# Biochemical Adaptation of *Pinus pumila* on Low Temperature in Mt. Seorak, Korea

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## ABSTRACT

**We tested the hypothesis that alpine plants have special physiological and biochemical mechanisms in addition to their structural adaptation in order to survive under extreme conditions. The photosynthetic organs of *Pinus pumila* were used to examine the seasonal changes in sugar concentration, antioxidative enzyme, and lipid peroxidation. The concentrations of sucrose, glucose, fructose and reducing sugar were the highest in the leaves in April. But sugar contents in buds and inner barks did not respond sensitively on temperature change. Meanwhile superoxide dismutase (SOD) activity responded sensitively on the change of temperature and SOD in all tissues maintained high activity in April. Meanwhile anthocyanin content increased rapidly in June but the increase of anthocyanin content was not enough to prevent their tissues from the damage by the exposure of high temperature or other stress. In conclusion, under low temperature condition, *P. pumila* increased the concentration of soluble sugars and SOD activity in their tissues in order to overcome extreme environmental condition. But in summer, these stress defense system against high temperature might be disturbed slightly. This results in the increase of malondialdehyde (MDA) contents in three tissues by lipid peroxidation.**

***Key words :*** Low temperature stress, *Pinus pumila*, MDA, SOD activity, Sugar

## INTRODUCTION

The Siberian Dwarf Pine (*Pinus pumila* Regel) has been preserved strictly by law of The Ministry of Environment and Korea Forest Service, since the number of these species is very limited in the world and could possibly be exterminated by habitat destruction and fragmentation. The understanding of physiological characteristics as well as ecological characteristics of the naturally inhabited areas for the endangered plant is essential for the establishment of effective and efficient conservation practices for a valuable genetic resources like *P. pumila*. Hong et al. (2004) suggested that considering not only the relatively low level of genetic diversity, small population size, and geographic isolation from other populations, but man and/or wildlife-caused disturbances, it is necessary to provide suitable conservation methods for the population study

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immediately.

High mountain plants, like *P. pumila*, must have a very effective carbon assimilation mechanism due to a very short growing period. The extreme climatic conditions of high mountain zone, high irradiance, low temperature, rapid temperature change and a reduced $CO_2$ partial pressure creates unfavorable conditions for photosynthesis (Streb *et al.*, 1998; Germino and Smith, 2000). Previous studies revealed that alpine plants are highly efficient in photosynthesis at low temperatures and are also adapted to high irradiance (Körner and Larcher, 1988).

The biggest damage caused by the high light intensity in plants is the inactivation of D1 protein (located on PS II) and the catalase (CAT) enzyme. Low temperature stress has a similar effect upon PS II and CAT (Streb *et al.*, 1998). In addition, the alpine plants have a much more effective protection mechanism against oxidative damage compared with the plants growing in lower altitude regions (Wildi and Lütz, 1996; Streb *et al.*, 1998; Polle *et al.*, 1999 ).

Meanwhile, seasonal dynamics of the *P. pumila* shoots were characterized by simultaneous replacement of old needles with new needles in the early autumn, thus avoiding any loss of canopy photosynthetic production during the growing season. Increases in needle longevity and fascicle density were associated with declining air temperature and increasing wind exposure. Needle longevity and fascicle density were characteristics of adaptive plasticity in *P. pumila* that prevent a reduction in growth potential in the stressful conditions of alpine regions (Kajimoto, 1993).

Therefore, we tested the hypothesis that alpine plants must have special physiological and biochemical mechanisms in addition to their structural adaptation in order to survive under extreme conditions. To testify the hypothesis, we examined the seasonal changes in carbohydrate concentration, antioxidative enzyme, and lipid peroxidation in the photosynthetic organs.

## MATERIALS AND METHODS

### Plant materials

The Siberian Dwarf Pine is a native to northeastern Asia, including Korea, Japan and Siberia. This shrubby pine ranges from 1-3 m in height, exceptionally up to 5 m, but may have individual branches that extend farther along the ground in length. The needles are formed in bundles of five and are about 4-6 cm long. The cones are 2.5-4.5 cm long, with large nut-like seeds. The seeds are harvested and dispersed by the spotted nutcracker.

