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Responses in the physiology and biochemistry of Korean pine (*Pinus koraiensis*) under supplementary UV-B radiation

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Abstract

The effect of supplementary UV-B radiation on Korean pine (*Pinus koraiensis* Sieb. et Zucc) was investigated. Compared with the control, the T1, T2, and T3 UV-B treatments increased by 1.40, 2.81, and 4.22 kJ m^{-2} d^{-1}, respectively. Gas-exchange parameters, photosynthetic pigment concentrations, contents of secondary metabolites, epicuticular wax, free radical, malondialdehyde (MDA), and the activities of antioxidant enzymes were determined after 40 d of exposure. The concentrations of chlorophyll (Chl) *a*, Chl *b*, total Chl, carotenoid (Car), and the ratio Chl *a*/*b* in the pine needles were in the following order: T1 > T2 > T3. Compared with the control, the contents of flavonoids and epicuticular wax significantly decreased in all levels of supplementary UV-B radiations ($p<0.05$). Moreover, the contents of hydrogen peroxide (H_2O_2) and MDA significantly increased with the enhanced UV-B radiations ($p<0.05$). Korean pine can increase the catalase, ascorbate peroxidase, and superoxide dismutase activities to prevent oxidative stress by supplementary UV-B radiation. However, its defence mechanism is not efficient enough to prevent UV-B-induced damage.

Additional keywords: epicuticular wax; Korean pine; photosynthesis; UV-B radiation.

Introduction

Over the last 50 years, stratospheric ozone has decreased by approximately 5%. This phenomenon is mainly caused by the release of ozone-destroying anthropogenic pollutants, such as chlorofluorocarbons (Pyle *et al.* 1997), which cause higher levels of UV-B (280–320 nm) radiation on the Earth′s surface (Surabhi *et al.* 2009). Failure of protection from UV-B may result in a wide range of morphological, physiological, and metabolic responses, such as altered plant growth, reduced yield, damage to photosystem II (PSII), and decrease in chlorophyll content (Saile-Mark and Tevini 1997, Germ *et al.* 2005).

UV-B radiation generally stimulates protective responses in plants. These responses increase the amounts of flavonoids (Lavola *et al.* 2003). Antioxidant enzymes, such as peroxidase (POD) and superoxide dismutase (SOD) are also activated. These enzymes scavenge free radicals from oxygen, and offer protection to lipids, proteins, and nucleic acids (Heinonen *et al.* 1998, Jain *et al.* 2004). The configuration of wax deposited on leaf surfaces influences radiation interception (Barnes *et al.* 1996). Certain wax surface structures (epicuticular wax crystalloids) increase the reflectance of solar radiation (Clark and Lister 1975, Schulze *et al.* 1980) and DNA-repair systems. However, these effects depend on the sensitivity of the species and their inherent abilities to attenuate the incoming UV-B radiation (Sullivan and Teramura 1988, Day 1993, Lavola *et al.* 2003). An examination of more than 200 plant species reveals that approximately 20% are sensitive, 50% are mildly sensitive or tolerant, and 30% are completely insensitive to UV-B radiation (Teramura 1983). Conifers are

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Abbreviations: P_N/PAR – light-response curves of photosynthesis; APX – ascorbate peroxidase; *ANOVA* – analysis of variance; Car – carotenoid; CAT – catalase; Chl – chlorophyll; CK – control; FM – fresh mass; H_2O_2 – hydrogen peroxide; I_C – compensation irradiance; I_m – saturation irradiance; MDA – malondialdehyde; ·OH – hydroxyl radical; PAR – photosynthetic active radiation; $P(I_m)$ – maximum net photosynthetic rate; P_{max} – light-saturated photosynthetic rate; P_N – net photosynthetic rate; POD – peroxidase; PS – photosystem; R_D – respiration rate; SEM – scanning electron microscopy; SOD – superoxide dismutase.
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considered to be tolerant to UV-B because the epidermal layer of mature needles effectively screens UV-B radiation (Day 1993). However, UV-B can penetrate into the needles during their development (DeLucia *et al.* 1992, Laakso *et al.* 2000) and damage the photosynthetic apparatus. Therefore, the photosynthetic capacity of needles is reduced (Sprtova *et al.* 1999). The penetration of UV-B depends on the thickness of the epidermis, as well as the concentration, quality, and location of UV-absorbing compounds (Day *et al.* 1993, Turtol *et al.* 2006).

Korean pine (*Pinus koraiensis* Sieb. *et* Zucc) is a native coniferous species in China. This pine is mainly distributed in the northeastern part of Asia, from the northeast of China throughout the Korean peninsula up to the centre of Japan (Xu and Yan 2001). Intensive studies on Korean pine have been performed because of the value of its good quality wood (Wu 1956, Li 1997). Unfortunately, the responses of Korean pine to supplementary UV-B radiation are not well understood. In fact, measurements of several physiological parameters, such as the gas exchange parameters, levels of UV-B absorbing compounds, and Chl contents, have been proved as useful indicators of UV-B tolerance or sensitivity (Caldwell *et al.* 1998). The different sensitivities of plants are partially explained by their abilities to respond to UV-B through the induction of defensive pathways (Creelman and Mullet 1997).

The present study aims to understand the physiological and biochemical characteristics of Korean pine under UV-B radiation, and to estimate its sensitivity and defence mechanisms under increased UV-B radiation.

