SEASONAL AND REGIONAL VARIATION IN CAROTENOIDS OF KOREAN FIR *(ABIES KOREANA)* ON MT. HALLA, SOUTH KOREA

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Abstract. Climate change affects the decline of coniferous forests in the subalpine zone in Korea, and causes significantly changes in the composition and content of carotenoids. In the present study, we analyzed the effect of temperature variation on carotenoid content in *Abies koreana* native to Mt. Halla National Park to establish a scientific basis for the conservation of the species. To understand habitat variation, fir leaves of *A. koreana* were collected from three different regions (Yeongsil, Witseoreum, and Jindallaebat) of Mt. Halla during April, July, and October to quantify lutein, α-carotene, and β-carotene. The total carotenoid contents in the regions were significantly high in October when the temperature was low compared to July. The carotenoid contents in October were 8.5 times, 4.7 times, and 4 times higher at Yeongsil, Witseoreum, and Jindallaebat each. The lutein content was significantly higher at the Witseoreum region where *A. koreana* is more abundant than in Jindallaebat where *A. koreana* is in decline. The result showed that changes in carotenoid contents were affected by temperature which could influence the *A. koreana* abundancy and health status at Mt. Halla. This study could contribute to understanding physiological changes of *A. koreana* caused by environmental changes on Mt. Halla.
Keywords: *subalpine, carotene, lutein, quantification, climate change*

Introduction

Many studies were reported on the global forest decline caused by climate change (Wildi and Lütz, 1996; Lim et al., 2006; Allen et al., 2010; Körner, 2012; Tsuyama et al., 2015). In particular, the Intergovernmental Panel on Climate Change (IPCC, 2014) pointed out the possibility of the widespread death of plants due to temperature increases and drought resulting from global warming, and Antos et al. (2008) predicted extinction risks of the genus Abies in alpine regions. Subalpine plant species found at 1,300 to 1,800 m above sea level are greatly affected by limited opportunities for genetic exchange and various environmental factors, including temperature rise and water stress (Kim et al., 2017).

The Korean fir (*Abies koreana*) is a native Korean species that was introduced to the world by British botanist Wilson in 1920. The subalpine plant is a climate-sensitive bioindicator and is only found in subalpine zones such as Mt. Halla and Mt. Jiri in South Korea. Mt. Halla has the only large colony of Korean fir in the world (Kim et al., 2017). The distribution of the Korean fir, a species of the genus Abies, decreased in 15.2% from 738.3 ha to 626.0 ha at Mt. Halla for 9 years (2006-2015). Because of continuous decline of the Korean fir, *Abies koreana* was listed as an internationally endangered species by the International Union for Conservation of Nature (IUCN, 2011). Several studies claimed the causes of the decline of Korean fir in various ways. Continuous temperature

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increase, heterogeneous invasion, weakening of the support base due to typhoons or droughts, and lack of water required for photosynthesis were found as factors hindering the growth of the Korean fir species (Koo et al., 2001, 2017; Song et al., 2014, 2020; Park et al., 2018). Of these, the temperature increase not only changes the moisture content in soil, affects plant growth and reproduction, and causes a decrease in nutrient intake capacity and yield, but is also a leading greenhouse gas contributor that triggers the release of carbon from the soil to the atmosphere (Körner and Paulsen, 2004; Lee et al., 2013; Hatfield and Prueger, 2015).

Variations in the habitat of trees induced physiological changes in genetic composition and chemical compounds (Hwang et al., 2018). Especially, plants in alpine regions produced large amounts of carotenoids for protection against antioxidants to survive in the harsh environment of low temperatures (Streb et al., 1997; Kim and Park, 2018). Some conifers produced a high level of carotenoids for survival during cold winters (García-Plazaola et al., 1999). The most common carotenoids include lutein, α-carotene, β-carotene, lycopene, zeaxanthin, and astaxanthin (Nisar et al., 2015). Study showed the carotenoid content in Korean fir seedlings and changes in photosynthetic apparatus caused by high luminous intensity and moisture stress (Je et al., 2018). Even though lutein and β-carotene contents were changed depending on the temperature changes with altitudes in the Jindallaebat region of Mt. Halla (Oh et al., 2013), the role of carotenoids for the Korean fir on Mt. Halla in a changing growth environment and the adaptation process are still largely unknown.

