PDHase) and trehalase (THase) were measured as molecular ecotoxicity biomarkers to explore the possible mechanisms of the resistance and response of mycorrhizae to acid precipitation from the viewpoint of mass transportation and energy translation.

MATERIALS AND METHODS

P. tinctorius, (obtained from Institute of Applied Ecology, the Chinese Academy of Sciences, Shenyang), was cultured in flasks (250 mL) containing 100 mL modified Melin Norkran medium (MMN) (Guttenberger et al. 1992). The flasks were on a rotator shaker at 25°C with continuous shaking (90 rpm) 5 d.

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Seeds of *P. massoniana* were surface sterilized with 30 % (w/v) H_2O_2 (2 h), washed twice with sterile water for 30 min, and incubated at 20 °C in dark for 4 d. Then the seedlings were transferred into chambers (15 cm×13 cm, 1.5 cm thick) containing sterilized vermiculite peat moss medium, to which MMN had been added. 8~12 seedlings were planted in each chamber. After two weeks, the fungal mycelia cultured were placed in the substrate near the roots. The chambers were kept under artificial light at a 14/10 (Light/Dark) cycle and 28°C for four weeks.

Artificial acid solutions were prepared by adding appropriate amounts of $Al_2(SO_4)_3$. Al concentrations were 75 and 150 μmol/L. The pH of the solutions was adjusted with 1.0 mol/L H_2SO_4 to 6.0, 4.0, 3.0 and 2.0. The seedlings were treated once every two days with 10 mL artificial acid solutions for four weeks.

After treatment, the seedlings were harvested and the roots were carefully examined for the existence of ectomycorhizae under a stereoscopic microscope. After being dried under vacuum with a vacuum drier (ADVANTAGE, USA) for 4 d, the dried seedlings in each chamber were weighed (W) and the number (n) recorded. The dry weight per plant was expressed as W/n. Chlorophyll was measured by the methods of Kong et al (1999).

The dry leaves, stems and roots were ground into fine powder in liquid nitrogen, extracted three times with solvent (ethanol: chloroform: water volume ratio =12: 5: 3) and centrifuged at 5 000×g for 20 min. The extracts were combined and water added. The mixture was centrifuged at 2 000×g for 20 min to separate chloroform. The water phase was adjusted to pH 7.0 with 0.1 mol/L NaOH, and concentrated at 50°C under vacuum. The sugar (trehalose, glucose and fructose) was determined with HPLC (WATERS 600, USA). Column condition was: mobile phase was pure water; 0.6 mL/min; C^{18} column; RI detector 156, and HP data system. The soluble sugar was measured with the methods of Zhang (1998).

Powdered dry roots were extracted with 1 mL Tris-H_3BO_3 buffer (0.01 mol/L Tris, 0.05 mol/L H_3BO_4, 0.05 mol/L EDTA) on ice for 10 min, centrifuged at 10 000× g for 20 min. The extracts were stored at -58℃. G6PDHase (EC1.1.1.49) and THase (EC 3.2.1.28) were measured by the method of Kong et al. (1999).

All analyses were determined with three measurements for each sample; the experiment was repeated twice. Statistical analysis was conducted using SPSS software package. Differences among means were detected using one way ANOVA by a multiple comparison test, taking $P<0.01$ as significant.

RESULTS AND DISCUSSION

It is shown in Fig.1a, when pH was lower than 4.0, the chlorophyll content decreased significantly with the decreasing pH (Table 1), especially at pH 2.0, chlorophyll contents decreased to 51.9 %, 64.1 %, 44.2 % and 26.1 % of the

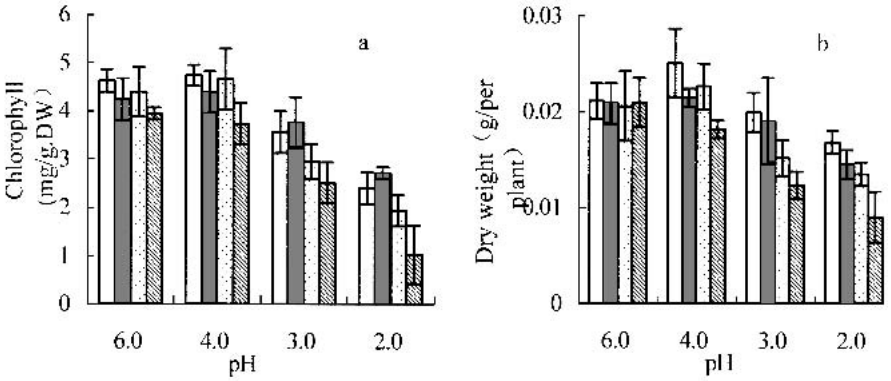

Figure 1. Effect of pH and Al on chlorophyll and dry weight of *P. massoniana*. □ Mycorrhizal seedlings treated with 75 μmol/L Al; ■ Mycorrhizal seedlings treated with 150 μmol/L Al; ▨ Non-mycorrhizal seedlings treated with 75 μmol/L Al ▧ Non-mycorrhizal seedlings treated with 150 μmol/L Al

