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Picea asperata pioneer and fibrous roots have different physiological mechanisms in response to soil freeze-thaw in spring

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Abstract

About 70 % of the total land area in the world are affected by soil freeze and thaw (FT) cycles. Root is the first organ of plant to sense soil environment and it is unclear how it copes with the soil FT. Based on the different functions of first-order pioneer and fibrous roots in woody plants, we hypothesize that pioneer and fibrous roots respond differently. The experiment was conducted in a growth chamber using *Picea asperata* seedlings. We designed the FT based on field observation data. The physiological responses in fibrous and pioneer roots were examined. Fibrous roots had higher root vitality and N content, whereas pioneer roots exhibited higher total nonstructural saccharide content. The accumulation of O_2^- under FT treatment was similar in the two types of roots. Pioneer roots showed higher osmolyte (especially proline) content, whereas fibrous roots had higher peroxidase activity. The present study confirmed that fibrous roots have stronger metabolism ability, whereas pioneer roots are the key storage organs. FT in the temperature range from -5 to 5 °C are mild and do not cause serious injury to roots. Pioneer roots have higher tolerance to soil FT in spring than fibrous roots. The roots have different strategies to FT: fibrous roots increase the antioxidant system, whereas pioneer roots accumulate more osmolytes. Such knowledge can help us to understand how roots of woody plants cope with soil FT.

Additional key words: antioxidants, nitrogen, osmotic adjustment, peroxidase, proline, root vitality, saccharides.

Introduction

Seasonal freeze and thaw of soil are one of the most significant temporal variability at high latitude and high altitude areas (Joseph and Henry 2008). Frequent freeze and thaw events occur mainly in late autumn and early spring in the subalpine/alpine forests (Tan *et al*. 2011, Zhu *et al*. 2012). The maximum extent of seasonally frozen ground is about 55 % of the total land area of the northern hemisphere (Zhang *et al*. 2003). Soil freeze-thaw (FT) cycles are common in this area, however, they received relatively less attention than in permafrost regions (Zhang *et al*. 2003). Global warming coupled with extreme and variable weather decreases snow cover (Easterling *et al*. 2000, Mellander *et al*. 2007), which can change the frequency of soil FT (Groffman *et al*. 2001, Henry 2008). These processes have considerable impacts on ecosystems (Kreyling 2010) including soil thermal and hydraulic properties (Smith *et al*. 2004, Kimball *et al*. 2006) and plant species compositions (Joseph and Henry 2008).

Damage to vegetation by a sudden frost is evident (Gorsuch and Oberbauer 2002), and a thaw can also be injurious to vegetation. Some researchers have found that fine root growth, longevity, and dynamics (Tierney *et al*. 2001, Repo *et al*. 2014), root-associated fungi (Kreyling

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Abbreviations: A - absorbance; ASA - ascorbic acid; EDTA - ethylenediamine tetraacetic acid; FT - freeze-thaw; MDA - malondialdehyde; O_2^- - superoxide radical; PCA - principal component analysis; POD - peroxidase; REL - relative electrolyte leakage; ROS - reactive oxygen species; SOD - superoxide dismutase; TNC - total nonstructural saccharides; TTC - triphenyltetrazolium chloride.
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et al. 2012), nitrogen uptake (Malyshev and Henry 2012, Campbell *et al*. 2014), and ecosystem composition and productivity (Schuerings *et al*. 2014) are affected by soil FT. The roots are usually more frost-sensitive than aboveground parts (Tierney *et al*. 2001, Gaul *et al*. 2008, Schaberg *et al*. 2008), and frozen soil often causes damage of root cells (Cleavitt *et al*. 2008). Fine roots are a highly heterogeneous system with a complex structure of branches (Pregitzer *et al*. 2002). Freeze tolerance of fine roots vary with their structure and age. Generally, mature woody roots are more frost tolerant than young roots and frost tolerance is lowest at the root tips (Havis 1976, Colombo *et al*. 1995). Based on Strahler's system, the most distal root tips were defined as the first order (Pregitzer *et al*. 2002). In woody plants, even the first-order roots are classified to fibrous roots (absorptive, short, or feeder) and pioneer roots (framework, long, or skeletal) (Horsley and Wilson 1971, Lyford 1980) by their roles. Polverigiani *et al*. (2011) reported that fibrous and pioneer roots have different responses to soil moisture deficits.