Materials and methods

Plant material and growth conditions: Three-year-old saplings of Korean pine were identified and transplanted from the Mao'er Mountain Forest Farm, Heilongjiang Province, China in October 2008. The morphological data of the Korean pine saplings are shown in Table 1. The plants were sown in 30 cm diameter cups containing loam soil [2.5% organic matter, 200 mg(N) kg^{-1}, 90 mg(P) kg^{-1}, and 200 mg(K) kg^{-1}] and cultivated in a greenhouse for nine months before exposure to UV-B radiation. A total of 100 cups of Korean pine were sown for each treatment. The samples were maintained in a 12 h day:12 h night cycle with the day/night temperature at 28/20°C, respectively. The relative humidity was between 60% and 70%. The plants were inspected daily and watered as required.

Under the control conditions (CK), the light intensities of photosynthetic active radiation (PAR) (400–700 nm) and the light intensity of UV-B (280–320 nm) were 2052.5 and 3.07 μW cm^{-2}, respectively. The daily biologically effective UV-B irradiance ($UV\text{-}B_{BE}$) of the control was 1.32 kJ m^{-2} d^{-1}. In comparison, under the T1, T2, and T3 UV-B treatments, the plants were exposed to increased supply of UV-B radiation using UV-B fluorescent tubes (303 nm, 40 W; *Beijing Electric Light Source Research Institute*, China) from 6:00 to 18:00 every day. Compared with that of the control, the daily biologically effective irradiance ($UV\text{-}B_{BE}$) of the T1, T2, and T3 UV-B treatments was increased by 1.40, 2.81, and 4.22 kJ m^{-2} d^{-1}, respectively. These values were calculated using the generalized plant action spectrum normalized at 300 nm (Caldwell 1971). The reported measurements were daily averages over the experimental period. The UV-B radiation was maintained at the specified levels (measured at the top of the seedlings) throughout the experiment by adjusting the lamp-to-plant canopy distance. The light intensity was determined using *AvaSpec 2048-2* (*Avantes BV*, Netherlands). Other environmental conditions in the treatments were kept constant. The UV-B fluorescent tubes were wrapped in cellulose acetate foil to prevent radiation from other UV ranges. The cellulose acetate foil was replaced twice each week with a fresh piece that had been pre-exposed for 12 h to avoid degradation effects. Gas-exchange parameters, as well as physiological and secondary metabolites, were determined after 40 d of exposure to UV-B radiation.

Light-response curves of photosynthesis (P_N/PAR): The youngest, fully expanded third or fourth needles from the main axis terminal were used to measure P_N/PAR curves after 40 d of treatment. Gas exchange was determined at nine levels of PAR (0, 50, 100, 200, 500, 800, 1,000; 1,500; and 2,000 μmol m^{-2} s^{-1}) at 400 ± 1 μmol mol^{-1} (CO_2) concentration, 28 ± 0.5°C leaf temperature, and 70% ± 1% relative humidity. Irradiance was gradually increased to reach the incident PAR of 2,000 μmol m^{-2} s^{-1}. Each PAR was left for 5 min to obtain a steady state prior to measurements. Three measurements were automatically recorded at 2-min intervals for each PAR per leaf. Gas exchange was measured using a portable photosynthetic gas analysis system with a LED radiation source (*LI-COR 6400*, *LI-COR*, USA). The P_N/PAR curves were fitted using a photosynthesis light-response curve-fitting model developed by Ye (2007).

Photosynthetic pigments and UV-B absorbing compounds: Pigments were extracted from the needles used for photosynthetic measurements. Pigment analysis was performed in accordance with the methods described by Wellburn (1994). A total of 10 mg of fresh mass (FM) of needles were extracted with 2 ml of dimethyl sulfoxide for 12 h in the dark at 45°C. Total Chl, Chl *a*, Chl *b*, and Car contents were calculated at the absorbance levels of 665, 649, and 480 nm, respectively.

UV-B absorbing compounds, mainly flavonoids (Kinnunen *et al.* 1999) were extracted from the needles

(0.5 g) by immersing the needles in 10 ml of acidified methanol (MeOH:H_2O:HCl = 79:20:1, v/v). The relative UV-B absorbing compounds of flavonoids were determined using a spectrophotometer (*Shimadzu UV-2550*, Japan) at 300 nm.

Hydroxyl radical (·OH): Spectrophotometry as previously described by Steiner and Babbs (1990) was used to measure ·OH. Approximately 1 g of needles prefed with 0.6 mM DMSO were frozen with liquid N_2. The resulting powder was extracted with 10 ml of distilled water, and centrifuged at 12,000 × *g* (*Sigma 3K30*, Germany) to remove solid substances. An equal volume of toluene/*n*-butanol (3:1, v/v) was added into the supernatant. The lower aqueous phase was transferred to a test tube containing 2 ml of 15 mM fast blue BB salt (freshly prepared and kept in the dark). Ten minutes were allowed for product development at room temperature without light. Then, 3 ml of toluene/*n*-butanol (3:1, v/v) were added and thoroughly mixed with the aqueous phase for 60 s. The lower phase was removed by aspiration and then discarded. The toluene/*n*-butanol phase was washed with 5 ml of *n*-butanol-saturated water. The samples were centrifuged at 500 × *g* for 3 min, and the upper phase was transferred to a cuvette. Colour was stabilized by adding 1 ml of pyridine. Absorbance was determined at 420 nm.