In this study, stem samples including needle leaves, buds, and inner barks were collected from five individuals of *P. pumila* that have inhabited in the neighborhood of Daecheong-Bong of Mt. Seorak in April, June and September, 2004. Altitude of this area is 1750 m, and annual temperature, precipitation, and snow showed in Fig. 1. Collected samples were carried to the laboratory in portable freezers (4℃).

### Ethanol-soluble sugars and water-soluble polysaccharides

Samples of leaves, buds, and inner barks were immediately frozen in liquid nitrogen, freeze dried and subjected to a triple extraction of ethanol-soluble sugars (ESS) by boiling in 80% ethanol. Sugar were analysed with high performance liquid chromatography (TSP, Ca, USA), on a Prevail Carbohydrate ES column (250 × 4.6㎜, 5㎛; Alltech Associates, Inc., USA), by isocratic elution with acetonitrile/$H_2O$ (72/28, v/v) for 30 min. Flow rate of the mobile phase was 0.8㎖ $min^{-1}$. Sugars were detected with ELSD detector (ELSD 200, Alltech Associates, Inc., USA).

Water-soluble polysaccharides were extracted with hot water from the pellets of the 80% ethanol extractions. The pellets were freeze-dried, then suspended in deionized water and kept at 60℃ for 60 min for pseudobulbs and 30 min for leaves. The samples were centrifuged at 10,000 × g and the soluble

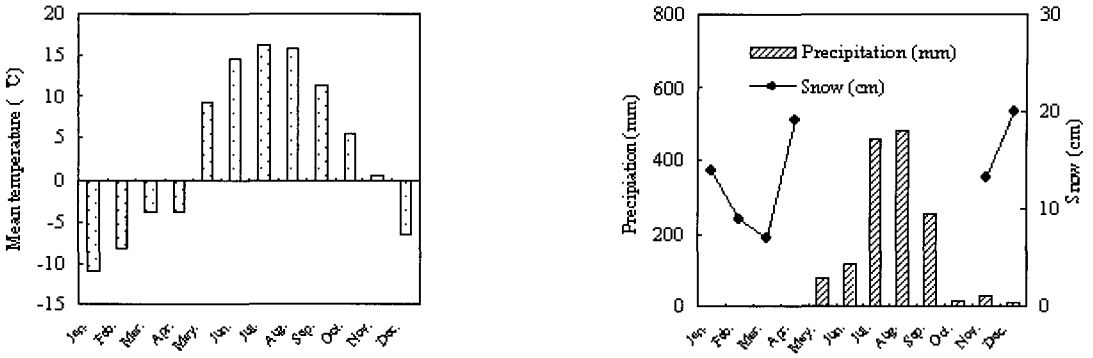



Fig. 1. Monthly changes of mean temperature, precipitation and snow at the Daecheong-bong of Mt. Seorak from January to December in 2004.

polymers in the supernatant were considered the water-soluble polysaccharide fraction and subsequently used for sugar determination and analysis. This procedure was repeated three times from the pellet suspension step. Acid hydrolysis of the water-soluble samples was performed with 4-5㎎ freeze dried extract in 72% (w/w) $H_2SO_4$ at 30℃ for 45 min, followed by 4% (w/w) $H_2SO_4$ for 1h at 120℃ (1.5 atm) in an autoclave. After acid hydrolysis, the samples were neutralized with $Ba(OH)_2$. The resulting monosaccharides were analysed by HPLC as described above.

**SOD activity and MDA content**

Leaves of the freshly harvested plants were used for the determination of SOD activity and MDA content. SOD activity assay per 100 mg protein was measured using the NBT (nitro blue tetrazolium)-xanthine oxidase method (Beauchamp and Fridovich, 1971). MDA assay followed the method of Ohkawa *et al.* (1979) and Kiyoshi *et al.* (1999), with a slight modification. A 0.5 g sample of primary leaves was ground with liquid nitrogen, then homogenized with 5mL of 50mM potassium phosphate buffer (pH 7.0) and 0.1% (w/v) butylated hydroxytoluene. The homogenate was centrifuged at 12,000 g for 20min at 4℃, and the resulting supernatant was used for MDA determinations. Lipid peroxidation was determined as the absorbance at 532 nm, and the amount of MDA was calculated by its extinction coefficient (155 $mM^{-1}$ $cm^{-1}$).