The subalpine coniferous forest ecosystems in the Eastern Tibetan Plateau located at the transition zone from the Qinghai-Tibet Plateau to the Sichuan Basin can be very susceptible to global climate change. Spruce (*Picea asperata* Mast.) is a dominant species and plays an important role in maintaining regional ecosystem stability and succession in subalpine coniferous ecosystems of western Sichuan. *P. asperata* is a shallow root species that is vulnerable to the damage caused by environmental changes (Hennon *et al*. 2008, Schaberg *et al*. 2008). Meanwhile, first-order roots were considered as sensitive indicators of environmental changes (Polverigiani *et al*. 2011, Yin *et al*. 2014). Based on functions of pioneer and fibrous roots in woody plants, we hypothesize that pioneer and fibrous roots also respond differently to FT, namely, the former would show higher tolerance to soil FT in spring than the latter. Besides, we also expect pioneer and fibrous roots have different physiological mechanisms in response to FT. It is well known that plants would try to alleviate oxidative stress and cellular damage through osmotic adjustment and antioxidant defense systems that include carotenoids, ascorbate, and enzymes such as superoxide dismutase (SOD), peroxidase (POD) and so on when exposed to environmental changes (Noctor and Foyer 1998, Acosta-Motos *et al*. 2014). Thus, the current study was conducted to expose the seedlings of *P. asperata* to soil FT, aiming to reveal the physiological responses of pioneer and fibrous roots and explore the potential protective mechanisms of fibrous and pioneer roots under FT. Such knowledge is necessary to understand how roots of woody plants cope with soil FT.

Materials and methods

Plants and experimental design: This study was conducted at Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, Sichuan, China. The soil FT was designed based on the observed meteorological data from Maoxian, a town in the west of Sichuan. From December 1, 2010 to March 31, 2011, the mean air temperature in this area was about 0.06 °C, diurnal air temperature fluctuations ranged mainly from -5 to +5 °C, and several FT occurred during the experimental period. In addition, with the increase in air temperature, great diurnal temperature fluctuations were observed, which led to rapid FT in early March 2013. Thus we designed typical air temperature profiles of the FT treatment (Fig. 1 Suppl.). Namely, at first, the seedlings were subjected to 3 diurnal FT fluctuations with air temperature varying between -5 and +5 °C (air temperature decreased or increased by 0.85 °C h^{-1}, minimum temperature at 3:00 and maximum temperature at 15:00); then maintained for 2 d to ensure complete freeze under the minimum air temperature -5 °C, and then the plants were exposed to +5 °C for 2 d after thaw. The target air temperatures for the entire experiment ranged from -5 to +5 °C, and 3 repeated FT were performed. The air temperature of the control treatment was maintained at 5 °C. The air and soil temperature (at 5 cm below ground) were recorded using *DS1921G Thermochron iButton* data loggers (*Dallas Semiconductor*, Sunnyvale, CA, USA) (Fig. 1 Suppl.).

A total of 30 healthy, uniform 2-year-old *Picea asperata* Mast. seedlings were selected based on plant height (about 28 cm) from a local nursery, and transplanted into plastic pots filled with homogenized soil (mountain brown earth with pH 5.85, 62.70 g kg^{-1} organic C, 3.66 g kg^{-1} total N, 0.43 g kg^{-1} total P, and 7.92 g kg^{-1} total K). The seedlings were grown in natural environment with routine management for about 5 months. Afterward, the seedlings were exposed to treatment in a growth chamber (*Percival LT36VL*, model *IA*, Iowa, USA) from 3rd to 27th March 2013. Fifteen *P. asperata* seedlings were exposed to FT and other 15 seedlings were grown as controls. In both control and FT treatments, daily irradiance (photosynthetically active radiation) was maintained at about 800 ± 50 μmol m^{-2} s^{1} (based on the maximal measured data at sunny days of winter in Maoxian), and a relative humidity at 65 - 70 %. Before each FT procedure, all plants were watered to the field capacity, *i.e.*, the soil water content was about 38 %. To avoid systematic errors resulting from the possible differences in microclimates caused by different place in the chamber, the pots were rotated every three days.

