J. Korean Wood Sci. Technol. 2020, 48(5): 591~607 pISSN: 1017-0715 eISSN: 2233-7180
https://doi.org/10.5658/WOOD.2020.48.5.591

Original Article

Profiling Patterns of Volatile Organic Compounds in Intact, Senescent, and Litter Red Pine (*Pinus densiflora* Sieb. et Zucc.) Needles in Winter[1]

Won-Sil CHOI[2] · Seung-Ok YANG[2] · Ji-Hyun LEE[2] · Eun-Ji CHOI[2] · Yun-Hee KIM[2] · Jiyoon YANG[3] · Mi-Jin PARK[3,†]

ABSTRACT

This study was aimed to investigate the changes of chemical composition of the volatile organic compounds (VOCs) emitted from red pine needles in the process of needle abscission or senescence. The VOCs in intact, senescent, and litter red pine needle samples were analyzed by headspace-solid phase microextraction gas chromatography-mass spectrometry (HS-SPME-GC/MS). And then, multivariate statistical interpretation of the processed data sets was conducted to investigate similarities and dissimilarities of the needle samples. Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were used to investigate the dataset structure and discrimination between samples, respectively. From the data preview, the levels of major components of VOCs from needles were not significantly different between needle samples. By PCA investigation, the data reduction according to classification based on the chlorophyll a / chlorophyll b (Ca/Cb) ratio were found to be ideal for differentiating intact, senescent, and litter needles. The following OPLS-DA taking Ca/Cb ratio as y-variables showed that needle samples were well grouped on score plot and had the significant discriminant compounds, respectively. Several compounds had significantly correlated with Ca/Cb ratio in a bivariate correlation analysis. Notably, the litter needles had a higher content of oxidized compounds than the intact needles. In summary, we found that chemical compositions of VOCs between intact, senescent, and litter needles are different each other and several compounds reflect characteristic of needle.

Keywords: *Pinus densiflora*, HS-SPME-GC/MS, VOCs, Multi-variate, PCA, OPLS-DA, Chlorophyll a / Chlorophyll b ratio

1. INTRODUCTION

Living stands in pine forest are the main source of a wide range of biogenic volatile organic compounds (VOCs) in the forest atmosphere. It is well known that plant VOCs have various direct and indirect defensive roles against various abiotic and biotic stresses, and show various biological activities and have been used as alternative treatments for diseases (Ahn *et al.*, 2018; Jeong *et al*., 2017). Most VOCs from trees contribute

[1] Date Received April 7, 2020, Date Accepted June 29, 2020
[2] National Instrumentation Center for Environmental Management, Seoul National University, Seoul 08826, Republic of Korea
[3] Division of Wood Chemistry, Department of Forest Products, National Institute of Forest Science, Seoul 02455, Republic of Korea
† Corresponding author: Mi-Jin PARK (e-mail: lionpmj@korea.kr, ORCID: 0000-0002-7748-3886)

to the characteristic atmospheric environment of forests and provide useful bioresources of essential oil for the flavor and fragrance industries (Kim *et al.,* 2017; Yang *et al.,* 2019; Venkatesan *et al*., 2020).

In pine trees, the VOCs are produced mainly in needle tissue via photosynthetic pathways, and therefore living intact needles are important for continuous emission and accumulation of VOCs. However, needle abscission or senescence of living stands is usually caused by environmental stressors, such as drought or flooding, atmospheric temperature fluctuation, soil salinity, heavy metals, etc. (Finkelstein, 2013). The premature senescence and shedding of leaves can be induced by a deficit of water. The loss of pine needles during droughts involves true abscission; in other plants, the leaves merely wither and die. As temperatures decline in fall, the leaves of trees stop producing chlorophyll, and some species that contain large amounts of carbohydrates and possess the hereditary potential to do so begin to form anthocyanins in their leaves. As chlorophyll synthesis stops, the chlorophyll already present in the leaves begins to degrade and the newly formed anthocyanins are unmasked (Krammer and Kozlowski, 1979).

