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MANGANESE ACCUMULATION AND ANATOMICAL CHANGES IN MASSON PINE (*PINUS MASSONIANA*) GROWING UNDER HIGH CONCENTRATIONS OF MANGANESE

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Abstract

Masson pine (*Pinus massoniana*) is especially good at accumulating manganese (Mn). Studying the manganese accumulation and anatomical changes in masson pine growing under high concentrations of Mn can provide a theoretical basis for exploring the adaptations and assessing the remediation effects of masson pine under Mn-contaminated environment. Using a sand culture method, a series of high manganese treatments were performed to study the growth, manganese accumulation in organs, and anatomical structure of needles and stems of masson pine when compared to masson pine that received Hoagland's nutrient solution. Our results showed that the parenchyma cells and vascular bundles in the needles of masson pine changed disproportionately to maintain growth under high concentrations of Mn, and similar patterns were observed for the xylem and pith in the stem. At low concentrations of Mn, masson pine stems and needles displayed an even and regular anatomical structure, but the cell arrangement became scattered with increasingly higher Mn treatments; especially in the 30 mmol·L^{-1} treatment, masson pine growth was inhibited and severe toxicity occurred. With higher Mn treatments, more Mn accumulated in masson pine. Needles, stems and roots of masson pine accumulated about 24, 24 and 27 times more Mn in the 30 mmol·L^{-1} treatment than in the normal treatment of 0.0091 mmol·L^{-1}. Results indicate that masson pine could be planted in Mn-contaminated environments to rehabilitate the soil.

Key words: Masson pine, Higher manganese environment, Manganese bioaccumulation, Anatomical structure of masson pine.

Introduction

Manganese (Mn) is a necessary micro nutrient for plants and has multiple purposes: maintaining chloroplast construction, releasing oxygen from photosynthesis (Shenker *et al*., 2004), acting in oxidizing and deoxidizing processes (Miller *et al*., 1990), activating 36 enzymes as a cofactor, and forming a component of 3 enzymes (Burnell, 1988).

However, Mn is also a toxic heavy metal element (He *et al*., 2005) and is an important factor in the crop yield decreases (Ma *et al*., 2009). The activity of Mn is much higher in acidic soil (He *et al*., 2005), and soil is contaminated by Mn during mine exploitation (Garcia-Arreola *et al*., 2015). Although Mn is important for physiological processes in humans, excess Mn absorption has toxic effects on neural systems, and may cause a parkinsonian-like syndrome (referred to as manganism)(Kwakye *et al*., 2015), and also lead to abnormal reactions of the male reproductive system and negative effects on the immune system may occur (Ponnapakkam *et al*., 2003; Brophy & Nolan, 2015).

Over 500 metal-hyperaccumulater species presently have been defined (Verbruggen *et al*., 2009; van der Ent *et al*., 2013), in which about 20 species have been confirmed as Mn-hyperaccumulators, and research on relationships between Mn and plants focuses on grasses and crops (Moosavi & Ronaghi, 2011), especially Phytolaccaceae (Xue *et al*., 2003; Xue *et al*., 2004; Xue *et al*., 2008; Pollard *et al*., 2009; Xu *et al*., 2009; Liu *et al*., 2010; Weng *et al*., 2013; Chen *et al*., 2015), but focuses less on trees (Fernado *et al*., 2007). Some Mn-hyperaccumulator trees have been reported, including *Schima superba* (Theaceae) (Yang *et al*., 2008), *Eucalyptus grandis* × *E. urophylla* (Myrtaceae**)** (Xie *et al*., 2015), *Gossia bidwillii* (Myrtaceae) *and Virotia neurophylla* (Proteaceae) (Fernando *et al*., 2006), *Maytenus founieri* (Celastraceae) (Fernando *et al*., 2007) *Austromyrtus bidwillii* (Myrtaceae) (Bidwell *et al*., 2002) and so on. Only 2 Mn-hyperaccumulator trees have been identified in china, *Schima superba* (Yang *et al*., 2008) and *Eucalyptus grandis* × *E. urophylla* (Xie *et al*., 2015). These Mn-hyperaccumulator plants could effectively improve soil environment. In addition, some other plants, such as mat rush (*Juncus effusus* L.) (Najeeb *et al*., 2015), could also play important roles in Mn- contaminated soil remediation.

