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*Full Length Article*

# Inhibitory Effects of Low Temperatures on the Rhizospheric Microorganisms and Apple Rootstock Growth

**Dongmei Lang[1,2], Deguo Lyu[1,2], Ying Wang[1] and Sijun Qin[1,2*]**
[1]*College of Horticulture, Shenyang Agricultural University*
[2]*Key Lab of Fruit Quality Development and Regulation of Liaoning province, Shenyang 110866, Liaoning, P.R. China*
[*]For correspondence: qinsijun1975@163.com

## Abstract

The contribution of nitrogen (N) supply is an essential source for the growth of apple trees from budding to fruiting. In new apple-producing regions of north China, such as the Shenyang area, air temperature rises rapidly, while in contrast, soil temperature rises very slowly. In such weather conditions, the amount of nitrogen absorbed by the roots is not sufficient for the growth and development of apple tree. In this research, the main attention has been paid to the effects of low soil temperature on rhizospheric microorganisms, soil enzyme activities, and the associated traits of seedlings such as nitrogen uptake and photosynthesis of *Malus baccata* Borkh. during early spring. Results showed that the bacteria and Actinomycetes activity decreased, while fungi activity in the rhizosphere increased. The bacterial ammonifiers and nitrogen-fixing bacteria activity increased, whereas nitrifying and denitrifying bacteria activity decreased in the seedling rhizosphere. Low temperature reduced protease activity but increased urease activity in the rhizosphere. No significant changes in phosphatase activity were observed. Nitrite reductase activity in the seedling roots decreased at low temperature, thereby reducing absorption of nitrogenous nutrients. Low temperature also reduced seedling chlorophyll content, thus reducing photosynthetic capacity. This finding implies that the low temperature of soil in early spring inhibited the metabolism of soil nitrogen in microorganisms and enzymes, and thus reduced the absorption ability and utilization of nitrogen in apple roots. As a result, physiological processes like photosynthesis were affected concomitantly. 

**Keywords:** Low temperature; Apple rootstock; Photosynthetic capacity; Root activity; Rhizospheric microorganism

## Introduction

Soil microbes and enzyme activities play important roles in the soil ecosystem, in particular, microbes and enzymes initiate processes such as plant litter decomposition, nutrient cycling, root nutrient uptake, growth, development, and health status (Edwards *et al*., 2006; Matthew *et al*., 2011). Thus, soil microbial populations and enzyme activities have gained increasing attention as the evaluation indexes of soil quality in the ecological environment become more complex (Enowashu *et al*., 2009). Soil microbial populations and enzyme activities are sensitive to low soil temperature (Vieira and Nahas, 2005; Matthew *et al*., 2011), plant species, and other factors. Low soil temperatures could influence the microbes by reducing their biomass, activities, as well as their community structure and diversity (Shishido *et al*., 2008). The variation in soil microbial populations and enzyme activities affects nutrient availability, root nutrient uptake, and plant growth (Rao and Tak, 2001).

The germination, blooming, and shoot growth of fruit trees require high amounts of nitrogen nutrients. Nitrogen fertilizers are usually applied to provide nutrition for budding and flowering of fruit trees, however, the absorption and utilization of soil nitrogen in apple roots is usually limited in cool apple-producing regions of North China because of the slow increase in ground temperatures in early spring and the reduced soil nutrient cycle and transformation efficiency caused by low microbe numbers and soil enzyme activity. This limits nutrient cycling and affects nitrogen and moisture absorption as well as root utilization (Boswell and Espie, 1998). Low soil temperature in early spring restricts apple production (Dong *et al*., 2001).

"Hanfu" apple, bred by Shenyang Agricultural University to resist to cold temperature, is cultivated in cool regions of North China over more than 70000 hectares. Cold areas such as Shenyang area, located in the north of the "Fuji" apple cultivation region, have become new regions of production in China. As the temperature of air rises rapidly, while the temperature of soil rises slowly, the growth and development process of apple trees is significantly different in these areas than that in warm areas, such as the Shandong province, there remains no scientific guidance for fertilization management in cold regions. We hypothesized that soil low temperature influences growth

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and development of orchard plants, perhaps the root has a decreased absorption ability, and the second reason is low temperature restrained nitrogen uptake ability of seedling root, which should be associated with soil enzyme activity of nitrogen transformation, and resulting in that low available nitrogen levels in soil. We further hypothesized that the various characteristic of tree seedling would be correlated with each other.