On the other hand, there are only a few studies whether the VOCs composition in needle are preserved or changed after needle abscission or senescence. The shed of aged or damaged needles in pine stands is important for the release of VOCs from the forest floor (Isidorov *et al.*, 2003, Wang *et al.*, 2018). Even when senescent or dead, pine needles contain high levels of terpenes and other precursors of the oxidized volatile compounds, formed as a result of enzymatic reactions (Isidorov *et al*., 2010; Jo and Kim, 2010; Zhang *et al*., 2018). Coniferous litter is also a good source of secondary metabolites that are released during chemical or biological decomposition processes. Intact Scots pine needles contain more than 70 organic compounds of different classes (Isidorov *et al*., 2003), as shown by solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS). They observed that the storage for four weeks on a conical floor stressed needles and caused their chemical compositions to vary.

It is obvious that volatile metabolomic patterns vary between intact and damaged or senescent needles, and statistical analysis should show which compounds constitute important discriminant variables. Besides the authentication and identification of needles, both marked and slight differences between the unique characteristics of needles can be studied with multivariate analysis. A practical and efficient assessment of biomass availability on pine forest floors could be made possible and easily accessible via the metabolomic interpretation of chemical components.

The objectives of the present study were to determine which VOCs in red pine needles change over the course of senescence or after shedding in relation to chlorophyll variation in the needles, and hence to reveal similarities and dissimilarities among intact, senescent, and litter needles.

2. MATERIALS and METHODS

2.1. Materials

The needles of red pines (*Pinus densiflora* Sieb. et Zucc.) were collected from standing trees at three site at Seoul National University, Seoul Campus, Republic of Korea (37°27'28.0"N 126°56'56.5"E) in the period of from November 2019 until January 2020. Intact needles were collected with an amount of 5-10 g for each stand and combined as a sample set, and senescent and litter needles were collected from broken branches and the upper layer of fallen needle on the wide-spread floor zone under the tree stands, respectively. Information on the samples is presented in Table 1, and sample code was named to reflect sampling site position and date. Collected needles (50-100 g in a

sample set) were stored at -80°C after collection for further analysis. The experiments were repeated three times by pooling samples.

2.2. Sample preparation

Frozen needles were crushed and milled in liquid nitrogen using a Freezer/Mill cryogenic grinder (SPEX 6875D, SPEX Co., USA) to avoid any chemical decomposition or change in the chemical composition of the needles after collection. Some of the milled needle samples were further freeze-dried and used for pigment analysis.

2.3. Pigment analysis

The chlorophyll a (Ca), chlorophyll b (Cb), and carotenoid (Cx-c) contents of the samples were determined according to the method described by Wellburn (1994). Briefly, 1 g sample mixed with 30 mL methanol in a 50 mL conical tube (polypropylene) was sonicated for 1 h and then shaken for 1 h. Samples were filtered with a 0.2 μm syringe filter. Absorbance was read at 666, 653, and 470 nm on a spectrophotometer (SpectraMaxⓇ ABS Plus, Molecular Devices LLC., USA) to determine Ca, Cb, and Cx-c content, respectively. The experiments were repeated three times. The amount of these pigments in the samples was calculated according to the formulae of Lichtenthaler and Wellburn (1983) as shown in the following equations, and expressed in microgram per unit dry weight gram of sample.

$$Ca = 15.65 \times A_{666} - 7.34 \times A_{653}$$
$$Cb = 27.05 \times A_{653} - 11.21 \times A_{666}$$
$$Cx\text{-}c = (1{,}000 \times A_{470} - 2.86 \times Ca - 129.2 \times Cb) / 245$$

where, A_{666}, A_{653}, and A_{470} are absorption values at 666 nm, 653 nm, and 470 nm spectral wavelength, respectively.

2.4. VOC analysis by headspace–solid phase microextraction gas chromatography–mass spectrometry

Next, the composition of VOCs emitted from the freeze-milled samples was investigated using a headspace-solid phase microextraction gas chromatog-

Table 1. Characteristics of red pine needle samples

Sampling Date	Needle Class	Needle Color with Naked Eye	Sample Name	Number of standing trees
2019.11.06	Intact	Green	G10	3
2019.11.06	Senescent	Yellowish-green	Y10	3
2019.11.06	Litter	Brown	B10	3
2019.11.06	Intact	Green	G20	3
2019.11.06	Litter	Brown	B20	3
2019.11.06	Intact	Green	G30	8
2019.11.06	Senescent	Yellowish-green	Y30	8
2019.11.06	Litter	Brown	B30	8
2020.01.22	Intact	Green	G31	8
2020.01.22	Senescent	Yellowish-green	Y31	8
2020.01.22	Litter	Brown	B31	8
2020.01.31	Intact	Green	G32	8
2020.01.31	Senescent	Yellowish-green	Y32	8
2020.01.31	Litter	Brown	B32	8