Hydrogen peroxide (H_2O_2): The contents of H_2O_2 in the needles were quantified using the method of Patterson *et al.* (1984). The needles (0.2 g) were homogenized in 3 ml of cold acetone, and the homogenate was centrifuged at 1,500 × *g* for 15 min at 4°C. The resulting supernatant was collected and added to a concentrated hydrochloric acid solution containing 0.1 ml of 20% (v/v) $TiCl_4$ and 0.2 ml of concentrated ammonia. After 10 min at 25°C, the reaction mixture was re-centrifuged at 1,500 × *g* for 20 min at 4°C. The pellets were washed twice with cold acetone and then 3 ml of H_2SO_4 (1 mM) were added. Absorption was observed at 410 nm. The content of H_2O_2 was quantified based on the standard curve.

Lipid peroxidation: MDA contents were measured to determine the lipid peroxidation. The MDA content in the test sample was measured after 40 d of UV-B treatment in accordance with the method of Heath and Packer (1968).

Antioxidant enzymes: Newly expanded needles (0.5 g) were ground using a mortar in 5 ml of ice-cold extraction buffer containing 1% (v/v) polyvinylpyrrolidone (PVP) in 50 mM phosphate buffer (pH 7.0). The homogenate was centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatants were used for enzyme assays. The catalase (CAT) activities, ascorbate peroxidase (APX), and SOD were measured in accordance with the method of Jiang *et al.* (2002).

Epicuticular wax: The quantity of epicuticular wax on the needle samples was determined using a modified method of Schuck (1976). The needles were washed in chloroform (30 ml) in glass tubes for 1 min. After the chloroform evaporated, the total amount of cuticular wax was expressed per total abaxial plus adaxial needle area. Based on Jenks *et al.* (1995), the chloroform-soluble cuticular wax extracts were evaporated under a nitrogen stream to dry. The dried residue was prepared for gas chromatography through derivatization for 15 min at 100°C using N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA). After the surplus BSTFA was evaporated under nitrogen, the sample was redissolved in chloroform for analysis. Wax samples were injected into a *Hewlett Packard 5989* gas chromatograph-mass spectrometer (GC-MS) (*Hewlett Packard*, USA). The GC was equipped with a 12 m, 0.2 mm *HP-2* capillary column with helium as the carrier gas. The GC was programmed with an initial temperature of 80°C and increased at 15°C min^{-1} to 260°C, at which the temperature remained constant for 10 min. The temperature was then increased at 5°C min^{-1} to 320°C, at which the temperature was held for 15 min. The mass spectrometer had a scan range of 40–800 atomic mass unit (amu) and was operated at a source temperature of 300°C. The wax chemical components are expressed as a percentage of the total wax content.

Scanning electron microscopy (SEM): The needle surfaces were examined using a scanning electron microscope (*Hitachi S-3400N*, Japan) at an accelerating voltage of 5 kV. The newly expanded needle samples were fixed in glutaraldehyde (2.5%, v/v), followed by osmium tetroxide (2%, v/v) in phosphate buffer (pH 6.8). The samples were then rapidly dehydrated with graded ethanol/acetone, and sputtered with gold-palladium under vacuum (*Hitachi E-1010*, Japan) before being examined using SEM in high-vacuum mode, in accordance with the methods described by Song *et al.* (2003).

Statistical analysis: Data of gas-exchange parameters, photosynthetic pigments, free radicals and MDA, activities of antioxidant enzymes, and UV-B absorbing compounds were expressed as means ± standard deviation. All data were subjected to *ANOVA* and *Tukey's* multiple comparison test ($p<0.05$) using the *SPSS 11.5* (*SPSS Inc*., USA) statistical package.

Results

Light-response curves of photosynthesis (P_N/PAR) and photosynthetic parameters: Under the control and T1 treatments, the net photosynthetic rate (P_N) of Korean pine needles increased with the increase in PAR intensity (Fig. 1). In comparison, under the T2 and T3 treatments, P_N decreased with the increase in PAR (> 500 μmol m^{-2} s^{-1}).