The object of this study was to compare the carotenoid content of Korean firs on Mt. Halla by area and season to identify the effect of temperature variation, and to analyze the correlation between carotenoid contents and temperatures. Considering the topographical and environmental differences of each slope on Mt. Halla, the carotenoid contents in Korean fir leaves collected from three colonies (Yeongsil, Witseoreum, and Jindallaebat) was quantified by season.

Materials and Methods

Study sites and sampling

The study was conducted in Mt. Halla National Park, Cheju Island, Korea. Study sites were selected in near hiking trails, 1,300 to 1,700 m above sea level, in three Korean fir colonies (YS: Yeongsil, WS: Witseoreum, JD: Jindallaebat) on each slope of Mt. Halla located in the southern part of the Korean Peninsula (*Fig. 1a*). The Korean fir leaves were harvested over three years from 2016-2018. Considering that the different seasons are based on temperature changes, Korean fir leaf samples were collected in three different seasons (April, July, and October). Based on the sample collection method of Oh et al. (2013), the samples were collected randomly from nine or more mature trees (≥ 2 m) with health conditions at a height of 1.5 to 2.5 m with sufficient sunlight. Weather data were collected from three or more portable thermo-hygrometers (HOBO®, Onset Corp., U23-001, Boston, MA, USA), and automatic weather systems were installed in each research area (*Fig. 1b*).

Standard materials and reagents

The study used lutein, zeaxanthin, α-carotene, and β-carotene produced by Sigma-Aldrich (Saint Louis, USA) as reference materials for carotenoids. For the extraction and

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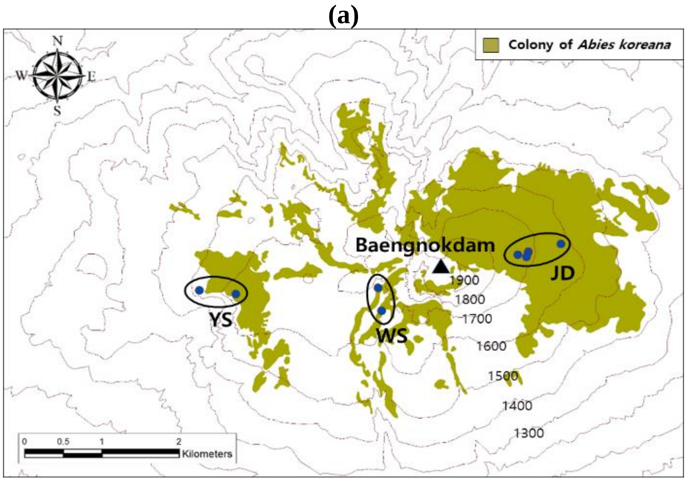
analysis of carotenoids, high-performance liquid chromatography (HPLC)-grade ethanol (EtOH), methanol (MeOH), ethyl acetate (EtOAc), potassium hydroxide (KOH), hexane, dichloromethane (DCM), acetonitrile (ACN), and water were used.

(a)



(b)

Location	Latitude	Longitude	Height (m)
YS	33.359494	126.499003	1,520
	33.359111	126.504050	1,627
	33.348320	126.496400	1,260
WS	33.360008	126.523842	1,690
	33.357236	126.524342	1,663
	33.362310	126.518100	1,668
JD	33.365553	126.549217	1,627
	33.364192	126.543231	1,735
	33.364575	126.544678	1,715
	33.364364	126.544617	1,716
	33.363911	126.544450	1,717

Figure 1. *(a) Map of the study sites (▲: Top of Mt. Halla, ●: Area of sampling locations) and (b) sampling locations in Mt. Halla*