control (pH 6.0) respectively. There was no significant difference for chlorophyll in mycorrhizal seedlings between 75 and 150 μmol/L Al stress, but in non-mycorrhizal seedlings, there was less chlorophyll under 150 μmol/L Al than 75 μmol/L Al; the existence of mycorrhizae induced chlorophyll under 150 μmol/L Al stress (Table1). At the same time, when pH was lower than 4.0, a decease of the dry weight was observed with decreasing pH (Fig.1b); the dry weight of non-mycorrhizal seedlings was diminished significantly by Al addition; under 150 μmol/L Al stress, the dry weight of mycorrhizal seedlings was much more than that of non-mycorrhizal seedlings (Table1). We also observed a good correlation between the chlorophyll content and dry weight of the seedlings ($r = 0.949$). The chloroplast is a sensitive tissue in plants, and under adverse stress its membrane is peroxidized (Araujo et al. 1989; Kong et al. 2000), resulting in a decrease of the chlorophyll content in leaves. The decrease of chlorophyll in leaves induces a decline of photosynthesis and inhibition of organic chemical synthesis such as sugars, results in inhibition of the accumulation of biomass. But inoculation of *P. tinctorius* increased chlorophyll content and dry weight.

The soluble sugar in leaves decreased significantly with the decreasing pH; there was more sugars in mycorrhizal leaves than in non-mycorrhizal leaves, and Al addition lessened soluble sugar (Fig.2; Table1). A good correlation was shown between soluble sugar and chlorophyll ($r = 0.897$). The seedlings synthesized soluble carbohydrates through photosynthesis and stored them in plants, so it indicated that the decreasing pH and Al stress inhibited photosynthesis and sugar accumulation directly. In stems, more soluble sugar was observed at pH 4.0 than at pH 6.0, thereafter a decreasing tendency with the decreasing pH; there was no significant variation between mycorrhizal and non-mycorrhizal stems (Table 1). In roots, a similar change of soluble sugar was observed as that in stems, but the content were less than that in stems significantly (two-way AVONA, $P<0.01$).

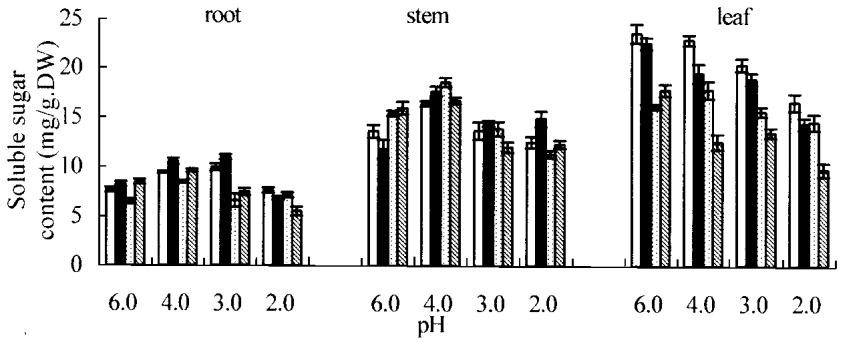

Figure 2. Effect of pH and Al on contents of soluble sugars in roots, stems and leaves of *P. massoniana* □ Mycorrhizal seedlings treated with 75 μmol/L Al; ■ Mycorrhizal seedlings treated with 150 μmol/L Al; ▨ Non-mycorrhizal seedlings treated with 75 μmol/L Al; ▧ Non-mycorrhizal seedlings treated with 150 μmol/L Al

Table 1. Results of one way ANOVA analysis for different variation in response to pH, Al concentrations and mycorrhizal inoculation to show the significance (*P*).