Masson pine (*Pinus massoniana*), a large fast-growing tree that is planted for harvesting and compensational use, is distributed in subtropical zones, especially in the south of China (Richardson & Rundel, 1998). It is an important industry species in China. The transformation of soil that is contaminated with heavy metals is enhanced by planting masson pine (Fu *et al*., 2005) because large amounts of heavy metals, such as As, Hg, Al, Cu, Zn, Mn, Pb, Cd, and Ni, can be accumulated in masson pine (Li & Hu, 2010; Chen *et al*., 2008; Fang *et al*., 2004). Masson pine is especially good at accumulating Mn (Qi *et al*., 2003; Guo & Zhou, 2010). In this study, the following questions were asked: 1) How severe of a Mn -contaminated environment can masson pine live in, and how does this affect its growth? 2) How much Mn can masson pine accumulate, and what is the distribution of Mn within the tree? 3) Is the anatomical structure of masson pine affected by high Mn content, and can masson pine be used to remedy Mn -contaminated soil? Our objectives were to investigate the adaptation of masson pine under Mn-contaminated environment and also to explore the potential of masson pine as a plant in repairing Mn-polluted soil.

Materials and Methods

Materials preparation: A sand culture method was used. Before planting, pure crystal sand (2-3 mm) was washed using pure water, immersed in a solution of 3% HCl for 1 week, washed again using pure water until no Cl^- was found, and washed using Hoagland's nutrient solution 2 times a day until the pH did not change for 24 hours. The sand was put in plastic buckets of 27cm×19cm×16cm to a height of 12cm. Masson pine seedlings were planted in the sand in December 2012. One week later, Hoagland's solution was applied. Mn treatments were applied when masson pine grew normally. A pre-experiment was carried out to define the experiment Mn density, and we found that masson pine was hard to survive when the concentration was over 50 mmol·L^{-1} Mn. Basted on the result, eight treatments were used: 0.0091 mmol·L^{-1} (CK: Hoagland nutrient solution), 1 mmol·L^{-1}, 5 mmol·L^{-1}, 10 mmol·L^{-1}, 15 mmol·L^{-1}, 20 mmol·L^{-1}, 30 mmol·L^{-1}, and 50 mmol·L^{-1}. $MnSO_4 \cdot H_2O$ was used. The experiment included three replicates of 15 buckets containing 3 seedlings in each bucket. Every four days, 250 ml of nutrient solution was added at the same time each day, and water was added when the sand was dry to ensure that the masson pine seedlings lived. The treatments were performed in the nursery garden of Guizhou University.

Growth indexes: The height and ground diameter of seedlings were measured using a ruler and vernier callipers before treatment and on a fixed time schedule until the end of the experiment.

Mn content: At the end of the experiment (360 days after treatments began), masson pine seedlings were harvested. Different organs were washed and dried on paper. Fresh roots, stems, and needles were weighed. Samples were killed when green by exposure to temperatures of 105 degrees Centigrade for 30 min and were then oven dried at 75 degrees Centigrade for 48 hours. Dry weight was measured. Samples were ground to pass through a 75 μm sieve and were digested using $HClO_4$-H_2SO_4. Mn was measured by AAS (PerkinElmer AAnalyst 400).

Anatomical structure: At the end of the experiment (360 days after the treatments began), fresh needles and stems were cut into small pieces for fixation in FAA fixative for 48 h. Samples were then immersed in water and then dehydrated in 50%, 70%, 85%, 95%, 100%, and 100% alcohol for 2 hours at each step, followed by dehydration in 1/2 dimethylbenzene + 1/2 alcohol free water for 2 h, pure dimethylbenzene for 1.5 h, and pure dimethylbenzene for 1.5 h at each step. Then, the samples were immersed in 1/2 ceresin wax (solid powder) + 1/2 dimethylbenzene at 40 degrees Centigrade for more than 12 h. The oven temperature was then increased to 60 degrees Centigrade. Pure ceresin wax was changed 3 times at 1- to 2-h intervals, after which the samples were put into a paper box on a 45-60 degree Centigrade board and filled with pure ceresin wax. One hour later, the paper box containing the samples was put in cold water until the wax was fixed and dried naturally. After microtomy, adhibit slice, dewax, pigmentation, and sealed, slices were then observed and photographed under a BM2000 microscope, and the images were processed using the JIFEI TECH software.

Statistical analysis: Each experiment was run on each sample at least three times, then we calculated mean of test results obtained from all samples and the data are presented as mean. All obtained data were analyzed using SPSS 20.0 statistical software with a two-way ANOVA at a significance level of 0.05. A Duncan multiple range test was carried out to determine if there were significant differences between individual treatments at $p<0.05$.