Extraction of carotenoids in Korean fir

Carotenoids were extracted for 5 min in a constant-temperature water bath (75 °C) with a 50 mL tube containing 0.5 g of dried Korean fir leaf powder and 5 mL of EtOH. An amount of 1.5 mL of 80% KOH was added to the extract, which was reacted for 10 min in a constant-temperature water bath (75 °C) and then cooled in ice for 5 min. Then, 2.5 mL of water and hexane were added to the tube once the reaction stopped and were well mixed. The mixture was centrifuged for 3 min (3,000 rpm, 4 °C). Thereafter, the supernatant was collected, and this process was repeated three times for a proper mix. After removing the solvent through lyophilization, the extract was dissolved in 1 mL of a MeOH:DCM (1:1, v/v) solution to be used as a sample for high-performance liquid chromatography (HPLC) analysis (Lee et al., 2017).

Carotenoids were analyzed using an Ultimate 3000 HPLC system (Thermo Dionex, Waltham, USA). The column used was INNO C-18 (4.6 × 250 mm, 5 µm, Youngjin

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Biochrom, Seongnam, Korea), and its temperature was maintained at 50 °C. Ten microliters of the sample was injected at a rate of 1 mL/min, and the wavelength of the detector was set to 440 nm. As regards the development conditions, water was used as mobile phase solvent A, ACN as solvent B, and MeOH:EtOAc (50:50, v/v) as solvent C. The slope conditions were as follows: 40% for solvent A, 10% for solvent B, and 50% for solvent C, maintained for 5 min. Solvent B was steadily raised to 50% up to 20 min. Solvents B and C were maintained at 50% to 30 min, followed by 40% for solvent A, 10% for solvent B, and 50% for solvent C up to 35 min. For quantitative analysis, an experiment was performed for five concentrations (10, 20, 50, 100, and 200 ppm) under the HPLC conditions established for the reference solution of each component, and a calibration curve was obtained. The correlation coefficient (R^2) of the calibration curve was > 0.99. Carotenoid contents were expressed as μg/g dry weight (DW).

Statistical analysis

This study conducted a multivariate analysis of the carotenoid content of Korean firs on Mt. Halla by region and season using R v3.6.1 (R Core Team, 2019), the stats and DescTools package version 0.99.28 (Signorell, 2019). A one-way ANOVA ($p < 0.05$) was performed using Scheffe's test to analyze the significance of carotenoids in Korean fir leaves by region and season, and a partial least squares-discriminant analysis (PLS-DA) and a variable importance in projection (VIP) score analysis were carried out to identify differences in the content of carotenoids based on the area, season, and health of trees.

As a method to determine which one of two or more populations a given sample was extracted from, the PLS-DA aimed to maximize the variance between groups using discriminant functions consisting of a combination of one or more discriminant variables. The VIP score has a higher value depending on the contribution to the variance between groups, and only those with a value of 1 or above were judged to have significance (Wilson, 2002). In addition, Pearson's correlation coefficient analysis ($p < 0.01$) was performed to analyze the correlation between the carotenoid content in Korean fir and temperature, and the correlation coefficient (r) was expressed as a value between -1 and +1.

Results and Discussion

Analysis of microclimate data in the Korean fir colony

After collecting data from the automatic weather systems in the research areas of Yeongsil, Witseoreum, and Jindallaebat on Mt. Halla, changes in the average temperature of the three Korean fir colonies were plotted on a graph (*Fig. 2*). During the research period (2016-2018), the average temperature ranged between 7.4 °C and 9.0 °C in Yeongsil, 6.38 °C and 7.4 °C in Witseoreum, and 6.4 °C and 7.6 °C in Jindallaebat. The average temperature in Yeongsil was at least 1 °C higher than that in Witseoreum and Jindallaebat. According to the average temperature data for 10 years (2010-2019) collected from the Korean Meteorological Administration, the average temperature in Yeongsil (8.9 ℃) was higher than that in Witseoreum (6.2 ℃) and Jindallaebat (7.5 ℃). Carotenoids, essential photosynthetic pigments, prevent damage protect to photosynthetic machinery under abiotic stress conditions such as temperature (Hatfield and Prueger, 2015; Nisar et al., 2015). Therefore, carotenoid contents and monthly mean temperature

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were displayed in *Figure 2*. Total carotenoid contents were between 1.17 and 28.15 μg/g. Interestingly; the level of total carotenoids was consistently higher in October from 2016 to 2018.