	Source of variation							
	pH				Al concentration		inoculation	
	My+75	My+150	Nm+75	Nm+150	My	Nm	75	150
Chlorophyll	<0.01	<0.01	<0.01	<0.01	0.59	<0.01	0.46	<0.01
Dry weight	<0.01	<0.01	<0.01	<0.01	0.25	<0.01	0.35	<0.01
sugar								
in leaves	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
in stems	<0.01	<0.01	<0.01	<0.01	0.42	<0.01	0.43	0.64
in roots	<0.01	<0.01	<0.01	<0.01	0.72	0.29	0.31	0.18
In roots								
Trehalose	<0.01	<0.01	----	----	<0.01	----	----	----
Glucose	0.69	<0.01	0.65	<0.01	<0.01	<0.01	<0.01	<0.01
Fructose	0.12	<0.01	0.09	0.26	<0.01	0.41	<0.01	<0.01
In stems								
Glucose	0.07	0.12	0.02	0.16	<0.01	0.71	<0.01	0.91
Fructose	0.48	<0.01	0.02	0.08	0.34	0.02	4.06	3.92
In leaves								
Glucose	<0.01	<0.01	<0.01	<0.01	0.11	0.03	0.46	0.43
Fructose	0.43	0.12	0.01	0.02	0.35	0.05	0.57	0.52
THase	0.12	<0.01	0.34	0.04	<0.01	0.09	<0.01	<0.01
GPDHase	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

My+75, My+150: mycorrhizal seedlings treated with 75 and 150 μmol/L Al
Nm+75, Nm+150: non-mycorrhizal seedlings treated with 75 and 150 μmol/L Al
My, Nm: mycorrhizal seedlings and non-mycorrhizal seedlings
75, 150: seedlings treated with 75 and 150 μmol/L Al

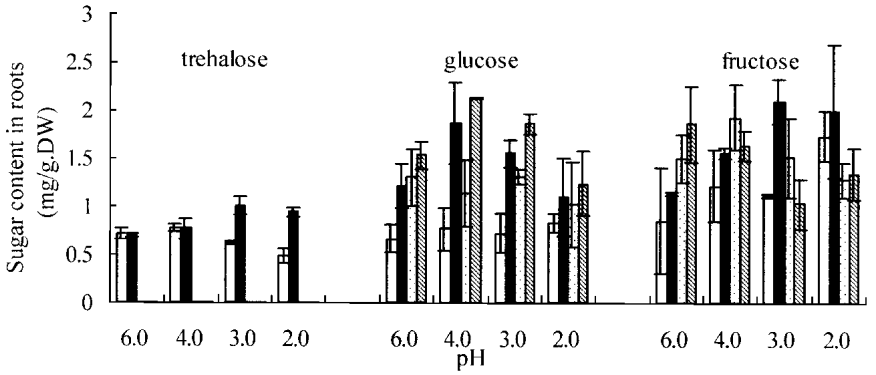

Figure 3. Effect of pH and Al on sugars in roots of *P. massoniana.* ☐ Mycorrhizal seedlings treated with 75 μmol/L Al; ■ Mycorrhizal seedlings treated with 150 μmol/L Al; ▨ Non-mycorrhizal seedlings treated with 75 μmol/L Al; ▧ Non-mycorrhizal seedlings treated with 150 μmol/L Al

Sugar serves as the fundamental unit storing energy produced through the process of photosynthesis in plants (Araujo et al. 1989). The storage of sugar in plants increases the cell water potential for the enzyme metabolism to protect the cells from peroxide injury (Christian et al. 1991). In this study, the light stress of acid and Al stress induced more sugar in stems and roots, but under severe stress, sugar content decreased significantly. This implied that the seedlings of *P. massoniana* synthesized sugars through photosynthesis and transported them to roots through the stems to supply the organic matter and energy that the roots needed. Plants transported more sugar to roots to meet the increased requirement under acid and Al stress, which stimulated the sugar storage in stems. But with the decline of photosynthesis and the sugar synthesis in leaves, the amounts of sugar transported to roots decreased too, therefore the sugar amount in stems decreased significantly. Under the strong acid stress, the sugar in stems were higher than that in roots, which suggested that the allocation of sugar down to roots was depressed between stems and roots, so the sugar supply to roots was absent.

It is shown in Fig.3, when pH was lower than 4.0, trehalose in mycorrhizal roots decreased under 75 μmol/L Al, but under 150 μmol/L stress, it increased and was significantly higher than that under 75 μmol/L Al to 146 % and 193 % at pH 3.0 and 2.0 (Table 1); No trehalose was detected in non-mycorrhizal roots. In the mycorrhizal roots, there was no significant difference for glucose content under 75 μmol/L stress (Table 1), but under 150 μmol/L stress, it increased greatly when pH decreased from 6.0 to 4.0, and thereafter, it decreased. In non-mycorrhizal roots, a peak of glucose content was observed at pH 4.0, and thereafter it decreased significantly (Table 1). It was illustrated that the light acid stress (pH 4.0) and Al stress stimulated glucose accumulation in mycorrhizal roots, but the strong acid stress (pH 2.0) reduced it. The change of fructose in roots was found to be a similar case to that of glucose. The existence of mycorrhizal symbionts could reduce the glucose and fructose in roots under light acid stress.