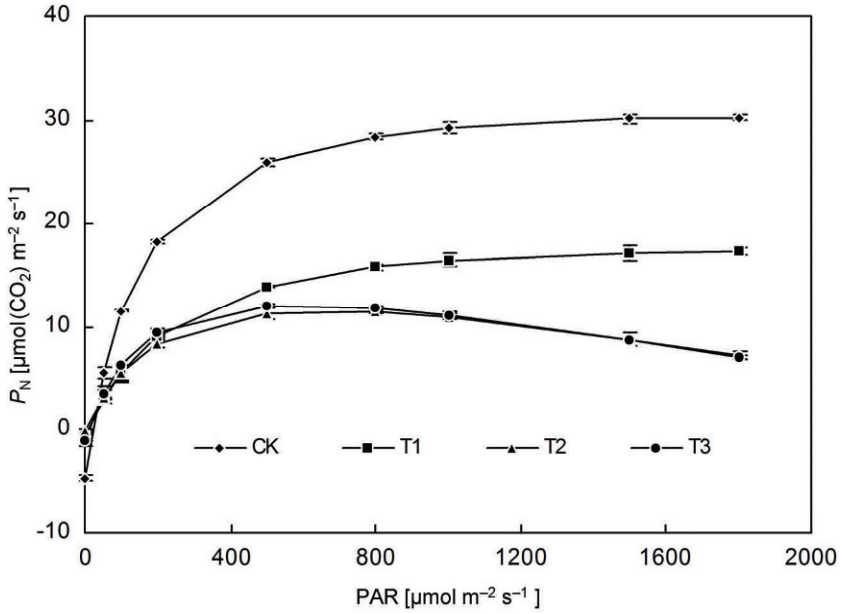

Fig. 1. Photosynthesis light-response curves (P_N/PAR) of needle subjected to different levels of UV-B radiation. CK – control, 1.32 kJ m^{-2} d^{-1}; T1 – increased UV-B radiation by 1.40 kJ m^{-2} d^{-1}; T2 – increased UV-B radiation by 2.81 kJ m^{-2} d^{-1}; T3 – increased UV-B radiation by 4.22 kJ m^{-2} d^{-1}.

Table 1. The data on morphology of Korean pine. Means ± SD (n = 80).

Seedling height [cm]	Root collar diameter [cm]	Shoot biomass [g]	Root biomass [g]	Leaf area [cm^2]	Root volume [cm^3]
9.51 ± 0.06	0.25 ± 0.004	16.26 ± 0.85	5.85 ± 0.07	11.38 ± 0.58	5.46 ± 0.05

The UV-B radiation significantly decreased (p<0.05) the P_N of Korean pine needles. Compared with the control, P_N decreased by 42.9% (T1 treatment), 61.8% (T2 treatment), and 60.0% (T3 treatment), as shown in Table 1. Respiration rate (R_D), maximum net photosynthetic rate $P(I_m)$, and compensation irradiance (I_C) declined significantly (p<0.05) with the increase in UV-B dosage (Table 2).

Photosynthetic pigments and UV-B absorbing compounds. After 40 d of exposure to UV-B radiation, the concentrations of Chl *a*, Chl *b*, total Chl, Car, and the ratio of Chl *a*/*b* in the needles of Korean pine were in the following order: T1 > T2 > T3 (Table 3). Compared with those in the CK, flavonoids in the needles were significantly decreased by all levels of supplementary UV-B radiations (p<0.05; Table 2). Moreover, the flavonoid contents decreased with the exposure of UV-B radiation. The average flavonoid content under the CK, T1, T2, and T3 treatments was 37.82, 33.42, 35.31, and 35.39 OD 300 g^{-1}(FM), respectively.

Free radical and lipid peroxidation: The contents of ·OH, H_2O_2, and MDA are shown in Table 2. The average ·OH content under the CK, T1, T2, and T3 treatments was 37.82, 33.42, 35.31, and 35.39 OD 420 g^{-1}(FM), respectively. The contents of H_2O_2 and MDA in Korean pine needles increased by enhanced UV-B radiation (p<0.05). The contents of H_2O_2 and MDA in the needles were in the following order: T3 > T2 > T1 > CK.

Antioxidant enzymes: The activities of CAT, APX, and SOD were measured (Table 2) and found to increase through supplementary UV-B radiation after 40 d of exposure (p<0.05). The average SOD activity under the CK, T1, T2, and T3 was 22.97, 22.99, 23.81, and 24.14 U g^{-1}(FM), respectively.

SEM and epicuticular wax: The SEM results showed that the wax stripes were fissured and broken after 40 d of UV-B radiation (Fig. 2). The quality of epicuticular wax in the Korean pine needles significantly decreased because of UV-B radiation exposure (p<0.05; Table 2). The average epicuticular wax content under the CK, T1, T2, and T3 treatments was 33.54, 33.13, 32.63, and 31.70 µg cm^{-2}, respectively. The most prominent substance class of Korean pine were alkanes, with C_{20} representing the dominant chain length. The alkanes represented more than 50% of the compounds (Table 2). The percentage of alkanes and phenols in Korean pine needles decreased with the enhanced UV-B radiation. The percentages of alkanes and phenols in the needles were in the following order: CK > T1 > T2 > T3. In contrast, the percentage of long-chain esters increased with the increase in UV-B radiation. The percentage of long-chain esters in the needles was in the following order: T3 > T2 > T1 > CK.

Table 2. Effects of UV-B radiation on the physiology and biochemistry characteristics of Korean pine. Respiration rate (R_D), maximum net photosynthetic rate, P(I_m), saturation irradiance (I_m), compensation irradiance (I_C), and the concentrations of chlorophyll (Chl *a* and *b*), carotenoids (Car), flavonoids, hydroxyl radical (·OH), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and the activities of catalase (CAT), ascorbate peroxide (APX) and superoxide dismutase (SOD), as well as the content of epicuticular waxes were measured in needles of Korean pine after 40 days of exposure to UV-B radiation. Means ± SD (n = 9). Means within a column with different superscripts lowercase letters are significantly different (p<0.05). Means within a column with the same lowercase letter are no significant difference (p>0.05) (*Tukey*'s multiple comparison test). CK – control, 1.32 kJ m^{-2} d^{-1}; T1 – increased UV-B radiation by 1.40 kJ m^{-2} d^{-1}; T2 – increased UV-B radiation by 2.81 kJ m^{-2} d^{-1}; T3 – increased UV-B radiation by 4.22 kJ m^{-2} d^{-1}.