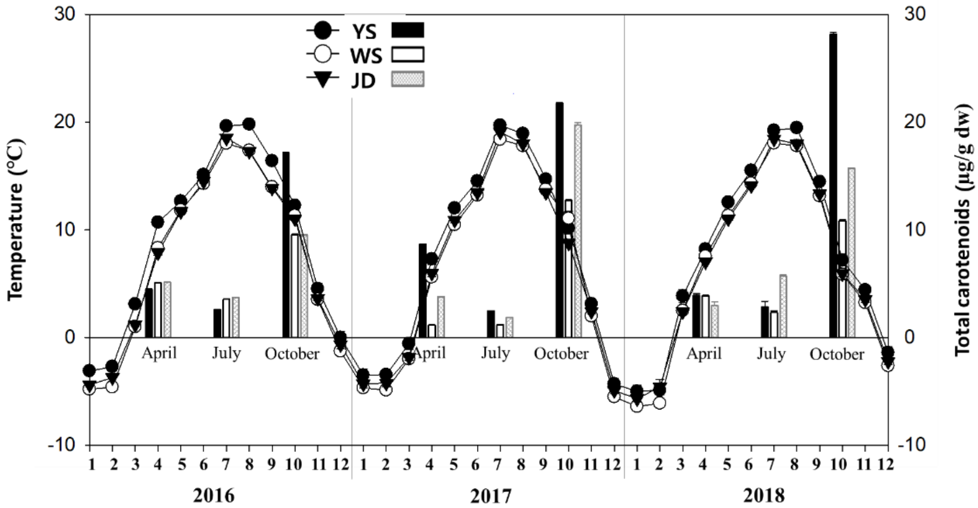

Figure 2. *Temperature changes and carotenoid contents at Yeongsil (YS), Witseoreum (WS), and Jindallaebat (JD) on Mt. Halla during 2016-2018*

Carotenoid compounds of Korean fir at different harvesting years

In this study, three types of carotenoids, namely lutein, α-carotene, and β-carotene were mainly found, and the Korean fir leaves contained relatively high levels of lutein compared to α-carotene and β-carotene in the years 2016-2018 (*Table 1*).

Zeaxanthin compound was expected in this study, but it was not found in the Korean fir. The content of lutein ranged from 0.82 to 23.30 μg/g, α-carotene between 0.02 and 2.01 μg/g, and β-carotene between 0.11 and 2.84 μg/g, and the total content of carotenoids was between 1.17 and 28.15 μg/g. Carotenoids are known as auxiliary pigments mainly present in the chloroplasts of plants and are involved in the photoprotection of plants by reducing excessive thermal energy generated during photosynthesis and keep reactive oxygen species from being produced by photooxidation (Howitt and Pogson, 2006). Therefore, it is expected that the health of the Korean fir trees could be predicted by the concentration of carotenoids, representatively, lutein, α-carotene, and β-carotene.

Carotenoids in Korean fir at different seasons and regions

A comparison of the carotenoids in Korean firs harvested in three different seasons and regions of April, July, and October, representing spring, summer and fall, respectively. The total carotenoids of lutein, α-carotene and β-carotene were significantly high in October and low in July in all research areas. Carotenoids in recorded 8.5, 4.7, and 4.0 times in October higher than that in July at Yeongsil, Witseoreum, and Jindallaebat, respectively (*Table 2*). The higher lutein concentrations could be attributed to the higher contents of carotenoids in Korean fir. Particularly, carotenoids increased in October when temperature is relatively low, compared to April and July.