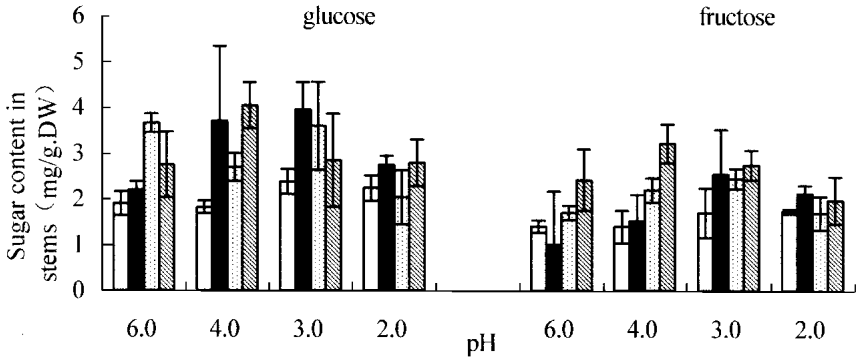

Figure 4. Effect of pH and Al on sugars in stems of *P. massoniana.* □Mycorrhizal seedlings treated with 75 μmol/L Al; ■ Mycorrhizal seedlings treated with 150 μmol/L Al;▨Non-mycorrhizal seedlings treated with 75 μmol/L Al; ▨ Non-mycorrhizal seedlings treated with 150 μmol/L Al

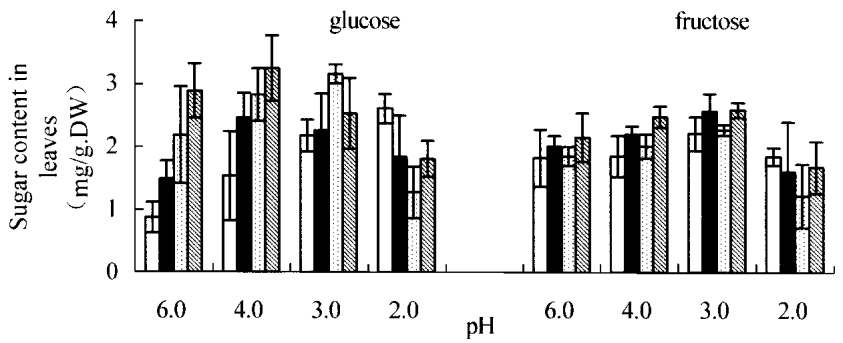

Figure 5. Effect of pH and Al on sugars in leaves of *P. massoniana.* □Mycorrhizal seedlings treated with 75 μmol/L Al; ■ Mycorrhizal seedlings treated with 150 μmol/L Al;▨Non-mycorrhizal seedlings treated with 75 μmol/L Al; ▨ Non-mycorrhizal seedlings treated with 150 μmol/L Al

Glucose content in stems showed no significant difference with the decreasing pH (Fig.4; Table1). In mycorrhizal stems, Al addition stimulated glucose accumulation, but in non-mycorrhizal stems, there was no significant difference between 75 and 150 μmol/L Al stress (Table 1); the change of fructose was similar to that of glucose. Fructose content in non-mycorrhizal stems was more than that of mycorrhizal stems when pH decreased from 6.0 to 4.0. In mycorrhizal leaves, glucose increased significantly under 75 μmol/L Al stress (Table 1); under 150 μmol/L Al stress, it increased when pH decreased from 6.0 to 4.0 and thereafter decreased. In non-mycorrhizal leaves, glucose content increased when pH was higher than 3.0 under 75 μmol/L Al stress, and it was found to be more than that in mycorrhizal leaves (Table 1); but when pH fell lower than 3.0, glucose content decreased steadily. The change of fructose content was similar to that of glucose (Fig.5), and there was no significant difference under acid and Al stress (Table1).