Treatment	CK	T1	T2	T3
R_D [μmol m^{-2} s^{-1}]	4.60 ± 0.01^a	0.65 ± 0.06^c	0.57 ± 0.04^d	0.98 ± 0.01^b
$P(I_m)$ [μmol(CO_2) m^{-2} s^{-1}]	30.18 ± 0.24^a	17.24 ± 0.35^b	11.53 ± 0.24^d	12.06 ± 0.08^c
I_m [μmol m^{-2} s^{-1}]	1896 ± 6^b	1924 ± 10^a	665± 4^c	591 ± 3^d
I_C [μmol m^{-2} s^{-1}]	19.39 ± 0.06^a	7.79 ± 0.05^c	7.31 ± 0.02^d	8.95 ± 0.06^b
Chl *a* [μg g^{-1}(FM)]	1545 ± 5^b	1984 ± 21^a	1424 ± 9^c	1205 ± 7^d
Chl *b* [μg g^{-1}(FM)]	491 ± 7^b	590 ± 11^a	505 ± 9^b	424 ± 6^c
Total Chl [μg g^{-1}(FM)]	2037 ± 9^b	2574 ± 12^a	1935 ± 12^c	1631 ± 2^d
Car [μg g^{-1}(FM)]	298 ± 3^b	348 ± 8^a	306 ± 4^b	263 ± 3^c
Chl *a*/*b* ratio	3.15 ± 0.04^b	3.36 ± 0.06^a	2.84 ± 0.05^c	2.82 ± 0.05^c
Flavonoids [OD 300 g^{-1}(FM)]	37.82 ± 2.15^a	33.42 ± 1.68^c	35.31 ± 0.98^b	35.39 ± 0.63^b
·OH [OD 420 g^{-1}(FM)]	0.44 ± 0.01^c	0.67 ± 0.01^b	0.73 ± 0.01^a	0.65 ± 0.01^b
H_2O_2 [μmol g^{-1}(FM)]	11.09 ± 0.03^d	11.48 ± 0.04^c	11.63 ± 0.01^b	12.56 ± 0.01^a
MDA [mmol g^{-1}(FM)]	114.05 ± 1.32^d	130.79 ± 0.82^c	143.43 ± 1.44^b	149.05 ± 0.83^a
CAT [U g^{-1}(FM)]	2.89 ± 0.05^c	3.22 ± 0.05^{bc}	4.79 ± 0.03^a	3.50 ± 0.06^b
APX [U g^{-1}(FM)]	7.93 ± 0.02^c	10.41 ± 0.04^a	9.01 ± 0.06^b	7.62 ± 0.05^c
SOD [U g^{-1}(FM)]	22.97 ± 0.08^c	22.99 ± 0.07^c	23.81 ± 0.08^b	24.14 ± 0.03^a
Wax amount [μg cm^{-2}]	33.54 ± 0.04^a	33.13 ± 0.05^b	32.63 ± 0.03^c	31.70 ± 0.05^d
Alkanes [%]	66.14	62.32	57.66	51.65
Long-chain esters [%]	4.25	10.24	13.21	20.33
Terpenes [%]	10.32	12.18	8.89	14.36
Phenols [%]	5.22	3.59	3.44	2.87
Primary alcohols [%]	4.47	3.98	3.1	4.88
Aldehydes [%]	2.09	-	4.1	-
Not identified [%]	7.51	7.69	9.61	5.92

Correlation analysis: Table 3 shows that the content of H_2O_2 had significant positive correlations with the contents of ·OH and MDA (p<0.01; r>0.8). The wax content had significant positive correlations with the concentrations of photosynthetic pigments (p<0.05), and significant negative correlations with the contents of H_2O_2 and MDA (p<0.01; r = –0.985 and r = –0.918). The index among photosynthetic pigments had significant positive correlations (p<0.05). The activities of antioxidant enzymes did not have significant correlations with other indices.

Discussion

In this study, the effects of supplementary UV-B radiation on the physiology and biochemistry of Korean pine were evaluated. Photosynthesis in various plants is affected by high light intensity, cold events, and especially by UV-irradiation (Zu *et al.* 2010). The results demonstrate that UV-B radiation apparently damaged the photosynthetic apparatus of Korean pine. The UV-induced inhibition of photosynthesis may have been caused by the decreased photochemical efficiency and repair rate. This effect is probably correlated with the concentration or activity of Rubisco, the key regulatory enzyme of the Calvin cycle (Gómez *et al.* 1998, Vagish *et al.* 2008). $P(I_m)$ is an important photosynthetic parameter that represents the maximal photon utilization capacity of plants; hence, $P(I_m)$ reflects the net primary productivity (Surabhi *et al.* 2009). In the present study, $P(I_m)$ decreased with elevated UV-B radiations. Previous studies have shown that plants attempt to reduce photochemical reactions to avoid potentially lethal photochemical damage (Malanga and Puntarulo 1995,