raphy-mass spectrometry (HS-SPME-GC/MS) system (TSQ 8000, Thermo Fisher Scientific Inc., USA). Five hundred milligram samples were placed into 25 mL glass vessels and 4 μL of internal standard solution (100 ppm 1,4-dichlorobenzene-d4 in methanol) was added. The necks of the vessels were tightly sealed with an elastic Teflon film. After incubation on an autosampler at 30°C for 10 min, the VOCs from the headspace were adsorbed on an SPME fiber coated with divinylbenzene/polydimethylsiloxane (DVB/PDMS, 50/30 mm, 10 mm fiber length, SUPELCO Co., USA). The total exposition time was 40 min and every 12 s the vessels with needle samples were slightly shaken to mix the gas phase for 10 s. After adsorption of the headspace, VOCs were desorbed by introducing the SPME fiber into the injector of the gas chromatograph with mass spectrometer. The injector temperature was set at 250°C. During injection, the fiber was maintained for 2 min in the injector, which was used in split mode (10:1). Components were separated on a DB-wax fused silica column (60 m length × 0.25 mm internal diameter × 0.5 μm film thickness). The carrier gas was helium at a flow rate of 2.0 mL/min. The oven temperature was altered as follows: the thermostat temperature was initially 40°C (holding time 2 min), then was increased to 150°C (holding time 10 min) at a rate of 4°C/min, then to 200°C (holding time 5 min) at a rate of 3°C/min, and finally to 240°C (holding time 5 min) at a rate of 10°C/min. The mass spectrometer transfer line temperature was 250°C and the ion source temperature was 250°C. Mass scanning was performed from 35 to 550 amu for with scan time of 0.2 s. Component qualification was conducted by comparison of the measured mass spectra with those from an NIST/EPA/NIH Mass Spectral Library (version 2.0 g, NIST, USA) and additional Kovats retention index information (the retention times of alkanes were obtained by direct injection of standard sol-

Table 2. Content of chlorophyll a (Ca), chlorophyll b (Cb), and carotenoid in red pine needles according to spectrophotometric determination

Samples	Needle Class	Chlorophyll a (μg/dwg[1])	Chlorophyll b (μg/ dwg)	Carotenoid (μg/ dwg)	Ca/Cb ratio[2]
G10	Intact	1,568.1 ± 9.7[3]	994.2 ± 2.4	4.0 ± 1.0	1.58
G20	Intact	1,351.7 ± 6.3	835.1 ± 6.9	44.5 ± 0.9	1.62
G30	Intact	1,396.3 ± 11.4	867.3 ± 7.9	2.9 ± 0.9	1.61
G31	Intact	914.8 ± 3.5	512.5 ± 2.3	150.2 ± 1.4	1.80
G32	Intact	1,233.1 ± 64.8	737.7 ± 38.5	192.9 ± 10.3	1.67
Y10	Senescent	161.5 ± 1.7	151.5 ± 2.3	45.2 ± 0.1	1.07
Y30	Senescent	78.2 ± 0.8	87.0 ± 1.3	49.8 ± 0.5	0.90
Y31	Senescent	561.4 ± 1.3	524.6 ± 2.8	10.7 ± 0.8	1.10
Y32	Senescent	1,118.7 ± 70.7	1,031.3 ± 63.1	20.4 ± 1.4	1.08
B10	Litter	55.2 ± 1.0	73.1 ± 1.6	10.7 ± 0.4	0.75
B20	Litter	55.8 ± 1.3	103.1 ± 1.6	11.9 ± 0.2	0.54
B30	Litter	71.2 ± 0.7	85.4 ± 0.5	25.2 ± 0.9	0.83
B31	Litter	51.8 ± 2.0	76.5 ± 2.7	19.0 ± 0.8	0.70
B32	Litter	90.9 ± 3.4	153.7 ± 2.3	22.1 ± 2.5	0.59

[1]dwg: g of dry weight
[2]Ca/Cb ratio: Chlorophyll a/Chlorophyll b ratio
[3]Data indicate an average ± standard deviation. (n=3)

ution under the same GC-MS conditions). To calculate retention time index, a standard solution containing C7-C30 normal alkanes in dichloromethane was used. The area of corresponding peaks was calculated by integration of peak intensity on the selected ion monitoring chromatogram from the total ion chromatogram (the parameter is shown in Table 3). The calculated area of each compound was normalized by dividing by the internal compound peak area and multiplying by 100 to give the semi-quantitative composition of VOCs. The two-array sample data sets were used for the following statistical analysis.