All plants were harvested at the last day of the experiment (27th March 2013). Roots obtained from the pots were immediately taken to the laboratory, cleared from adhering soil particles and organic matter, rinsed with deionized cold water (4 °C), and dissected into fibrous and pioneer roots. The root parts were stored in plastic bags in a refrigerator at 4 °C and processed within 4 d. In each replication, root samples from 5 seedlings were mixed, so all measurements were carried out in triplicate.

Root vitality assay was performed using a modified triphenyltetrazolium chloride (TTC) test (Li *et al*. 2000). The root samples were blot-dried by filter paper and then cut into 1 - 2 mm long segments. Root vigour was expressed according to triphenylformazan reduction per 1 g of fresh mass per min.

Enzyme activities and ascorbic acid content: Crude enzyme extract was prepared according to Prochazkova *et al*. (2001). Briefly, root samples (0.2 g) were placed in an ice bath to preserve proteolytic activity and ground with 8 cm^3 of 0.1 M phosphate buffer (pH 7.5) containing 0.5 mM ethylenediamine tetraacetic acid (EDTA) and 1 mM ascorbic acid (ASA). The homogenate was then centrifuged at 15 000 *g* and 4 °C for 20 min and the supernatant was used to determine superoxide dismutase (SOD; EC 1.15.1.1) and peroxidase (POD; EC 1.11.1.7) activities. The SOD activity was determined by measuring the ability to inhibit the photochemical reduction of nitroblue tetrazolium, and one unit of enzyme activity was expressed as the amount of enzyme that reduced absorbance to 50 % compared with tubes that lacked the enzyme (Costa *et al*. 2002). The POD activity was assayed by estimating the increase in absorbance due to the formation of tetraguaiacol. One unit of enzyme activity was defined as the increase of absorbance (A_{470}) by 0.01 per minute. ASA was determined as described by Hodges *et al*. (1996).

Superoxide radical production, malondialdehyde content, and relative electrolyte leakage: The rate of O_2^- production was analyzed following the method of Sairam *et al*. (1997). Malondialdehyde (MDA), as a marker for lipid peroxidation, was determined according to Hodges *et al*. (1999) and calculated by the formula: MDA content = $6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$. Relative electrolyte leakage (REL) was measured as an indicator of cell damage. The roots (about 0.2 g) were rinsed three times with deionized water to remove the surface-adhered electrolytes, placed in tubes containing 20 cm^3 of deionized water, and then incubated on a shaker at 25 °C for 30 min. Electrical conductivity of the bathing solution (EC_1) was determined by conductometer *LC116* (*Mettler-Toledo Instruments*, Shanghai, China). The tubes were then incubated in a boiling water bath (100 °C) for 10 min, cooled to room temperature, and the electrical conductivity (EC_2) was measured. The REL was calculated as: $EC_1/EC_2 \times 100$ according to Martin *et al*. 1987.

Analyses of the content of free proline, proteins, sugars, carbon, and nitrogen: Proline was quantified by a colorimetric reaction with ninhydrin according to Bates *et al*. (1973) and determined from a standard curve of 0 to 20 µg of proline. The amount of soluble protein was quantified as described by Bradford (1976) using bovine serum albumin as a standard. Soluble sugars and starch were estimated spectrophotometrically using an anthrone-sulphuric acid reagent as described by Hansen and Moller (1975). Total nonstructural saccharides (TNC) was calculated as the sum of soluble sugars and starch. Root samples were oven-dried at 105 °C for 1 h, followed by drying at 70 °C to a constant mass. In the ground tissues, total carbon and nitrogen were analyzed using a *CHN* analyzer (Model *2100*, *Perkin-Elmer*, Norwalk, USA).

Statistical analyses: Two-way analysis of variance (*ANOVA*) was conducted to evaluate the effects of soil FT treatment and root types. The full factorial model was employed to find the interaction between soil FT and root type. Individual differences among means were determined by Duncan's test at $P < 0.05$. Principal component analysis (PCA) was used to reduce dimension, to show similarities as well as differences in the individual characteristics and responses of fibrous and pioneer roots to FT. The first two components of PCA were considered, for their cumulative eigenvalue was 82.58 %. All statistical analyses were carried out using the software package *SPSS v. 16.0* (*SPSS Inc.*, Chicago, USA).