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***Table 1.** Carotenoid contents in Abies koreana collected from three regions on Mt. Halla*

(μg/g DW)

Month	Place	Year	Lutein	α-Carotene	β-Carotene	Total[a)]
Apr	YS[b)]	2016	3.54±0.08	0.17±0.00	0.70±0.02	4.42±0.10
		2017	7.41±0.08	0.21±0.01	1.02±0.01	8.65±0.07
		2018	3.22±0.13	0.15±0.01	0.54±0.03	3.91±0.17
	WS	2016	4.04±0.03	0.25±0.00	0.80±0.01	5.09±0.02
		2017	1.03±0.01	0.02±0.00	0.11±0.01	1.17±0.01
		2018	3.28±0.04	0.15±0.00	0.44±0.01	3.87±0.06
	JD	2016	3.87±0.01	0.48±0.00	0.78±0.01	5.14±0.02
		2017	3.24±0.01	0.11±0.01	0.44±0.01	3.80±0.01
		2018	2.58±0.31	0.10±0.01	0.29±0.03	2.97±0.35
Jul	YS	2016	1.59±0.03	0.49±0.01	0.51±0.01	2.59±0.05
		2017	1.67±0.03	0.41±0.01	0.38±0.01	2.47±0.02
		2018	1.94±0.36	0.46±0.09	0.44±0.08	2.84±0.54
	WS	2016	2.28±0.05	0.61±0.00	0.67±0.00	3.56±0.05
		2017	0.82±0.04	0.15±0.01	0.19±0.01	1.17±0.04
		2018	1.60±0.10	0.29±0.02	0.45±0.03	2.34±0.15
	JD	2016	2.16±0.02	0.70±0.01	0.88±0.01	3.74±0.03
		2017	1.27±0.02	0.26±0.01	0.32±0.01	1.85±0.03
		2018	3.76±0.09	0.98±0.02	1.01±0.02	5.74±0.14
Oct	YS	2016	13.42±0.12	1.45±0.01	2.27±0.04	17.13±0.09
		2017	18.12±0.11	1.37±0.03	2.25±0.02	21.74±0.08
		2018	23.30±0.05	2.01±0.06	2.84±0.08	28.15±0.19
	WS	2016	8.71±0.06	0.36±0.02	0.46±0.05	9.54±0.07
		2017	11.33±0.03	0.48±0.02	0.92±0.04	12.73±0.06
		2018	9.90±0.06	0.33±0.01	0.60±0.04	10.83±0.10
	JD	2016	8.12±0.01	0.65±0.04	0.77±0.03	9.53±0.06
		2017	15.97±0.12	1.75±0.03	2.03±0.08	19.75±0.21
		2018	12.83±0.01	1.18±0.04	1.71±0.02	15.72±0.05

[a)] All data are shown as mean±SD (n=3)
[b)] YS: Yeongsil, WS: Witseoreum, JD: Jindallaebat

Being structurally more stable than chlorophyll, carotenoids are sustained at a relatively high level during leaf senescence caused by temperature changes, which cause leaves to turn yellow (Biswal, 1995; Merzlyak and Solovchenko, 2002). The total content of carotenoids was the highest in Yeongsil (22.34 μg/g), followed by Jindallaebat (15.00 μg/g), and Witseoreum (11.03 μg/g). The major of lutein content was also high in the order of Yeongsil (18.24 μg/g), Jindallaebat (12.30 μg/g), and Witseoreum (9.98 μg/g) (*Table 2*). This is due to the topographical differences in the slopes, including periglacial landforms in the subalpine climate of Mt. Halla, which frequently cause weathering and erosion resulting from freezing, fragmentation, and turf destruction (Kim, 2008). Because of such topographic features, each slope of Mt. Halla presents a different environment (such as exposed rock, broad-leaf bamboo coverage, and vegetation), as well as

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meteorological conditions, including temperature, precipitation, and wind. A study showed that variation in the lapse rate throughout the year was also different: West slope (Yeongsil), −0.62 ℃/100 m, south slope (Witseoreum), −0.55 ℃/100 m, east slope (Jindallaebat), −0.45 °C/100 m, and north slope, −0.48 °C/100 m (Choi, 2011). Therefore, it proved our result that carotenoids concentration was high in the order temperature for each slope of Mt. Halla.