**Anthocyanin**

A 0.1 g sample of sliced leaves, buds and inner barks were taken from stems and extracted for 4 h in 10 ml of 0.1% HCl-MeOH at room temperature. Absorption of the extracts at three wavelengths, 650, 620 and 530 nm, were determined, and the absorbance of the anthocyanin extract was determined by means of the formula ($A_{530}$-$A_{620}$)-0.1($A_{650}$-$A_{620}$) (Proctor, 1974). The anthocyanin content was determined by using a molar extinction coefficient of $3.43 \times 10^4$ (Francis, 1982).

## RESULTS

**Ethanol-soluble sugar and water-soluble polysaccharides**

Our results for soluble sugars and polysaccharides in the tissues of *P. pumila* showed significant differences among parts and months, and interaction of parts and months presented (Table 1). Exceptionally sucrose, non-reducing sugar, represented significant difference among not parts but months. Sucrose content was highest (64.8 mg/g) in the leaves of *P. pumila* in April

Table 1. Changes in soluble sugar content in leaves, buds, and inner barks of *P. pumila* collected from Mt. Seorak on April, June and September, 2004. Each data point represents the mean of five replicates±standard deviation.

| Sugars | Month | Leaves | Buds | Inner barks | Significant level |
|---|---|---|---|---|---|
| | | mg/g | | | |
| Sucrose | April | 64.8±11.0 | 54.0±14.3 | 49.1±6.4 | Part (P) : N. S. |
| | June | 47.0±8.5 | 59.8±19.0 | 48.6±6.0 | Month (M) : *** |
| | September | 40.3±2.9 | 32.0±10.8 | 46.0±3.4 | P×M : N. S. |
| Glucose | April | 30.3±6.3 | 2.9±0.9 | 6.6±2.2 | Part (P) : *** |
| | June | 24.2±2.0 | 8.8±2.7 | 13.5±2.4 | Month (M) : ** |
| | September | 14.5±2.0 | 7.4±1.9 | 13.8±1.1 | P×M : *** |
| Fructose | April | 1.5±1.1 | 70.5±5.7 | 59.9±5.9 | Part (P) : *** |
| | June | 1.4±0.6 | 21.4 ±8.7 | 30.7±5.4 | Month (M) : *** |
| | September | 0.1±0.04 | 32.1±7.2 | 15.2±3.6 | P×M : *** |
| Reducing sugar | April | 132.7±10.4 | 175.5±22.1 | 75.0±8.7 | Part (P) : *** |
| | June | 122.7±9.3 | 76.2±9.3 | 80.7±3.2 | Month (M) : *** |
| | September | 93.5±1.7 | 127.8±8.7 | 72.3±4.2 | P×M : *** |

N. S. : not significant, ** : P<0.01, *** : P<0.001

and lowest (32.0 mg/g) in buds in September. Glucose level was observed highest in leaves and lowest in buds. Glucose content in leaves was highest (30.3 mg/g) in April, but it decreased progressively together with seasonal change and finally showed the lowest content (14.5 mg/g) in September. However, glucose in inner barks, unlike leaves, represented higher content in June and September than April.

Fructose in leaves was detected only by a little amount, but fructose in buds and inner barks represented high level. In the buds, fructose content was highest, 70.5 mg/g, in April, but its content decreased largely in June and September. In inner barks, fructose content was also highest in April (59.9 mg/g), after that decreased progressively up to 15.2 mg/g in September. Reducing sugar in leaves showed the highest level (132.7 mg/g) in April, and decreased linearly together with seasonal change. Meanwhile reducing sugars in buds showed rapid reduction in June, and increased again in September. Reducing sugar content in inner barks was maintained consistently during the whole season, and their contents were in the range of 72.3~80.7 mg/g.