In order to study the effect of low soil temperature in early spring on orchard soil and plant, we characterized the physiology and metabolism of fruit trees at low temperatures by studying the effect of low temperature on microbial community, nitrogen-transforming bacteria, soil extracellular enzyme activity, seedling root absorption, and photosynthetic performance. Apple stocks of *Malus baccata* Borkh. widely used in cold areas, were cultured under controlled temperature conditions in order to develop basic guidelines for orchard soil management.

**Experimental Site and Design**

The experiment was conducted in an apple orchard at Shenyang Agricultural University (123°23`E`, 41°48`N; 50–60 m altitude), Liaoning province, China, in 2009. The area is cold, with a mean temperature of 7.7°C, and an accumulated temperature about 3414.2°C, or ≥10°C per year, with the highest monthly average temperature of 24.6°C, minimum monthly average temperature of -11.7°C (January), extreme minimum temperature of-30.6°C, and a frost-free period of about 153 d.

The apples blossom in early April and bud in early May. The experiment was conducted at the Shenyang Agricultural University (41°83′ N, 123°56′ E), and its elevation, averages of accumulated temperature, annual sunshine h, annual frost-free period, and mean annual precipitation were 76.2 m, 32.81°C, 2372 h, 146–163 d, and 721 mm, respectively. Seeds of *M. baccata* Borkh. were pre-soaked at 4°C for vernalization, sown in substrate on a plastic tray, and placed in a greenhouse in February 2009. The seedlings were transplanted into pots (12 cm × 13 cm, one seedling per pot) at the 5 leaf stage. The matrix for seedling cultivation was a mixture of orchard soil for "Hanfu"/GM256/*Mulus baccata* Borkh. (Rootstock-scion combination) and manure (farmyard manure from pig manure composting), at a 3:1 volume ratio. The chemical properties of the experimental soil were as follows: pH, 6.89±0.08; electric conductivity, 183.4±4.2 $\mu S \cdot cm^{-1}$; organic matter content, 52.7±0.7 $g \cdot kg^{-1}$; Nitrogen (N), 224.0±1.3 $mg \cdot kg^{-1}$; available Phosphorus (P), 278.3±3.9 $mg \cdot kg^{-1}$; and available Potassium (K), 296.6±4.6 $mg \cdot kg^{-1}$. All soil analyses were performed according to Soil Sampling and Methods of Analysis (Hart *et al.*, 1994). The strong seedlings were transferred to an artificial climate box about a month later, and grown under the following conditions: temperature, 25°C (day)/15°C (night); light intensity, 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$; illumination time, 12 h about 70–80% air humidity; The low-temperature treatments were conducted after 10 d in the artificial climate box. *M. baccata* Borkh. seedlings were grown under low temperature conditions at 15°C (day)/2°C (night). For the control, temperature was maintained at 25°C (day)/15°C (night). Experimental groups included *M. baccata* Borkh. at normal (BC) and low temperature (BL).

The experiments were conducted using a randomized block design with three replicates and one plot was sampled for each replicates, eighteen plants for each treatment and measured the indices for six times.

**Soil Sampling**

The soils were sampled at 0 to 5 d of each treatments and controls. The rhizosphere samples consisted of soil loosely adhered to the roots and soil that could be brushed or scraped off the root surface. Each treatment had four replicates of three plants per replicate (n=3). Samples for microbial analysis were placed inside plastic bags and sent to the laboratory. All visible root and fresh litter materials were removed from the samples then homogenized, sieved through 2 mm mesh, and used for soil microbial, soil enzyme and other index assay immediately.