Table 2. *Carotenoid compounds (μg/g DW) of Abies koreana leaves harvested from different seasons and locations*

Place	Month	Lutein	α-Carotene	β-Carotene	Carotene	Total[a)]
YS[b)]	Apr	4.73±2.02[a]	0.18±0.03[a]	0.75±0.22[a]	0.93±0.24[a]	5.66±2.25[a]
	Jul	1.73±0.28[a]	0.45±0.07[b]	0.44±0.07[b]	0.90±0.14[a]	2.63±0.37[a]
	Oct	18.28±4.28[b]	1.61±0.30[c]	2.45±0.30[c]	4.06±0.60[b]	22.34±4.79[b]
WS	Apr	2.78±1.35[a]	0.14±0.10[a]	0.45±0.30[a]	0.59±0.40[a]	3.38±1.74[a]
	Jul	1.57±0.64[a]	0.35±0.20[b]	0.43±0.21[a]	0.78±0.41[ab]	2.36±1.04[a]
	Oct	9.98±1.14[b]	0.39±0.07[b]	0.66±0.21[a]	1.05±0.27[b]	11.03±1.39[b]
JD	Apr	3.23±0.59[a]	0.23±0.19[a]	0.50±0.22[a]	0.74±0.40[a]	3.97±0.97[a]
	Jul	2.40±1.09[a]	0.64±0.31[a]	0.74±0.32[a]	1.38±0.63[a]	3.78±1.69[a]
	Oct	12.30±3.42[b]	1.19±0.48[b]	1.50±0.57[b]	2.70±1.04[b]	15.00±4.46[b]

[a)] Means with the different letter are significantly different from each other (P<0.05)
[b)] YS: Yeongsil, WS: Witseoreum, JD: Jindallaebat

Variable importance in projection (VIP) score analysis

Among the three research areas on Mt. Halla, Jindallaebat (east slope) is most severely affected by typhoons as it is directly affected by heavy rains caused by counter-clockwise water advection when a typhoon approaches (Choi, 2011). The decline in the Korean fir colony in Jindallaebat between 2006 and 2015 accounted for 71.8% of the total decline of Korean fir colonies on Mt. Halla (Kim et al., 2017). In this study, assuming the Witseoreum as a relatively vulnerable area and Jindallaebat as a relatively healthy area for Korean fir colonies, PLS-DA was performed. Witseoreum and Jindallaebat were significantly distinguished due to concentration of lutein and total carotenoids manifested in Korean firs in October, suggesting that lutein and total carotenoids are the most important discriminatory variables (*Fig. 3, Table 3*).

The VIP score analysis showed that lutein and total carotenoids are the distinguished indicators for the seasonal variations in the three areas (Yeongsil, Witseoreum, and Jindallaebat). Lutein as a major component of carotenoids showed a significant difference in Korean fir leaves according to seasonal changes, regardless of region (*Table 3*). This study demonstrated an increasing pattern of carotenoid components in October, and relatively healthy and declined regions for Korean firs were classified based on the carotenoid concentrations. Although it could be difficult to determine the degree of healthy regions of trees including Korean firs only measured by carotenoids analysis, it seems that changes in carotenoid contents caused by temperature variation are considered to be related to the health of Korean firs. The results provide knowledge on the physiological responses of declining Korean firs' habitats and could provide clues or basic materials for preparing future conservation measures.