Polysaccharides in the leaves, buds and inner barks represented the highest level in June, but decreased largely in September (Fig. 2). In special, polysaccharides level in inner barks was higher than leaves and buds in April and June, but it became lower than level in leaves in September.

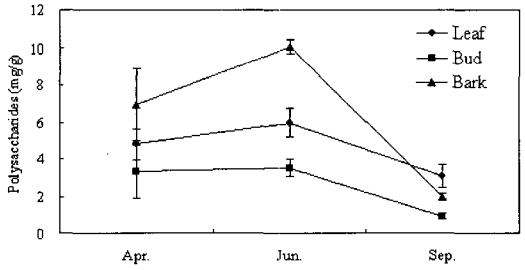



Fig. 2. Changes in polysaccharides content in leaves, buds, and inner barks of *P. pumila* collected from Mt. Seorak on April, June and September, 2004. Each data point represents the mean of five replicates±standard deviation.

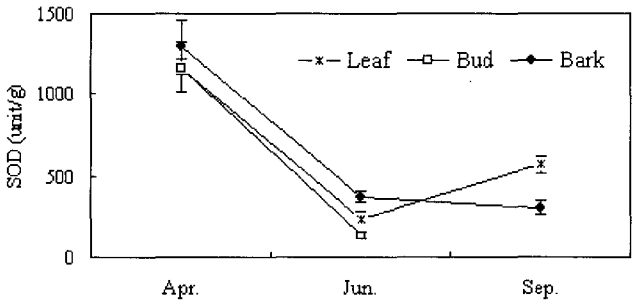
**Biochemical parameters**

SOD activity in the tissues of *P. pumila* represented highest in April, decreased largely in June, and increased again in September (Fig. 3A). SOD activity in inner barks was higher in comparison with leaves and buds in April and June, and in September SOD activity was higher in leaves than in inner barks.

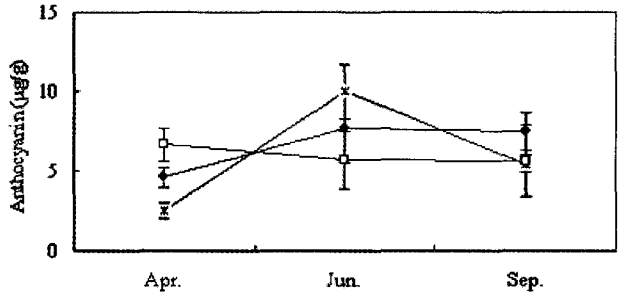
Anthocyanin content was significantly different among sampling months and parts (Fig. 3B). Anthocyanin content in leaves was highest (9.94 μg/g) in June and lowest (2.49 μg/g) in April. In addition, anthocyanin content in inner barks was lowest (4.61 μg /g) in April like content in leaves. However anthocyanin content in buds wasn't correlated with seasonal changes.

MDA content showed relatively high level in whole

(A) SOD activity




(B) Anthocyanin




(C) MDA

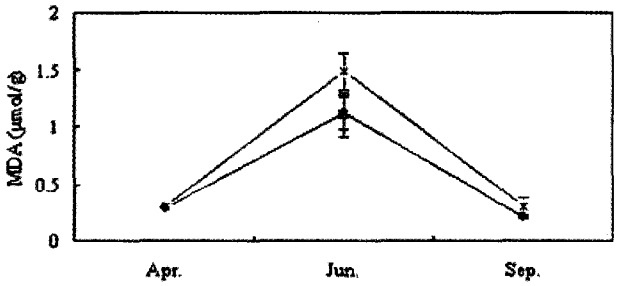



Fig. 3. Changes in SOD activity, anthocyanin content and MDA content in leaves, buds, and inner barks of *P. pumila* collected from Mt. Seorak on April, June and September, 2004. Each data point represents the mean of five replicates± standard deviation.

parts in June, and it was in the range of 1.10-1.48 $\mu$ mol/g (Fig. 3C). In special, MDA content in the leaves represented highest in June. Meanwhile, MDA content in tissues was not significantly different between April and September.