**Soil Microbial Community Analysis**

Bacteria, fungi, actinomycetes, azotobacteria, and ammonifiers were isolated using the dilution plate technique (Woomer, 1994) with beef-protein media, Rose Bengal agar media, Gause's synthetic agar media, modified Ashby nitrogen-free media, and beef-protein agar media, respectively. The most probable number method (Zuberer, 1994) was used to count nitrobacteria and denitrobacteria in liquid media. Modified Stephenson media and peptone semisolid media were used to isolate nitrobacteria and denitrobacteria, respectively. Bacteria, fungi, actinomycetes, azotobacteria and ammonifiers were incubated between 25 and 28°C for 5 to 7 d to determine an accurate colony count. The nitrobacteria and denitrobacteria were incubated at the same temperature, for 15 d. Results were calculated and expressed as colony forming units (CFU) per gram dry weight soil ($CFU \cdot g^{-1}$ dry weight).

**Enzymatic Assays**

Urease, phosphatase and proteinase activities were assayed according to previously described methods (Wu *et al.*, 2004). In brief, 5 g of air-dried soil was mixed with 1.5 mL methylbenzene, 10 mL buffer, and 10 mL substrate solution in a reaction flask. The mixture was incubated at 37°C for 24 h. The following buffer and substrate solution were used: 0.1 mol $L^{-1}$ citrate buffer (pH 6.7) and 10% (w/v) urea solution for urease; 0.1 mol $L^{-1}$ citric acid buffer (pH 7.0) and 0.5% (w/v) disodium phenyl phosphate for phosphatase; and 0.2 mol $L^{-1}$ phosphate buffer (pH 7.4) and

1% gelatin solution for proteinase. After incubation, distilled water was added to produce 100 mL solution at 38°C, mixed carefully, and filtered. An aliquot of the filtrate (1 mL) was added to the reaction solutions with 4 mL of 0.1 mol $L^{-1}$ phenolate and 3 mL of 0.9% hypochlorite solution for urease; 5 mL of 0.0125 mol $L^{-1}$ boric acid buffer (pH 9.6) and 1 mL of 0.2% (w/v) 2, 6-dibromoquinone chloride for phosphatase; and 1 mL of 2% ninhydrin solution for proteinase. After color development for 30 min, absorbance was measured spectrophotometrically at 578, 660 and 560 nm, respectively. The soil urease, phosphatase, and proteinase activities were expressed as mg $NH_3$-N, mg phenol, and mg glycine produced by 1 g of dried soil per 24 h, respectively. Catalase activity was determined via the potassium permanganate ($KMnO_4$) titration method (Johnson and Temple, 1964). A solution of peroxide (0.3% $H_2O_2$) was added to the soil as the substrate. Activity was detected by potassium permanganate titration and expressed as 0.1 mol $L^{-1}$ $KMnO_4$ produced by 1 g dried soil for 20 min (mL $g^{-1}$ soil·20 $min^{-1}$).

Nitrate reductase (NR) activity in roots was determined according to Baki *et al.* (2000). The reaction medium (total volume, 1 mL; pH 7.8) contained 50 mM sodium phosphate, 10 µM flavin adenine dinucleotide, 1 mM dithiothreitol, 5 mM $KNO_3$, and 20 mM ethylenediamine tetra acetic acid. The reaction was induced by adding 200 µL extract and terminated after 5 min by adding 125 µL of 0.5 M zinc acetate solution. Nitrate formation was determined colorimetrically by adding 750 µL of 1% sulfanilamide in 3 M HCl and 750 µL of 0.02% N-naphtyl ethylenediamine hydrochloride. Absorption was then determined at 546 nm.

### Plant Indicators Assays

Root activity was determined using the 2,3,5-triphenyl tetrazolium chloride (TTC) reduction method of Yoshida (1996). Reduction of TTC is widely used as a simple and rapid assay for plant tissue respiration (Leigh and Mazur, 1975). The TTC is reduced by dehydrogenase in root tissue. Reduced TTC (which is red) is extracted with ethyl acetate and measured using a spectrophotometer at 485 nm. Root DH activity was expressed as the amount of TTC reduced per gram fresh root mass per h (µg $g^{-1}$ $h^{-1}$) and the chlorophyll content was calculated according to Arnon (1949). A CIRAS-2 portable photosynthesis analyzer (PP-system, USA) was used to determine the net photosynthetic rate. Handy-PEA (Hansatech, Britain) was used to determine the kinetics of chlorophyll fluorescence.