Results

Growth of masson pine: The survival rate of masson pine climbed from 44.0% in the 0.0091 mmol·L^{-1} Mn treatment to 50.8% in the 5 mmol·L^{-1} Mn treatment and then decreased to 11.1% in the 50 mmol·L^{-1} Mn treatment (Table 1). Compared to the highest survival rate of 50.8%, the survival rate of masson pine was extremely low in higher Mn treatments. The ground diameter of masson pine was higher in the 5-20 mmol·L^{-1} Mn treatments compared with the CK treatment (0.0091 mmol·L^{-1} Mn), but it was lower in the 30 and 50 mmol·L^{-1} Mn treatments. The lowest ground diameter was only 54% in the 50 mmol·L^{-1} Mn treatment, and the highest ground diameter was 126% in the 20 mmol·L^{-1} Mn treatment, compared with the CK treatment. The height of masson pine showed the same pattern as ground diameter: increasing up to the 10 mmol·L^{-1} Mn treatment and then decreasing in the 50 mmol·L^{-1} treatment compared to the CK treatment. The root activity of masson pine was markedly different among the treatments. The patterns of root activity showed the same changes that occurred in the aboveground indicators, first increasing and then decreasing. The highest total active absorbed root area was 888.3 $cm^2$and 304.7cm^2 for the treatment of 10 mmol·L^{-1} Mn and 1 mmol·L^{-1} Mn, respectively, and the lowest was 535.4 cm^2 and 191.6 cm^2 for the treatment of 30 mmol·L^{-1} Mn. Meanwhile, root activity in the 20 and 30 mmol·L^{-1} Mn treatment was lower than root activity in the 0.0091 mmol·L^{-1} Mn treatment.

Mn content: Mn content in the needles, stems and roots of masson pine as the treatments significantly increased from 1 to 30 mmol·L^{-1}, but Mn content in those organs was the fastest in the 20 mmol·L^{-1} Mn treatment. Mn content was highest in needles, followed by roots and then stems except 10 mmol·L^{-1} treatment. Compared with control (0.0091 mmol·L^{-1} Mn treatment), Mn content in the needle, stem and root of masson pine under 30 mmol·L^{-1} Mn treatment were about 24, 24, 27 times, respectively (Table 2).

Anatomical structure of needles: Figure 1 shows the anatomical structure of cross sections (10×10) of masson pine needles from different Mn treatments. In the 0.0091 mmol·L^{-1} treatment and the 10 and 20 mmol·L^{-1} Mn treatments, the anatomical construction of pine needles was clear, and the epidermis, hypodermis, and stratum corneum demonstrated full integrity. Needle tissue was severely damaged and fragmented in the 30 mmol·L^{-1} Mn treatment.

Table 1. Growth of masson pine under high Mn concentrations.

Mn treatment /mmol·L^{-1}	Survival rate/%	Change in ground diameter/mm			Change in height/cm			Root vigor/cm^2	
		Begin	End	Growth	Begin	End	Growth	Total	Active
0.0091(ck)	44.0	2.26	3.50	1.24	17.3	24.8	7.5	644.8 e	253.5 a
1	41.0	2.40	3.60	1.20	15.6	23.8	8.2	652.4 c	304.7 b
5	50.8	2.21	3.65	1.44	17.1	25.2	8.2	850.4 b	291.4 c
10	45.0	2.45	3.70	1.25	17.6	25.9	8.3	888.3 a	256.5 d
15	27.1	2.42	3.73	1.31	15.8	23.7	7.9	645.2 d	252.11 e
20	23.8	2.22	3.78	1.56	16.5	24.4	7.7	591.2 f	212.5 f
30	20.8	2.33	3.41	1.08	17.9	25.6	7.7	535.4 g	191.6 g
50	11.1	2.12	2.80	0.68	16.8	24.0	7.2	—	—

Values with different lowercase letters within a column are significantly different (Duncan's Multiple Comparison Test, $p<0.05$)

Table 2. Mn content in the needles, stems and roots of masson pine under high Mn concentrations.