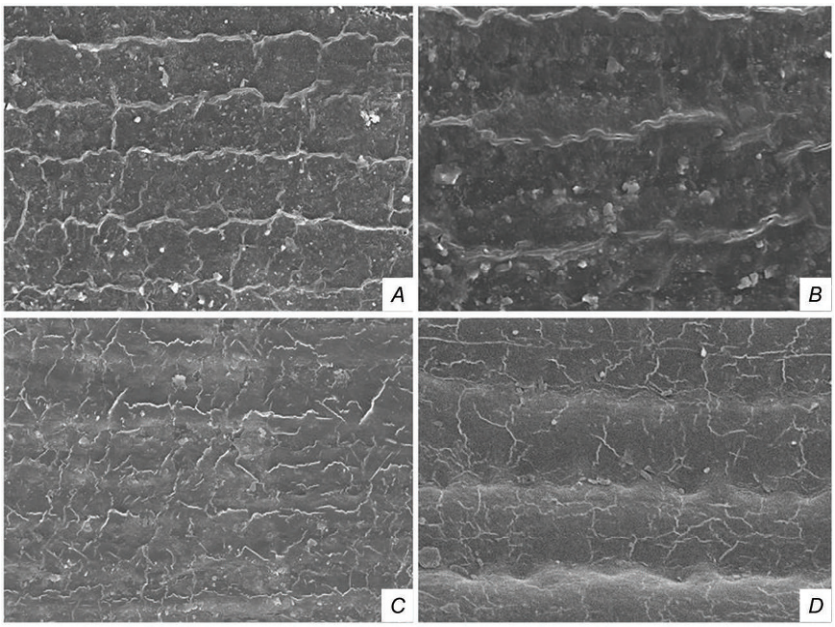

Fig. 2. The wax surface of Korean pine needles exposed to control (*A*), T1 treatment (*B*), T2 treatment (*C*) and T3 treatment (*D*). The scale for A, B, C and D was 20 μm. CK – control, 1.32 kJ m^{-2} d^{-1}; T1 – increased UV-B radiation by 1.40 kJ m^{-2} d^{-1}; T2 – increased UV-B radiation by 2.81 kJ m^{-2} d^{-1}; T3 – increased UV-B radiation by 4.22 kJ m^{-2} d^{-1}.

Table 3. Correlation on the physiological indexes of Korean pine seedings under the UV-B stress. CAT – catalase; APX – ascorbate peroxidase; SOD – superoxide dismutase; Car – carotenoid; Chl – chlorophyll; * – $p<0.05$, ** – $p<0.01$.

Physiological index	MDA	H_2O_2	·OH	Flavonoids	CAT	APX	SOD	Car	Chl *a*	Chl *b*	Total Chl	Chl *a*/*b*
H_2O_2	0.857**											
·OH	0.500	0.849**										
Flavonoids	–0.279	–0.499	–0.734**									
CAT	0.345	0.301	0.076	–0.108								
APX	0.368	0.312	0.154	–0.143	0.606**							
SOD	–0.003	0.205	–0.053	0.148	–0.031	–0.396						
Car	–0.622*	–0.375	0.164	–0.509	–0.156	–0.079	–0.021					
Chl *a*	–0.620*	–0.485	–0.027	–0.454	–0.315	–0.323	–0.183	0.954**				
Chl *b*	–0.648*	–0.380	0.165	–0.474	–0.332	–0.336	–0.141	0.989**	0.934**			
Total Chl	–0.631*	–0.481	0.008	–0.462	–0.293	–0.295	–0.182	0.966**	0.998**	0.958**		
Chl *a*/*b*	–0.478	–0.609*	–0.356	–0.261	–0.140	–0.133	–0.227	0.628*	0.847**	0.623**	0.820**	
Wax amount	–0.985**	–0.918**	–0.568	0.252	–0.328	–0.340	–0.219	0.645**	0.687*	0.657**	0.686**	0.616*

Gómez *et al.* 1998). The decrease in $P(I_m)$ under UV-B radiation is in agreement with that of other reports (Percy *et al.* 1994, Zhao *et al.* 2004, Koti *et al.* 2007).

Studies also have shown that the decrease in Chl concentration is a common symptom of biochemical damage related to UV-B stress. In the present study, Chl concentrations in the Korean pine needles significantly decreased with supplementary UV-B radiation (increased UV-B radiation by 4.22 kJ m^{-2} d^{-1}). The decrease in total Chl concentration induced by UV-B radiation was probably due to the destruction of chloroplast structure, inhibited synthesis of new Chl, and increased degradation of Chls (Table 3) (Sakaki *et al.* 1983). The Chl *a*/*b* ratio significantly increased with UV-B radiation in the Korean pine needles. Chl *a*/*b* may reflect the relative ratio of stacked to unstacked regions, which are inversely proportional to the stacking degree of thylakoids (Ibañez *et al.* 2008). Thus, an increased Chl *a*/*b* ratio may indicate that UV-B radiation causes the decomposition of the stacked regions of thylakoids (Day *et al.* 1999). Moreover, higher levels of Car in leaves may provide a protective function for the plants (Bornman *et al.* 1997). The negative effect of long-term enhancement of UV-B radiation (+25%) on photosynthetic gas exchange and photosynthetic pigment was observed in *Pinus banksiana* and Norway spruce (Stewart and Hoddinott 1993, Šprtová *et al.* 1999).

UV-B induces the production and scavenging free radicals; moreover, the interactions of free radicals on cell membranes have been well documented (Rao and Ormrod 1995, Prasad *et al.* 2005). The results of the present study show that increased UV-B radiation significantly affected the concentrations of H_2O_2 and ·OH in Korean pine needles. UV-B-induced free radical formation also has been demonstrated in leaves and isolated chloroplasts (Hideg and Vass 1996). The

increase in lipid production caused by stress may have occurred because of the accelerated formation of reactive oxygen species (ROS) [*i.e.*, singlet oxygen ($^1O_2$) and ·OH]; ROS attack lipids, particularly unsaturated fatty acids, and the accelerated formation of ROS results in the formation of peroxidation products, the main one of which is MDA (Forman and Fisher 1981). The reaction of such radicals with macromolecules, particularly lipoproteins, can cause faster peroxidative damages as observed from the destruction of membrane lipids (Asada 1992).