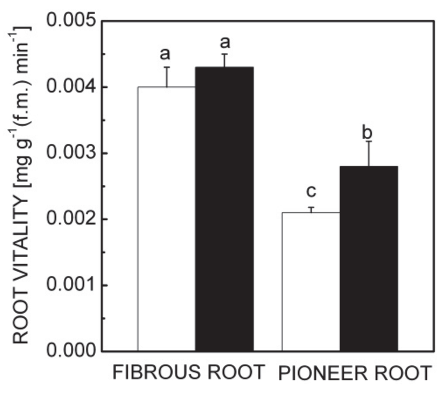

Fig. 1. Effect of soil freeze-thaw (FT) cycle on root vitality of the fibrous and pioneer roots. Means ± SDs, $n = 3$; different letters indicate significant differences ($P < 0.05$, Duncan test). Results of *ANOVA*: effect of FT at $P < 0.05$, effect of root type at $P < 0.001$, nonsignificant interactions.

Results

Root vitality was significantly affected by FT and root type, but no significant interaction of treatment × root type was observed (Fig. 1). FT treatment in spring increased root vitality of both fibrous and pioneer roots, but more in pioneer roots (by 36.1%). Nevertheless, the fibrous roots had significantly higher root vitality than pioneer roots.

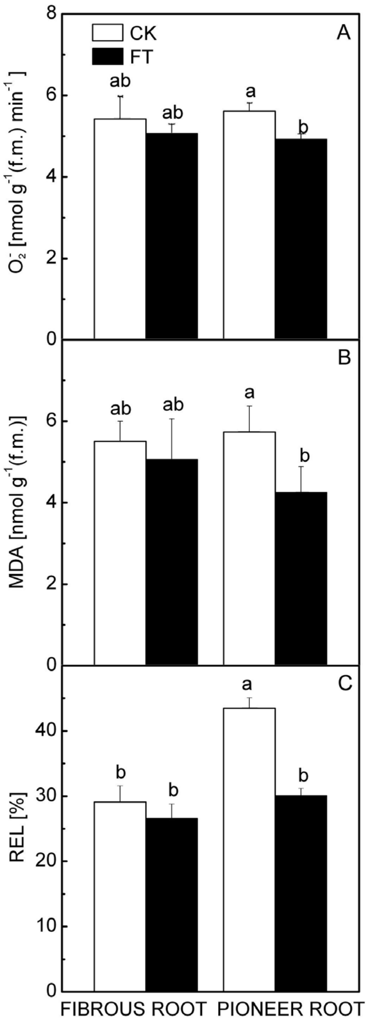

Fig. 2. Rate of superoxide radicals (O_2^-) production (*A*), malondialdehyde (MDA) content (*B*), and relative electrolyte leakage (REL) (*C*) of the fibrous and pioneer roots under soil freeze-thaw (FT) cycles. Means ± SDs, *n* = 3; different letters indicate significant differences ($P < 0.05$, Duncan test). Results of *ANOVA*: effect of FT for O_2^- and MDA at $P < 0.05$ and for REL at $P < 0.001$; effect of root type for for O_2^- and MDA non-significant, for REL at $P < 0.01$; interaction for O_2^- and MDA non-significant, for REL at $P < 0.001$.

The O_2^- production and MDA content were only affected by the FT treatment, and not by root type and their interaction (Fig. 2*A*,*B*). FT significantly reduced the rate of O_2^- production and MDA content. The FT treatment also decreased REL and pioneer roots had higher REL than fibrous roots. Moreover, the REL of pioneer roots in FT treatment was 43.5 % lower than that of control (Fig. 2*C*).