Mn treatment /mmol·L^{-1}	Needle /mg·kg^{-1}	Stem /mg·kg^{-1}	Root /mg·kg^{-1}
0.0091(ck)	294 e	150 d	183 e
1	779 d	259 d	453 d
10	2593 c	1400 c	1095 c
20	6015 b	2810 b	3339 b
30	6934 a	3655 a	4895 a

Values with different lowercase letters within a column are significantly different (Duncan's Multiple Comparison Test, $p<0.05$)

Figure 2 shows the anatomical structure of cross sections (40×10) of masson pine needles from different Mn treatments. Needles were greatly affected by high Mn concentrations. Compared with the 0.0091 mmol·L^{-1} treatment, parenchyma thickness in needles markedly increased in the 10 and 20 mmol·L^{-1} treatments but decreased in the 30 mmol·L^{-1} treatment. Vascular bundle area was lower in the 30 mmol·L^{-1} treatment than in the 0.0091 mmol·L^{-1} treatment. Mesophyll area showed a trend of increasing to declining as the treatments increased in Mn concentration. Based on the date in Table 3 the shorter radii, longer radii, perimeter and area of vascular bundle of masson pine needles were gradually reduced as the increasing of Mn concentration; nevertheless, these parameters of mesophyll tissue increased at first and decreased afterwards. The shorter radii, longer radii and perimeter of mesophyll tissue were the highest in the 10 mmol·L^{-1} treatment, but the maximum of mesophyll area appeared in 20 mmol·L^{-1} treatment.

Anatomical structure of stems: The growth of masson pine stems was greatly affected by higher Mn treatments. With increasing Mn concentrations, xylem of masson pine stems became scattered. In comparison, in the 0.0091 mmol·L^{-1} treatment xylem structure was uniform, simple and regular and consisted of large number of tracheids and few wood parenchyma, wood rays, or resin canals (Fig. 3). The perimeter, radii, and area of masson pine stem pith increased as Mn concentrations increased (Table 4). The xylem area of masson pine stems increased in the 20 mmol·L^{-1} treatment, but markedly decreased in the 30 mmol·L^{-1} treatment.

Table 3. Characteristics of anatomic structure of masson pine needles under high Mn concentrations.

Mn treatment /mmol·L^{-1}	Vascular bundle				Mesophyll tissue			
	Shorter radii /μm	Longer radii /μm	Perimeter /μm	Area /μm^2	Shorter radii /μm	Longer radii /μm	Perimeter /μm	Area /μm^2
0.0091(ck)	134	235	650	25,443	168	240	610	93,657
10	117	205	580	20,492	227	280	819	105,600
20	87	203	509	14,008	219	270	746	113,854
30	81	97	286	6171	123	246	633	48,785

Table 4. Characteristics of anatomic structure of masson pine stems under high Mn concentrations.

Mn treatment /mmol·L^{-1}	Pith			Xylem		
	Radius/ μm	Perimeter/ μm	Area/ μm^2	Radius/ μm	Perimeter/ μm	Area/ μm^2
0.0091(ck)	64	405	13,061	388	2438	891,794
10	317	1993	315,964	348	2184	1072,139
20	411	2582	530,385	277	1743	958,103
30	287	1801	258,069	178	1118	420,185

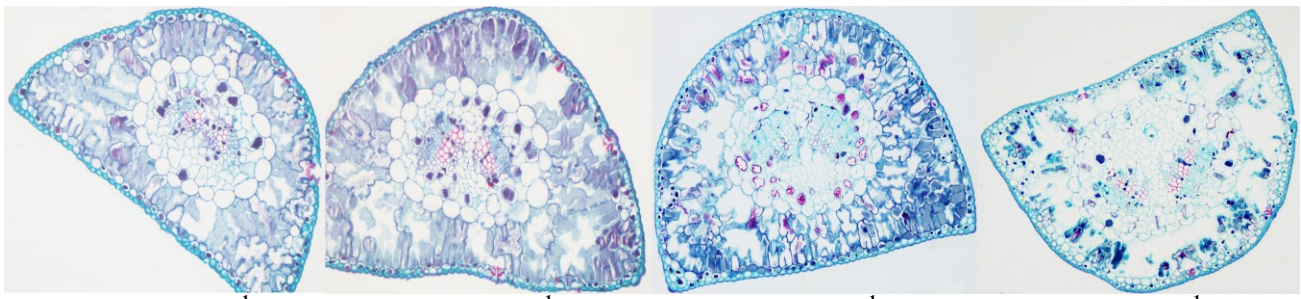

Fig. 1. Paraffin slices showing masson pine needles grown under high Mn concentrations (10×10).