Different plants can protect themselves from stresses through different biochemical adjustments (Ahmad *et al.* 2008). ROS scavenging is a common response to most stresses, and it depends on the detoxification mechanism provided by an integrated system of nonenzymatic and enzymatic antioxidants (Dai *et al.* 1997). In plants, the system of eliminating free radicals is very complex. A change in an enzyme or a substance in the system cannot represent changes in the overall protection in the body.

The essential function of Car in protecting the photosynthetic system from photooxidative damage is well documented. The Car of the xanthophyll cycle plays a major role in photoprotection by safely dissipating excess excitation energy (Yamamoto and Bassi 1996). The efficacy of Car in protecting photosystems is likely due to their function as efficient quenchers of high-energy short-wave radiation (Demmig-Adams and Adams 1992).

Flavonoids compounds have effective radical-scavenging capabilities and can directly contribute to enhanced photoprotection against UV-B radiation. The increases in UV-B absorbing compounds, mainly in flavonoids, are recognized as a general response to UV-B stress (Flint *et al.* 2004). In Scots pine, UV-B radiation ($UV\text{-}B_{BE}$ 4.8 kJ m^{-2} d^{-1}) has been found to induce flavonoid biosynthesis (Schnitzler *et al.* 1997). However, in the present study, flavonoid concentrations in Korean pine needles significantly decreased with supplementary UV-B radiation. A lower amount of UV-absorbing compounds were surprisingly found through supplementary UV-B, although the same results were reported in *Phlomis fruticosa* (Petropoulou *et al.* 1995) and *Umbilicaria amaricana* (Swanson and Fahselt 1997). The pathway that stimulates the synthesis of protective pigments may already have been saturated (Deckmyn *et al.* 1994), as Flint *et al.* (1985) previously hypothesized. The decrease in the UV-B absorbing capacity may be interpreted as a UV-B-induced acceleration of phenylpropanoid pathway and a concomitant UV-B-induced decomposition of phenolics in *Cistus laurifolius* as observed by Vogt and Gul (1994). The needles under supplementary UV-B may also have responded through some other defence mechanisms, *e.g.*, by cell-wall-bound pigments, such as ferulic acid and other bound phenylpropanoids (Kinnunen *et al.* 2001). Plants have formed a complex system of self-protection in a long evolutionary process to adapt to the changing living environment. Under environmental UV-B radiation stress, the enhanced synthesis of flavonoids *in vivo* is an important protective mechanism.

CAT, APX, and SOD are key enzymes of the antioxidant defence system. The SOD, POD, APX, and CAT activities are also associated with UV-B exposure, as these enzymes act as antioxidant compounds to help reduce photooxidative damage in plant leaves. SOD accelerates the conversion of superoxide to H_2O_2, whereas CAT and APX catalyze H_2O_2 breakdown (Zancan *et al.* 2008, Wang *et al.* 2010). The results of the present study indicate that the CAT, APX, and SOD activities were positively affected by supplementary UV-B radiation. Therefore, a preferential synthesis/activation of this enzyme by Korean pine needles counteracts oxidative stress. The increase in the CAT, APX, and SOD activities are frequently observed under stressful conditions (Yazici *et al.* 2007, Mishra *et al.* 2009). Lu *et al.* (2009) reported that UV-B and exogenous ABA stresses significantly increased the activities of CAT, SOD, and GPX. The present result suggests that antioxidative systems play an important role in protecting Korean pine against increased UV-B damage. Baumbusch *et al.* (1998) showed that UV-B radiation (1.2 kJ m^{-2} $UV\text{-}B_{BE}$) in the presence of ambient O_3 caused an increase in total SOD activity in spruce but had no effects on antioxidants in pine. Twice ambient O_3 levels together with low UV-B radiation counteracted the O_3-induced increases in ascorbate and CAT in pine, but not in spruce. Pine needles exposed to UV-B and elevated O_3 levels showed elevated lipid peroxidation, suggesting that this species is less protected and suffers higher oxidative stress than spruce.

Wax amount, composition, and homologue distribution patterns are known to vary considerably between, and even within, plant species. These characteristics may be degraded by a variety of environmental factors, such as acid mist and solar radiation quality (Tevini and Steinmueller 1987, Esch and Mengel 1998). In *Pinus pinea* and Scots pine needles, the enhanced UV-B (6.3 kJ m^{-2} and 7.5 kJ m^{-2} $UV\text{-}B_{BE}$) causes an increase in the thickness of the epidermal layer (Manetas *et al.* 1997, Laakso *et al.* 2000). However, the results of the present study show that the content of epidermis wax was reduced by supplementary UV-B radiation. The wax stripe was fissured and broken by the increased UV-B radiations. Similarly, Jeremy *et al.* (1996) reported that enhanced UV-B radiation ($UV\text{-}B_{BE}$ 4.54 kJ m^{-2} d^{-1} and 5.66 kJ m^{-2} d^{-1}) significantly ($p<0.05$) reduced the amount of wax deposited on the adaxial leaf surface of a UV-B-sensitive tobacco genotype and modified the wax composition and homologue distribution patterns on the adaxial surface of leaves. Wax structure is largely determined by its chemical composition (Percy *et al.* 1994). To our knowledge, no other study has compared the wax properties and chemical composition of Korean pine under different levels of UV-B radiation. However, no evidence was found to suggest that exposure to UV-B