### Statistical Analysis

All the data are expressed as the mean of 3 replicates, except for the date of maximum photochemical efficiency (Φpo) and performance index (PI), for which 10 replicates were used to calculate the mean. Data are expressed as mean ± standard error. Statistical analysis was performed with SPSS 11.5 (SPSS 11.5 for Windows; SPSS Inc., Chicago, IL, USA) and compared using the Duncan's new multiple range test ($P<0.05$). Differences between values at $P < 0.05$ were considered statistically significant.

## Results

### Soil Microbial Activities in the Rhizosphere

The bacteria activity was reduced significantly in BL treatment for 1 d and 2 d after low-temperature treatments, and subsequently increased in 3 d under low temperature treatment (Fig. 1a). The bacteria activity was lower at low temperatures than at control temperatures. The bacteria activity was reduced by 26.1% in *M. baccata* Borkh. after 5 d treatment under low temperature (Fig. 1a).

The actinomycetes activity in *M. baccata* Borkh. rhizospheres initially increased, and then decreased under low temperature (Fig. 1b). After 5 d, the actinomycetes activity in *M. baccata* Borkh. decreased by 26.9% compared with the control treatment. The fungi activity in the *M. baccata* Borkh., rhizosphere rapidly increased at low temperature, then plateaued. After 5 d low temperature treatment, the fungi activity in *M. baccata* Borkh. increased by 49.9% compared with the control treatment (Fig. 1c).

### Nitrogen-transforming Rhizosphere Bacteria at Low Temperature

The ammonifier activity in *M. baccata* Borkh. rhizospheres increased sharply about 2.4-fold versus the control treatment at 1 d (Fig. 2a). However, this number decreased rapidly and became stable after 3 d of the low temperature treatment. After 5 d at low temperature, the quantity of ammonifiers in *M. baccata* Borkh. decreased by 16.8% in comparison to the control. The effect of low temperature was similar for nitrogen-fixing bacteria and ammonifiers, an initial increase was followed by a decrease (Fig. 2b). The azotobacteria activity in *M. baccata* Borkh. decreased by 24.5%, after 5 d under low temperature, compared with the control. Nitrifying bacteria oxidize ammonia to nitrite or nitric acid and release energy. The nitrifying bacteria activity in *M. baccata* Borkh. increased by 93.2% after 5 d at low temperature (Fig. 2c). The nitrobacteria activity in *M. baccata* Borkh. increased by 93.2% after 5 d at low temperature. Denitrifying bacteria participate in the reduction of nitrate or nitrite. The population trends of denitrobacteria in *M. baccata* Borkh. were similar to those of the nitrobacteria (Fig. 2d). The denitrobacteria activity in *M. baccata* Borkh. rapidly increased then decreased continuously in the low-temperature treatment. The lowest denitrobacteria activity was obtained at 3 d, after which the population steadily increased. The denitrobacteria activity in *M. baccata* Borkh. increased by 169.9% at low temperature.

**Table 1:** Effect of low temperatures on the soil enzyme activities in *M. baccata* Borkh. rhizospheres

| Treatments | Urease $NH_3$-N (g dw 24 $h^{-1}$) | Phosphatase mg Phenol (g dw 24 $h^{-1}$) | Proteinase mg Glycine (g dw 24 $h^{-1}$) | Catalase 0.1 mol $L^{-1}$ $KMnO_4$ (mL $g^{-1}$ soil 20 $min^{-1}$) |
|---|---|---|---|---|
| *Malus baccata* Borkh. at low temperature(BL) | 13.5 ± 0.2a | 0.44 ± 0.02a | 17.0 ± 0.7b | 26.3 ± 1.3a |
| *M. baccata* Borkh. at normal temperature(BC) | 12.3 ± 0.2b | 0.47 ± 0.03a | 22.1 ± 2.1a | 27.5 ± 1.2a |

BL and BC represent M. baccata Borkh. under low temperature, normal temperature, respectively. Error bars in this and the next figure represent one standard deviation (n = 4).This table summarizes data from a 5 day treatment. Different letters above the columns indicate significant differences between the means by Duncan's multiple range test ($P < 0.05$)