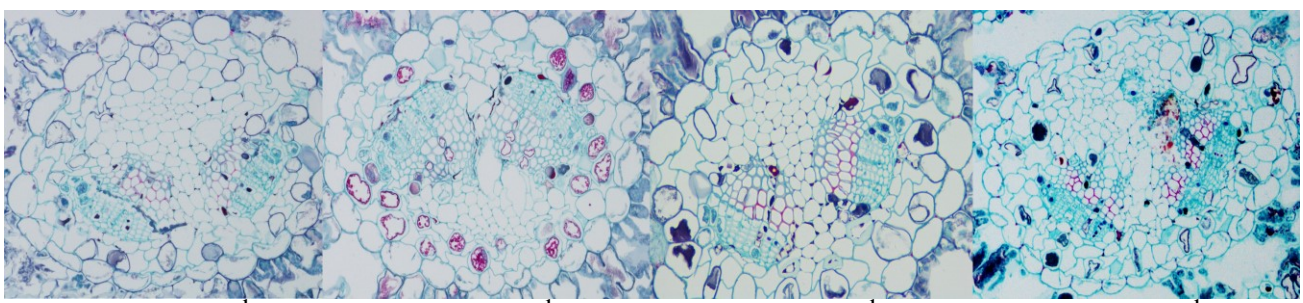

Fig. 2. Paraffin slices showing mason pine needles grown under high Mn concentrations (40×10).

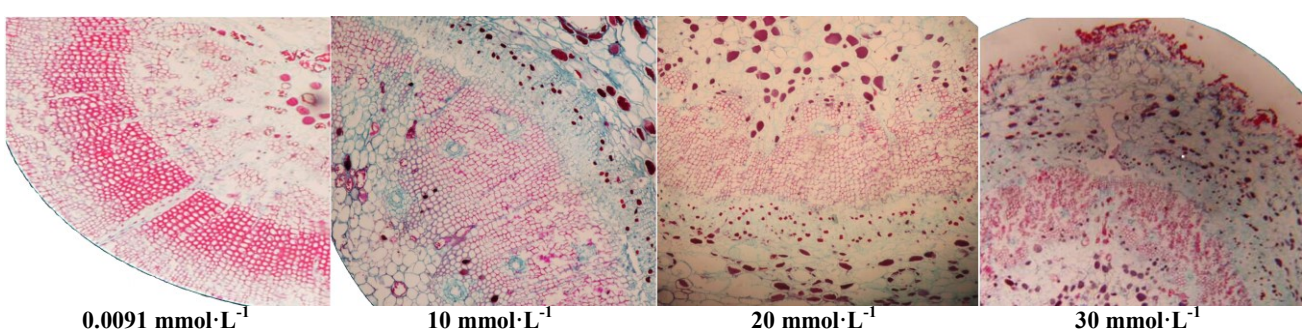

Fig. 3. Paraffin sections showing masson pine stems grown under high Mn concentrations (10×10).

Discussion

The growth of trees directly reflects their environmental conditions. The height and ground diameter reflect the leaf area, photosynthetic capacity, transpiration area, and growth rate. The larger the height and ground diameter are, the better trees grow (Shen, 2001). In this study, the annual growth in height and ground diameter of trees in the Mn treatments was first higher than trees in the control treatment and then decreased, but the changes in height and ground diameter were not linear: the rate of growth in height was higher in the 30 mmol·L^{-1} treatment than in the control (0.0091 mmol·L^{-1}) treatment, but the rate of growth in ground diameter was higher in the 20 mmol·L^{-1} treatment. Lower root activity occurred in the 20 mmol·L^{-1} treatment compared to the control treatment. The growth of masson pine was inhibited only in the treatment with the highest Mn concentration. The much lower survival of masson pine in the experimental treatments compared to the control treatment was not due to higher Mn; for one, the highest survival rate of masson pine was only 50.8% in this experiment because of the high temperatures (more than 45 degree Centigrade) that occurred in the greenhouse for many days. In a 2010 experiment, the survival rate of masson pine was 100% (Gong *et al*., 2012). Second, the survival rate of masson pine in the control treatment was only 44%. However, the masson pine that survived under high Mn and high temperature indicated that masson pine might grow in soils with Mn concentrations above 5000 times higher than those of typical soils and still have a normal growth rate.