Ascorbic acid (ASA) content was slightly affected by FT in pioneer roots but not in fibrous roots (Fig. 3*A*). SOD activity was significantly affected by FT treatment and root type, but no significant interaction was observed. The SOD activity was decreased by FT but significantly only in fibrous roots, and fibrous roots had higher SOD activity than pioneer roots (Fig. 3*B*). Similarly, the POD

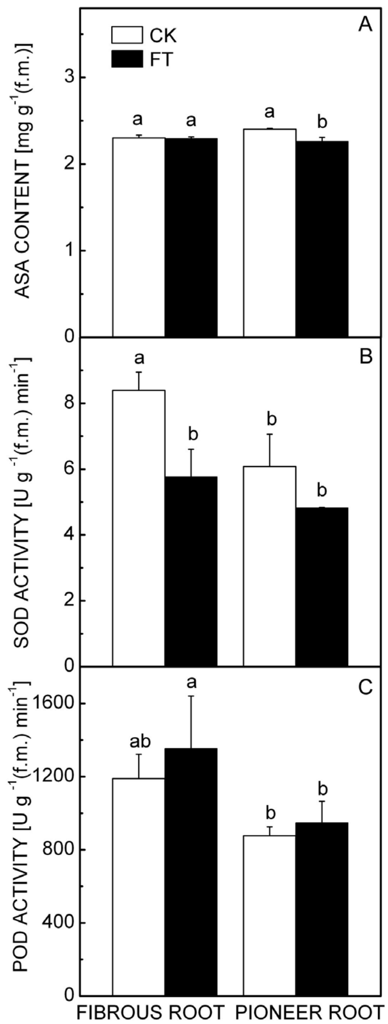

Fig. 3. Ascorbic acid (ASA) content (*A*), superoxide dismutase (SOD) activity (*B*), and peroxidase (POD) activity (*C*) of the pioneer and fibrous roots under soil freeze-thaw (FT) cycles. Means ± SDs, *n* = 3; different letters indicate significant differences ($P < 0.05$, Duncan test). Results of *ANOVA*: effect of FT for ASA content and SOD activity at $P < 0.01$, for POD non-significant; effect of root type for ASA non-significant, but for SOD and POD at $P < 0.01$; interactions for ASA and SOD at $P < 0.01$, for POD non-significant.

activity in fibrous root was higher than that in pioneer roots. The POD activity was affected only by root type, and not significantly by FT treatment and their interaction (Fig. 3*C*).

Compared with fibrous roots, pioneer roots had higher free proline, soluble sugar, and protein content. Especially, free proline content of pioneer roots was twice as much as in fibrous roots under FT treatment (Fig. 4*A*). Whereas, FT treatment increased free proline content, it decreased soluble sugar and protein content of pioneer roots, but no significant changes were observed in fibrous roots (Fig. 4*B*,*C*).

Significant effects of root type, FT treatment, and their interaction were observed in N content, C/N ratio, starch content, and TNC content (Fig. 5). Fibrous roots had higher N content than pioneer roots. FT treatment had different effects in two root types. In particular, FT treatment increased the N content in fibrous roots but decreased it in pioneer roots (Fig. 5*A*). The opposite trends were observed in C/N ratio, starch, and TNC content (Fig. 5*B*,*C*,*D*). The pioneer roots had higher values of these parameters than fibrous roots.

Discussion

Root systems have a complex organization with different roots having different functions for resource acquisition or storage (Waisel and Eshel 2002). Root vitality is an important metabolism index and has a direct relationship to the ability of resource acquisition. Besides, high N content and low C/N ratio are also the indices that reflect the strong metabolic activity of roots (Guo *et al.* 2004). Our results showed that fibrous roots have greater root vitality (Fig. 1), higher N content, and lower C/N ratio (Fig. 5*A*,*B*) than pioneer roots, indicating that fibrous roots have higher metabolic ability than pioneer roots. In contrast, pioneer roots exhibited higher soluble protein (Fig. 4*B*), soluble sugar (Fig. 4*C*), starch (Fig. 5*C*), and TNC content (Fig. 5*D*) than fibrous roots, which highlighted that pioneer roots were key storage organs. The results confirmed that fibrous and pioneer roots have great physiological differences. Fibrous roots primarily guarantee favourable water and nutrients supply (Xia *et al.* 2010, Zadworny and Eissenstat 2011), whereas pioneer roots accumulate sugars and proteins to facilitate growth of fibrous roots (Lyford 1980). Additionally, it was proved that pioneer roots have stronger growth plasticity than fibrous roots (Polverigiani *et al.* 2011).