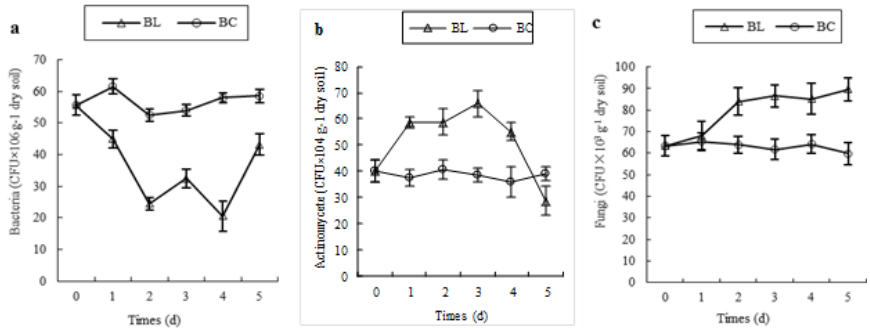


**Fig. 1:** Effect of low temperature on the number of bacteria (a), fungi (b), and actinomycetes (c) in soil of rhizospheres of *M. baccata* Borkh. BL and BC represent M. baccata Borkh. at low temperature, normal temperature, respectively. Error bars in this and the next figure represent one standard deviation (n = 4)

### Soil Enzyme Activities in the Rhizosphere at Low Temperature

Decrease in temperature increased urease activity by 9.7% in *M. baccata* Borkh. rhizospheres, but proteinase activity significantly decreased by 23.1% ($P<0.05$) (Table 1). Phosphatase activity and catalase activity in *M. baccata* Borkh. were unaffected at low temperature.

### Effect of Low Temperatures on Roots Nitrate Reductase Activity

Nitrate reductase (NR) activity increased in the roots of *M. baccata* Borkh. at 1 d, then decreased rapidly under low temperatures. At 5 d, the NR activity in *M. baccata* Borkh. decreased by 34.8% in comparison to the control (Fig. 3a). Root activity reflects the nutrient and moisture absorption capacity of the roots. Low temperature reduced root activity in *M. baccata* Borkh. seedlings over time (Fig. 3b). At 5 d, the root activity of *M. baccata* Borkh. decreased by 52.9% in comparison to the controls.

### Leaf Photosynthetic Capacity Lowered at Low Temperatures

Chlorophyll content decreased over time in the leaves of *M. baccata* Borkh. seedlings at low temperature (Fig. 4a). At 5 d, chlorophyll content in the leaves of *M. baccata* Borkh. seedlings decreased by 39.1%. ΦPo represents the maximum quantum efficiency of photosystem II (PSII). The net photosynthetic rate (Pn) of *M. baccata* Borkh. was significantly lower than that in normal temperature (Fig. 4b). At low temperature, Pn of *M. baccata* Borkh. seedlings decreased by 42.6%. The changes in ΦPo and Pn were similar in *M. baccata* Borkh. at normal temperature. ΦPo of *M. baccata* Borkh. seedlings decreased by 50.9% after 5 d at low temperature (Fig. 4c). The performance index ($PI_{ABS}$) is sensitive to drought, low temperature, and other abiotic stresses. $PI_{ABS}$ accurately reflects the state in the PSII light system. Low temperature decreased $PI_{ABS}$ values of M. baccata Borkh. seedlings by 37.2% at 5 d (Fig. 4d).

## Discussion

Soil temperature can strongly influence soil environment, development and growth of plants. The biological factors were affected significantly in the soil, while the effect on plants may have two ways, one is a direct impact on physiological processes (physiological metabolic processes are enzymatic reaction, with temperature three basis points), another roots uptake nutrition and water from soil which is restricted by substrate level, while substrate level is derived and restricted by soil microorganisms, enzymes and the other factors.

Rhizosphere microorganisms are an important component of the fruit tree ecosystem. The microbial population, activities, and community structure are closely related to the rhizosphere soil environment, such as humidity and temperature. Soil bacteria account for the majority of the rhizosphere microecosystem and have an important function in the transformation of material and energy. Many studies (Töwe *et al*., 2010; Yang *et al*., 2012) have indicated that reduced soil temperature inhibited reproduction of soil microorganisms in the wheat rhizosphere, including bacteria, fungi, and actinomycetes.