radiation results in a shortening of chain lengths, such as that reported for esters by Day *et al.* (1996). The results of the present study suggest that the chemical composition of the cuticular waxes vary in Korean pine under different UV-B treatments. Interspecific differences clearly exist in response to UV-B radiation in terms of leaf wax content and chemical composition (Pilon *et al.* 1999). The epicuticular waxes of certain species (*e.g. Alnus glutinosa*) are known to contain unbound flavonoids and flavones (Kolattukudy *et al.* 1980). Hence, the well-developed wax layer can prevent UV-B penetration (Kinnunen *et al.* 2001).

The index among the contents of H_2O_2, ·OH, MDA, wax, and the concentrations of photosynthetic pigments had significant correlations in the process of Korean pine needles under UV-B stress. CAT is suggested to be involved in mass scavenging of H_2O_2 of cells. CAT was mainly distributed in the peroxisome, glyoxysome of plant cells, and cytoplasm. A small number of CAT was distributed in mitochondria. The H_2O_2 in chloroplasts is removed through the Halliwell-Asada pathway, where APX and glutathione reductase (GR) play an important role (Gu *et al.* 2006). Therefore, H_2O_2 levels are not correlated with changes in H_2O_2-scavenging enzymes.

The wax layer acts as an interface between the environment and internal leaf structures (Koti *et al.* 2007). Wax and photosynthetic pigment concentrations, MDA content, and H_2O_2 had significant correlations in the needles of Korean pine under UV-B stress. Wax biosynthesizes in epidermal cells. Wax synthesis requires coordinated activities of a large number of enzymes. Most of these enzymes are membrane-bound proteins. Cuticular wax is mainly composed of long-chain aliphatic compounds derived from fatty acids with very long chains. Wax biosynthesis begins with fatty acid synthesis in the plastid (Kunst and Samuels 2003). Lipid peroxidation refers to the unsaturated fatty acids of biofilm occurring as a series of radical reactions. Denaturation of proteins and nucleic acids can lead to decreased membrane fluidity and increased membrane permeability. The accumulation of MDA *in vivo* may lead to further disturbance of cell metabolism (Hodges *et al.* 1999). This effect may block the key steps of wax biosynthesis and may indirectly affect the synthesis and transport of epidermal wax. H_2O_2 may attack biomolecules such as lipids, proteins, and DNA (Mackerness *et al.* 2001). H_2O_2 is relatively stable and diffusible through the membrane, and can function as a signalling molecule mediating a range of responses to environmental stresses, including UV-B radiation (Foyer *et al.* 1997, He *et al.* 2005). H_2O_2 may also act as signalling molecule inhibiting the expression of waxy synthesis genes. The ·OH generated *in vivo* is a multi-pathway. ·OH is the most lively active oxygen radical with no specific scavenging enzymes. The reaction rate of ·OH with any biological molecule is very fast. The life of ·OH in organisms is very short, and the reaction force is very strong. Thus, ·OH is difficult to detect *in vivo* (De Zwart *et al.* 1999). The established Babbs method of detecting ·OH is lengthy and cumbersome. This limitation may be the reason waxes are not correlated with ·OH. Flavonoids in plants are found near the main stem and leaf epidermal cells, leaf wax, leaf hairs, and new seedlings or young leaves. Flavonoids of leaf wax are different from the water-soluble flavonoid glycosides in epidermal cells. The flavonoids are in an unbound state and not combined with glycosyl (Li *et al.* 2001). In the present study, the total content of flavonoids in leaf tissue was also detected, whereas the flavonoids in leaf wax were only partly. Therefore, wax content is possibly not correlated with the content of flavonoids.

UV-B affects the needle surface, causing changes in the contents of free radicals, wax, flavonoids, and Chl concentrations. However, limited information is available to understand whether the effect is in response to stress or is a protective mechanism. Thus, fully and accurately reflecting the strengths and weaknesses against UV-B with a single index is difficult. Only the physiological indexes were integrated to accurately reflect the ability of Korean pine in resisting UV-B.

The responses of plants to supplementary UV-B radiation have interspecific and intraspecific differences. However, the mechanisms of this diversity need to be further studied. Tolerance and avoidance of increased UV-B radiation are manifested in a range of plant strategies. In addition to absorbing and reflecting UV-B radiation by the epidermal cells, trichomes, and wax, another important protection mechanism is the elimination of free radicals by enzymes or by nonenzymatic systems. Meanwhile, the UV-B absorbing compound and secondary compounds of Korean pine needles play important roles in protecting against UV-B radiation damage. The present research shows that supplementary UV-B radiation decreased the photosynthetic rate and wax content, but increased the free radical and MDA contents. This observation indicates that Korean pine exhibits significant sensitivity to supplementary UV-B radiation. Although the antioxidative system of Korean pine needles could significantly protect the plant against increased UV-B damage, the defence mechanism is not efficient enough to prevent the UV-B-induced damage.

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