Even if TTC reduction in this study was relatively lower than that found in other literature (Ruf and Brunner 2003), the root samples were alive based on their colour and texture. The lower root vitality might be caused by low temperature (Sardans *et al.* 2007) in spring and by treatments. Electrolyte leakage has good correlation with survival under freeze stress (Sutinen *et al.* 1992). Electrolyte leakages of both fibrous and pioneer roots were less than 50 % (Fig. 2*C*), which implied that FT treatment did not cause serious injury to the roots. Our findings are in line with the results of those employing moderate amplitude of FT cycles (Larsen *et al.* 2002, Sjursen *et al.* 2005). Similarly, Tierney *et al.* (2001) found that mild freeze temperatures (-5 °C) are insufficient to directly injure the winter-hardened fine roots, and Repo *et al.* (2014) detected that Norway spruce fine roots were not strongly affected by increasing frost during winter. In addition, root cold tolerance differs

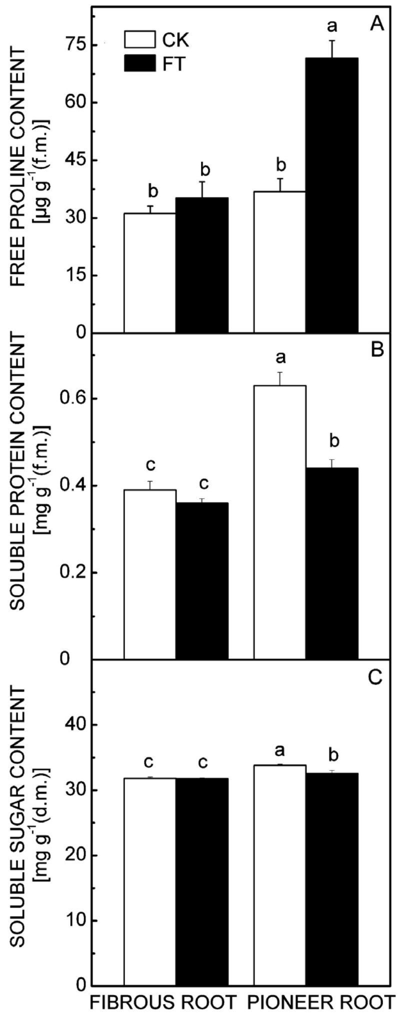

Fig. 4. Free proline content (*A*), soluble protein content (*B*), and soluble sugar content (*C*) of the first-order pioneer and fibrous roots under soil freeze-thaw (FT) cycles. Means ± SD, $n = 3$; different letters indicate significant differences ($P < 0.05$, Duncan test). Results of *ANOVA*: effect of FT, root type, and interactions on free proline content and soluble protein content at $P < 0.001$, on soluble sugar content at $P < 0.01$, 0.001, and 0.01, respectively.

among species; the whole root systems of Norway spruce, white spruce, Serbian spruce, and black spruce have a maximum cold tolerance up to -20 °C (Bigras *et al.* 2001). Our study was conducted in spring, and temperature was controlled in range of -5 °C to 5 °C; thus, the FT scenarios should belong to the mild FT treatment.

The FT treatment significantly decreased the rate of O_2^- production, MDA content, and electrolyte leakage (Fig. 2), accompanied by increased root vitality (Fig. 1) and proline content (Fig. 4*A*) of pioneer roots, but no significant effect of FT on these parameters was found in fibrous roots. These results confirmed that mild FT did not cause serious injury to the roots. Unexpectedly, root vitality was enhanced and REL was reduced in pioneer roots under FT. The latter result agrees with previous study that REL is reduced in pioneer roots by soil moisture deficit (Polverigiani *et al.* 2011), but differs with the finding of Kreyling *et al.* (2012). The main reason might be that the first-order pioneer and fibrous roots samples were used in this study and by Polverigiani *et al.* (2011), whereas a whole of fine roots were used by Kreyling *et al.* (2012). Moreover, the season (spring *vs.* winter), the cycle length, and soil temperature were also different.