Mn is one of the essential trace elements required during plant growth. The most important function of Mn is in the metabolism of plants. Mn may accumulate in plants in the range of 20-500 mg·kg^{-1} (dry weight) in normal conditions, and although the accumulation of Mn differs among plant species, few plants exceed 1000 mg·kg^{-1} (Baker & Brooks, 1989). After long-term evolution and natural selection, a few special plants growing on highly contaminated soil might accumulate Mn exceeding 10,000 mg·kg^{-1} to be defined as a Mn-hyperaccumulator (Baker & Brooks, 1989). Under normal conditions, needles might accumulate Mn at a level of 294 mg·kg^{-1} (Table 2); nevertheless, masson pine in all treatments accumulated Mn at less than 10,000 mg·kg^{-1}.

Mn in the needles accumulated in experimental plants at levels that were about 3, 9, 20 and 24 times higher than those observed in the control plants, thus clearly demonstrating that masson pine accumulated abundant Mn. Compared with control, masson pine root under 30 mmol·L^{-1} Mn accumulated Mn at a more rate than stem and needle, suggesting that masson pine could be a root hoarding plant and accumulate Mn in the roots to lower its toxic effects on stems and leaves (Zhu *et al*., 2012), and also appear to have a high tolerance to Mn. Typically, higher Mn concentrations are found in mine tailings; Mn concentrations from 8819 to 17,789 mg·kg^{-1} were found in Liangcheng mining area in southeastern China (Xie *et al*., 2015), which is much higher than in normal soils (450-4000 mg·kg^{-1} Mn) (Foulds, 1993; Marschner, 1995). Nevertheless, masson pine could survive in the soil containing 17,789 mg·kg^{-1} Mn (Xie *et al*., 2015). Due to the bioavailability of wood and the lack of secondary pollution, masson pine could be planted in and used to treat Mn -contaminated soil.

Photosynthesis mainly occurs in the mesophyll, which contains parenchyma cells with abundant chloroplasts (Adams *et al*., 2013). We observed that needle morphology and structure was complete and that needles grew normally in the 0.0091 mmol·L^{-1} treatment; however, mesophyll tissue suffered from different degrees of damage in higher Mn treatments, especially in the 30 mmol·L^{-1} treatment (Figs. 1-3). Mesophyll area were larger in the 10 mmol·L^{-1} and 20 mmol·L^{-1} treatments than in the control treatment, and photosynthesis was higher. Mesophyll cells were severely injured in the 30 mmol·L^{-1} treatment, causing the growth rate of masson pine to decrease, consistent with the conclusion of growth (Table 1).

Needle tissue structure was affected by higher Mn treatments. The thickness of parenchyma cells increased, and the area of vascular bundles decreased. Consequently, nutrient supply was affected when compared to seedlings in the 0.0091 mmol·L^{-1} treatment. In particular, the Mn concentrations were too large to impede nutrient supply, and masson pine was injured and its growth rate restricted (Lee *et al*., 2011). Masson pine could adapt to higher Mn environments by increasing the thickness of parenchyma cells and decreasing the area of vascular bundles.

The growth of pith and xylem was also affected by higher Mn treatments. The pith area increased, possibly to store materials such as starch granules to maintain the required nutrients to adapt to environmental change. The growth rate of masson pine was enhanced because the area of xylem increased to accelerate absorption of water and ions (Jeschke & Pate, 1995). However, when the Mn concentrations were too large, the xylem cell arrangement became disorganized, and the xylem area was drastically reduced, thereby decreasing the absorption of water and mineral ions and threatening the growth of masson pine. Higher concentrations of Mn affected masson pine growth by affecting the growth of the xylem and pith in the stem.

Conclusions

In a series of high manganese treatments, the growth indexes of masson pine were not the same. Masson pine grew normally on less than 20 mmol·L^{-1} manganese, was constrained on more than 30 mmol·L^{-1} manganese, but could still live on 50 mmol·L^{-1} manganese, implying that masson pine had a high tolerance for manganese. The manganese contents in different organs of masson pine varied markedly; the order of Manganese content, from high to low, was needle, root and stem. In the 30 mmol·L^{-1} treatment, needles, stems and roots of masson pine accumulated manganese about 24, 24 and 27 times when compared with Hoagland's nutrient solution treatment, implying high manganese accumulation in masson pine. Vascular bundles in masson pine needles were markedly smaller under the 30 mmol·L^{-1} manganese treatment, parenchyma cells were decreased and fragmented, and stem xylem was more scattered and decreased in area, implying that masson pine growth was constrained. Based on the high manganese tolerance and accumulation in masson pine, this species could be planted on manganese-contaminated soil to efficiently remedy contamination.

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