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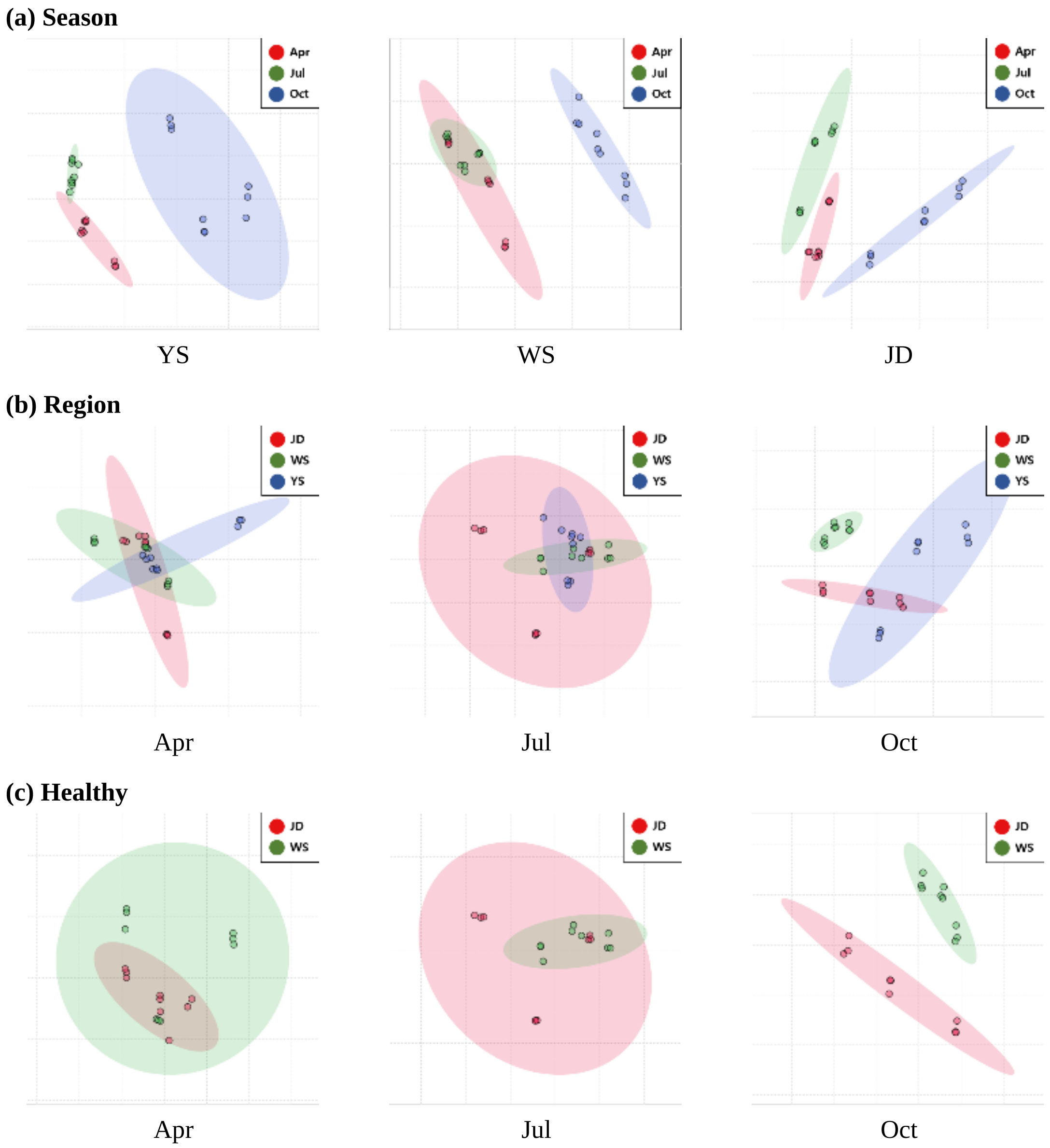

Figure 3. *Partial least squares discriminant analysis (PLS-DA) score plot for carotenoid contents of Abies koreana on Mt. Halla. (YS: Yeongsil, WS: Witseoreum, JD: Jindallaebat)*

Carotenoid content and temperature interactions for Korean firs

An analysis of the correlation coefficients between the carotenoid content of Korean fir leaves and the monthly average temperature of each area and season ($p < 0.01$) showed that the total content of lutein (–0.58), β-carotene (–0.54), and carotenoids (–0.56) were significantly negatively correlated with temperature in Yeongsil (*Table 4*).