In the present study, the proline content increased significantly in pioneer roots under FT treatment (Fig. 4*A*), whereas fibrous roots had higher antioxidant (mainly POD) activity than pioneer roots (Fig. 3*A*,*C*). Soluble sugars, proline, and some soluble proteins are the major constituents of osmoregulation in many plants (Xu *et al.* 2011, Zhang *et al.* 2012), and have crucial protective functions in plant adaptation to stresses (Jiang *et al.* 2013, Radic *et al.* 2013). Besides, soluble sugars are involved in reactive oxygen species (ROS) balance (Couee *et al.* 2006) and proline helps to protect the membrane integrity of plants (Kim *et al.* 2004), prevents protein denaturation, and acts as a free radical scavenger (Ben Ahmed *et al.* 2009, Kohler *et al.* 2009). Antioxidants have important roles in eliminating ROS, *e.g.*, SOD catalyzes the dismutation of O_2^- to O_2 and H_2O_2, and H_2O_2 is subsequently detoxified by POD and ASA (Halliwell and Gutteridge 1989). Our results suggest that fibrous roots increased the activity of antioxidant system in response to unfavorable environment, whereas pioneer roots accumulated osmolytes. In addition, the fibrous roots had higher SOD activity than pioneer roots under the control treatment (Fig. 3*B*), suggesting that more effective O_2^- detoxification occurred in fibrous roots. The results of PCA showed: TNC, starch, proline and N content, and C/N ratio were related on component 1, which represented mainly osmotic adjustment ability; whereas REL, root vitality, O_2^- , MDA and ASA content, and POD activity were related on component 2, which illustrated primarily the extent of damage and capacity of antioxidants (Fig. 2 Suppl.).

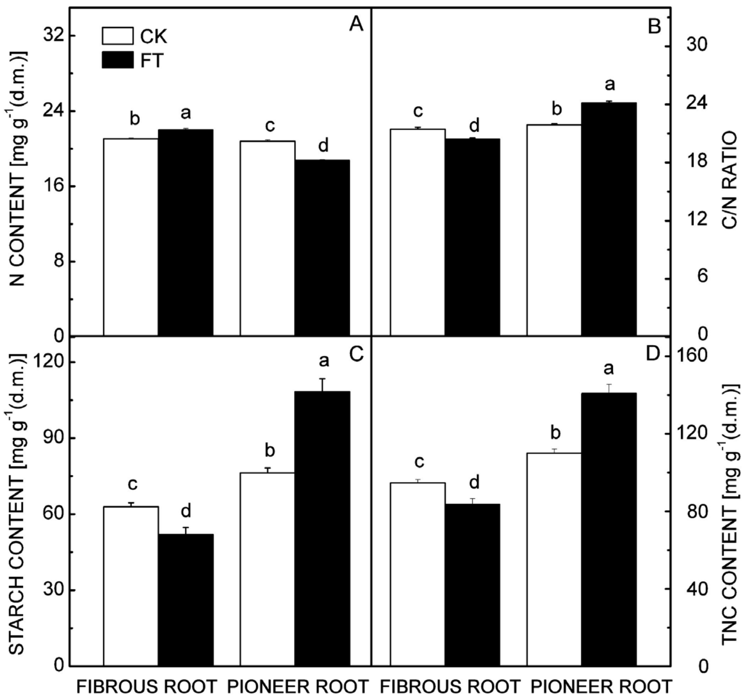

Fig. 5. Nitrogen content (*A*), C/N ratio (*B*), starch content (*C*), and total non-structural saccharide (TNC) content (*D*) of the first-order pioneer and fibrous roots under soil freeze-thaw cycles. Means ± SDs, *n* = 3; different letters indicate significant differences ($P < 0.05$, Duncan test). Results of *ANOVA*: effect of FT on C/N ratio, N content, starch, and TNC content at $P < 0.001$.

Conclusions

The present study confirmed that fibrous roots had higher metabolic ability than pioneer roots, whereas pioneer roots were the key storage organs. Under FT treatment, electrolyte leakages of both fibrous and pioneer roots were less than 50 %, which implied that FT scenarios of temperature in range of -5 °C to 5 °C were mild and did not cause serious injury to roots. Pioneer roots have higher tolerance to soil FT in spring than fibrous roots. They had different responses to FT: fibrous roots increased the activity of antioxidant system, whereas pioneer roots accumulated more osmolytes. Such knowledge can help us understand how roots of woody plants cope with soil FT cycles.

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