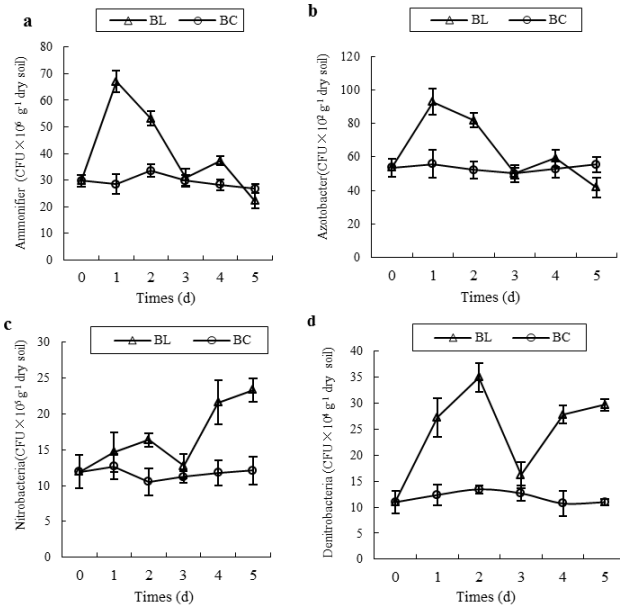


**Fig. 2:** Effect of low temperature on the number of ammonifiers (a), azotobacteria (b), nitrobacteria (c), and denitrobacteria (d) in *M. baccata* Borkh. rhizosphere. BL and BC represent M. baccata Borkh. at low temperature, normal temperature, respectively. Error bars in this and the next figure represent one standard deviation (n = 4)

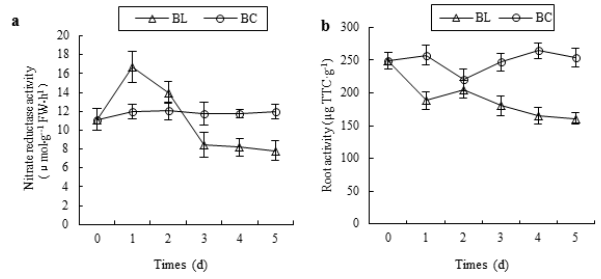


**Fig. 3:** Effect of low temperature on nitrate reductase activity (a) and root activity (b) of *M. baccata* Borkh. BL and BC represent M. baccata Borkh. at low temperature, normal temperature, respectively. Error bars in this and the next figure represent one standard deviation (n = 4)

We demonstrated that the number and proportion of rhizosphere bacteria decreased at low temperature, which is consistent with previous findings (Vieira and Nahas, 2005). In contrast, the number and proportion of fungi increased at low temperature. The comparison experiment of temperature effects on bacterial and fungal growth rates was reported previously, which indicated that fungi were more adapted to low temperature conditions than bacteria (Krumins *et al*., 2009). Nitrogen is one of the most important nutrients in soil. The microbial nitrogen groups, which affect nitrogen uptake, growth, and development of plants, are directly or indirectly involved in soil nitrogen

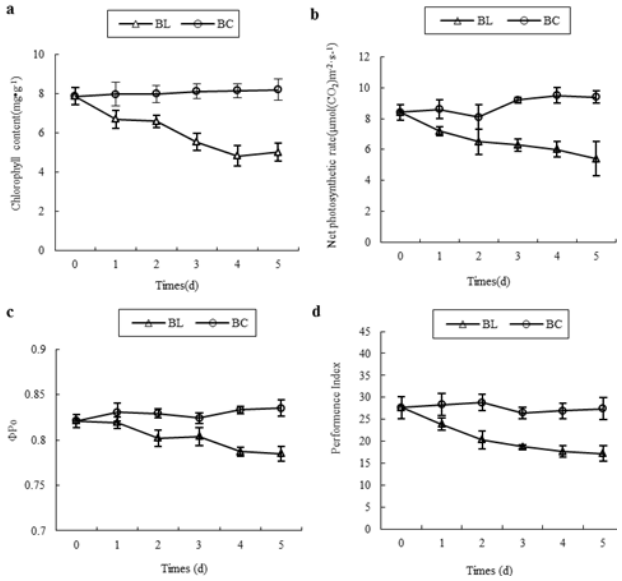


**Fig. 4:** Effect of low temperature on chlorophyll content(a), net photosynthetic rate (b), maximum photochemical efficiency(φpo) (c), photosynthetic performance index (d) of *M. baccata* Borkh. leaves