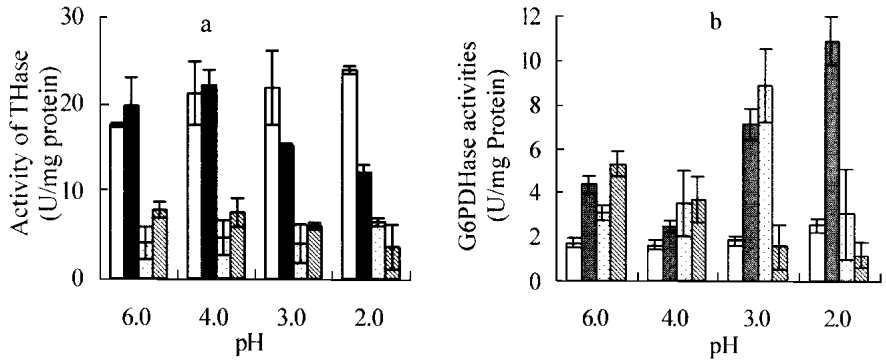

Figure 6. Effect of pH and Al on THase and G6PDHase in roots of *P. massoniana*. □ Mycorrhizal seedlings treated with 75 μmol/L Al; ■ Mycorrhizal seedlings treated with 150 μmol/L Al; ▨ Non-mycorrhizal seedlings treated with 75 μmol/L Al; ▧ Non-mycorrhizal seedlings treated with 150 μmol/L Al

Previous studies of Christian (1991) and Kong et al. (2000) have demonstrated that Environmental stress stimulated the decomposition of starch and sucrose to glucose to enhance the resistance to adverse stress. But the severe stress inhibited sugar accumulation. Therefore the glucose content showed a tendency that it increased under light acid and low Al stress but reduced under high Al and strong acid stress. At the same time, the mycorrhizal fungi could convert the glucose into trehalose, a special carbohydrate in fungi and stored in mycorrhizal roots (Dighton et al. 1991, Kong et al. 1999). Zhou et al (1993) revealed that the carbon source in fungus was mainly supported by the leaves of mycorrhizal plants, and high content of soluble sugar in mycorrhizal roots could stimulate trehalose and mannitol synthesis. This hypothesis was unclear in the mycorrhizal research field, but the results of our experiment agreed with his findings. Under light acid stress, both of glucose and trehalose in mycorrhizal roots increased. But under serious acid stress, the content of glucose was reduced while the trehalose still increased.

THase activity increased slowly with the decreasing pH under 75 μmol/L Al stress in mycorrhizal roots (Fig.6a), but under 150 μmol/L Al stress, it declined significantly when pH was lower than 4.0 (Table 1). In non-mycorrhizal roots, it remained low. Thus it suggested that acid stress promoted the THase activity under 75 μmol/L Al stress, and then catalyzed the degradation of trehalose to glucose, which caused a fall of the content of trehalose. Kong (1995) found that under the combined stress of acid and Al stress, THase activity decreased, which slowed the degradation of trehalose and preserved the resistance of mycorrhizal roots. In this study, high Al concentration reduced the activity of THase, leading to a pronounced increase in trahalose content. The results suggested a good correlation between the THase activity and the trahalose content (r = -0.857).

It is shown in Fig.6b, under 75μmol/L Al stress, G6PDHase activity showed a

significant increase with the decreasing pH in mycorrhizal roots (Table 1); in non-mycorrhizal roots, it increased significantly as pH decreased, only at pH 2.0 did it decrease obviously, which indicated that under strong acid stress, the enzymatic activity was lessened. Under 150 μmol/L Al stress, G6PDHase activity was observed lowest at pH 4.0 and then a sharp increase was recorded at pH 3.0 and 2.0 in mycorrhizal roots; but it decreased in non-mycorrhizal roots. Al addition induced G6PDHase activity in mycorrhizal roots, but in non-mycorrhizal roots, Al addition inhibited G6PDHase activity significantly (Table 1).

G6PDHase is the enzyme responsible for catalyzing G-6-P to 6-P-gluconate and the key enzyme associated with the oxidative pentose that provides the NADPH to catalyze glucose-6-phosfate to pentane-phosphoric acid pathway (Kong 1995). The increase of G6PDHase activity would decompose more glucose to produce enough energy to sustain the resistance to environmental stress. This study found that environmental stress induced G6PDHase activity to consume more glucose. However, the severe acid stress was found to inhibit the enzymatic function, which indicated that less glucose would be consumed. On the contrary, the content of glucose didn't increase but decreased with the decreasing G6PDHase's activity. One explanation was that the glucose transportation from leaves to roots was inhibited under the severe stress. So in our experiments, the light acid and low Al stress was observed to stimulate G6PDHase activity while the glucose content increased. Under the severe stress, not only did the sugar decompose, but the transportation of glucose was interrupted also. Hence, there was no negative correlation between the glucose content and G6PDHase activity in roots.

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