## DISCUSSION

The high mountain vegetation consists of plants, which have developed adaptations to low temperature and high irradiance conditions. These adaptations are combined with the rapid metabolic changes and biochemical rearrangements, which include antioxidant equipment (Wildi and Lütz, 1996).

In stress tolerance of plants, the soluble compounds containing sugars, proline, soluble proteins and phenolics have an important role. In drought and low temperature conditions, sugar (Chatterton *et al.*, 1987) and proline (Thomos and James, 1993; Oncel *et al.*, 2000) concentrations showed an increase.

In our results, the concentrations of sucrose, glucose, fructose and reducing sugar were the highest in the leaves of *P. pumila* in April that monthly mean temperature was very low. The result means that sugar concentration in leaves responded sensitively on the change of temperature, and leaves of *P. pumila* increased sugar contents in order to overcome low temperature stress.

In nature, plant cells subjected to chilling temperatures induce the accumulation of soluble sugars consisting mainly of sucrose, glucose and fructose (Gounaris, 2001; Kontunen-Soppela *et al.*, 2002), and it is well established that in *Pinus* species there is a relationship between carbohydrate concentration and frost hardness (Aronsson *et al.*, 1976; Ögren *et al.*, 1997; Greer *et al.*, 2000).

However, unlike leaves, sugar contents in buds and inner barks did not respond sensitively on temperature change. The results demonstrated that their morphological and anatomical features had great capacity for enduring cold stress under winter conditions.

In a similar way to sugar content, SOD responded sensitively on the change of temperature (Bowler *et al.*, 1992; Leng and Qi, 2003). In our study, SOD in all tissues maintained high activity in April, so that the superoxide radicals accumulated in cells under stress during the winter could be scavenged rapidly to prevent the tissues of *P. pumila* from oxidative damage caused by superoxide radicals. High activity of SOD protected the tissues of *P. pumila* to some degree from low temperature injury. In addition, the fact that SOD activity was higher in alpine plants is related to defense against the free radical formation and photoinhibition triggered by the low temperature stress (Streb *et al.*, 1998).

However, *P. pumila* maintained at relatively low SOD activity during the summer, with very high temperature and drought wind. This results indicated that SOD in the tissues of *P. pumila* was not sensitive on high temperature, and the alpine plant had the other defence mechanisms in order to tolerate against high temperature stress.

In general, anthocyanin is one of the flavonoid compounds that have strong reducing power, and can be induced to accumulate rapidly under low temperatures (Parker, 1962; Leng *et al.*, 1993, 1995). In addition, anthocyanin accumulates rapidly in plant tissues from autumn to winter, and reached its maximum level in mid-December (Leng *et al.*, 2000), and low temperatures increase and elevated temperatures decrease anthocyanin concentration (Christie *et al.*, 1994; Dela *et al.*, 2003). In our study, anthocyanin content increased rapidly in June (Fig. 3), but the increase of anthocyanin content was not enough to prevent their tissues from the damage by the exposure of high temperature or other stress.

Therefore, the increase of MDA content in the leaves

during the summer means that active oxygen radicals by high temperature or other stress was not removed completely in tissues (Fig. 3). When plants are exposed to low temperature stress, active oxygen species (AOS) such as superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen, can accumulate in vivo and can lead to lipid peroxidation (Wise and Naylor, 1987; Hariyadi, 1993). MDA, the final product of lipid peroxidation, can make proteins intercross, and be conjugated. This destroys seriously in the lipid structures and functions and disrupts normal metabolism. The MDA is often used as an index of cell oxidative damage under environmental stress (Shen and Wang, 1997).

Meanwhile anthocyanin content in buds and inner barks, unlike SOD, didn't showed seasonal changes. That is, buds and inner barks did not respond sensitively due to buffering effects of structural and morphological characteristics, like the results of sugars.

In conclusion, we found out that alpine *P. pumila* has been influenced by the low temperature stress in winter and high temperature stress in summer. Under low temperature condition, *P. pumila* increased the concentration of soluble sugars and SOD activity in their tissues in order to overcome extreme environmental condition. But in summer, these stress defense system against high temperature might be disturbed slightly. This results in the increase of MDA contents in three tissues by lipid peroxidation.

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