BL and BC represent *M. baccata* Borkh. at low temperature, normal temperature, respectively. Error bars in this and the next figure represent one standard deviation (n = 4)

cycling and energy flow (Dalias *et al*., 2002; Smith *et al*., 2010). The microbes that mediate soil nitrogen metabolism are sensitive to temperature. Therefore, nitrogen transformation in soil by nitrogen-transforming bacteria depends on temperature (Wang and Cai, 2004). In our study, low temperature increased the nitrobacteria and denitrobacteria activity but significantly inhibited nitrification and denitrification, similar to the findings in red soil (Zhang *et al*., 2009). However, other research by Bhatia *et al.* (2009) indicated that the nitrobacteria activity decreases at low temperature. The azotobacteria activity at low temperature decreased after 5 d, which is consistent with previous results (Lü *et al*., 2010). This study also showed that the ammonification of organic nitrogen in the soil decreased, whereas nitrification and denitrification increased, due to an increase in ammonifiers and an increase in nitrifying and denitrifying bacteria. Furthermore, due to the low level of ammonifiers, the transformation of organic residues into amino acids and ammonia was inhibited at low temperature. However, organic residues can easily be transformed into nitrate with sufficient nitrifying bacteria in the soil, which increases nitrogen loss. Therefore, low temperature reduced the nitrogen absorption capability of the roots (Feng *et al*., 2007), resulting in the loss of nitrogen in the soil and causing lower nitrogen utilization efficiency in cool regions in early spring.

Soil enzyme activities characterize soil fertility and are closely related to soil nutrient cycling. These processes are an important index for the soil ecosystem.

Urease, invertase, and phosphatase activities in Abies faxoniana reach their peak in summer and decrease in winter (Enwall *et al*., 2005). Our results show that urease activity in *M. baccata* Borkh. was increased, while proteinase activity decreased at low temperature, and the phosphatase activity was not temperature sensitive. Microorganisms are a major source of soil enzymes and changes in soil urease and protease activity may be attributed to changes in soil microbial communities associated with nitrogen transformation, the increase in urease activity at low temperature may be due to an increase in the population of denitrifying bacteria. A previous study showed that long-term addition of different fertilizers affected both the activity and the structure of the denitrifying communities in arable soil (Salvagiotti *et al*., 2008). Low temperatures constraints may have limited the supply of N or its uptake by the plant (Kudoh and Sonoike, 2002) which is similar to our results.

Low temperature influenced the microorganisms and nitrogen metabolism enzyme activities, limiting the transformation and utilization of available nitrogen in the soil. Low temperature also directly reduced the root uptake capacity. Nitrogen deficiency at low temperature also reduces chlorophyll synthesis, accelerates chlorophyll degradation, and inhibits photosystem function. Therefore, low temperature reduced photosynthetic performance in *M. baccata* Borkh. seedlings. Our previous study in the same apple orchard showed that nitrogen fertilization in autumn accompanied by applying a film covering in cold areas can improve the soil microorganism community structure and soil enzyme activity promote transformation and utilization of nitrogen in the soil, and enhance nitrogen storage in the tree. These results also indirectly indicated the effect of low temperature on soil microorganisms, enzyme activity, and physiological of plants.

## Conclusion

Low temperatures in early spring influence microorganisms and enzyme activities associated with nitrogen metabolism in *M. baccata* Borkh. seedlings. Available nitrogen transformation and utilization in the soil were also limited by low temperature, as was nitrogen absorption in the roots and photosynthetic performance in the leaves, thereby affecting plant growth and development. Since low temperatures in early spring could inhibit microbial nitrogen metabolism, root absorption, and nitrogen utilization in cold regions such as Shenyang, we recommend using polyethylene film to cover the soil, thus increasing soil temperature in early spring, and promoting nitrogen transformation and absorption in soil. Nitrogen storage in the trees is also effectively improved if fertilization occurs in the previous autumn. These two measures can be used to meet the demands of nitrogen supply during developmental processes such as sprouting, blossoming and leafing.

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