The changes in carotenoid content caused by temperature variation differ according to plant species; as a result of this study, in general, the lower the temperature, the higher the carotenoid contents (Massacci et al., 1995; Ormrod et al., 1999; Lisiewska et al., 2004; Lefsrud et al., 2005; Rivera-Pastrana et al., 2010; Lee et al., 2014). According to Oh et al. (2013), the lutein content of Korean firs in Jindallaebat on Mt. Halla increased with increasing altitude and decreasing temperature. In this study, the carotenoid contents of

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Korean firs on Mt. Halla was remarkably low in July when the temperature was high, and increased as the temperature decreased in October, showing a negative correlation with temperature. This is because carotenoids, including lutein, contribute to the removal of free radicals generated during the oxidation process by light in the photosynthetic process, and loss increases upon exposure to excessive light and heat (Ahmad et al., 2013). It also seems to be for the purpose of photoprotection and the maintenance of efficient photosynthetic function in conifers during winter, when photosynthesis is minimal due to low temperatures (Öquist and Huner, 2003; Oh et al., 2013).

Table 3. *VIP scores by PLS-Discriminant Analysis*

Division		**Lutein**	**α-Carotene**	**β-Carotene**	**Total**
Season	YS	1.3880	0.1466	0.1739	1.7085
	WS	1.5293	0.0529	0.0447	1.6269
	JD	1.4006	0.1483	0.1543	1.7032
Region	Apr	1.4653	0.0510	0.2449	1.6591
	Jul	1.0232	0.2914	0.4504	1.7650
	Oct	1.3887	0.0973	0.2205	1.7066
Healthy	Apr	1.3115	0.2599	0.1628	1.7342
	Jul	1.0272	0.3642	0.3759	1.7673
	Oct	1.0346	0.3568	0.3761	1.7674

Means are significant at score > 1.0000, YS: Yeongsil, WS: Witseoreum, JD: Jindallaebat

Table 4. *Correlation analysis (Abies koreana carotenoids and temperature)*

Division			**Lutein**	**α-Carotene**	**β-Carotene**	**Carotene**	**Total**[a)]
Temperature	Season	Apr	+0.2279	+0.3242	**+0.4734***	**+0.4588***	+0.2787
		Jul	- 0.2176	- 0.1401	- 0.2602	- 0.2037	-0.2155
		Oct	- 0.2590	- 0.1409	- 0.1111	- 0.1247	-0.2318
	Region	YS[b)]	**- 0.5866****	- 0.2731	**- 0.5420****	**- 0.4367***	**-0.5640****
		WS	- 0.3808	**+ 0.3983***	- 0.0445	+0.1427	-0.3442
		JD	**- 0.4485***	- 0.0086	- 0.2033	- 0.1119	**-0.3947***

a) Significant at *P<0.05, **P<0.01 (n=9)
b) YS: Yeongsil, WS: Witseoreum, JD: Jindallaebat

Conclusions

Temperature greatly affects the survival of alpine plants. However, interactions in the ecosystem occur organically through various abiotic and biotic factors such as precipitation and soil characteristics. Thus, it is still difficult to predict the cause of the rapid decline and change in the distribution of the Korean fir. In this study, the carotenoid content of Korean firs on Mt. Halla was measured by area and season, and the results confirmed a significant increase in carotenoids in October. It was also found that Witseoreum and Jindallaebat regions had significantly different carotenoid contents because of temperature characteristics, suggesting possibilities to assess health. There are still insufficient studies on the various secondary metabolites involved in the growth of

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trees, including carotenoids. Therefore, further studies are needed to elucidate physiological changes and environmental variables for a comprehensive approach to the preservation of Korean firs on Mt. Halla.

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