2.5. Statistical data analysis

Principal component analysis (PCA) was implemented on the two-way array to overview data structure and relation trend between data sets. Results were presented as score plots. For further discriminant analysis, the orthogonal partial least square discrimination analysis (OPLS-DA) was conducted. OPLS-DA model is a useful discriminant model for metabolic fingerprinting studies of chromatography data sets (Bernal *et al*., 2016). An OPLS-DA model was therefore constructed for classification of the samples with chlorophyll variables to determine the important compounds among samples. The PCA and OPLS-DA models were created using SIMCA 15 software (Umetrics Inc.), and bivariate correlation analyses were conducted using IBM SPSS Statistics (IBM Co., ver 25.0).

3. RESULTS and DISCUSSION

3.1. Pigment analysis

The amounts of Ca, Cb and Cx-c in needle samples are shown in Table 2. The average amount of both Ca and Cb was lowest in litter needles and highest in intact needles, while there was no obvious trend or pattern for Cx-c. During the growing season, chlorophyll is constantly broken down and replaced. According to Taylor and Whitelaw (2001), abscission is frequently associated with senescence as both processes are initiated in evergreen plants by many of the same developmental as well as environmental and stress factors, such as changed photoperiod and temperature. Abscission involves the shedding of aged or diseased organs and often occurs as a consequence of senescence related to the death of an organism or some part of it. The low efficiency of photosynthesis below a certain threshold causes the senescence of needles and abscission following cessation of photosynthesis and an endogenous decline in plant hormones (Thimann, 1985). As the photoperiod decreases in the fall, the synthesis of new chlorophyll is prevented by an abscission layer of cork-like material that forms due to the rapid division of cells near the leaf stem (Webster and Leopold, 1972). This abscission layer prevents transport of carbohydrates and minerals to and from leaf, causing chlorophyll to be broken down but not replaced. Therefore, a lower amount of Ca and Cb in needles is related to senescence or abscission, although this relationship is not straightforward because environmental and stress factors can also influence Ca and Cb levels.

In the present study, the Ca/Cb ratio was 1.7 ± 0.1 in intact green needles, 1.0 ± 0.1 in senescent yellowish-green needles, and 0.7 ± 0.1 in brown litter needles, as shown in Table 2. The Ca/Cb ratio was high in intact needles and low in litter needles because in many species Ca is destroyed more rapidly than Cb in the fall and under water stress (Krammer and Kozlowski, 1979). Therefore, the Ca/Cb ratio was regarded as a good indicator of three levels of senescence (intact, senescent, litter), whereas Ca, Cb, and Cx-c values appear to be poor indicators of senescence as they varied greatly within the same needle sample. Therefore, the Ca/Cb ratio was used to investigate variation in VOCs among needle samples.

Table 3. The VOCs identified in needle of red pine tree by HS-SPME-GC/MS analysis

Retention Time (min)	Quantitation ion (m/z)	Component Name	Kovats Retention Index[1]
11.9	81	2-Ethyl furan	915
13.5	57	2-Methyl-3-pentanone	974
13.8	58	4-Methyl-2-pentanone	984
14.5	93	Tricyclene	1005
15.1	93	α -Pinene	1021
16.5	74	2-Methyl-1-propanol	1060
16.7	93	Camphene	1064
18.3	93	β -Pinene	1104
18.6	93	Sabinene	1114
18.7	91	2,4(10)-Thujadiene	1117
19.0	57	1-Penten-3-ol	1127
19.4	69	4-Methyl-2-pentanol	1136
19.9	93	β -Myrcene	1149
20.3	93	l-Phellandrene	1159
20.6	74	Methyl hexanoate	1167
20.7	83	2-Methyl-2-butenoate	1170
20.8	121	α -Terpinene	1173
20.9	70	Isopentanol	1175
21.1	83	Cyclohexene oxide	1180
21.4	45	2-Hexanol	1188
21.5	67	*d*-Limonene	1191
21.8	41	(*E*)-2-Hexenal	1198
22.0	93	β -Phellandrene	1202
22.5	68	Isoprenol	1217
24.0	119	*p*-Cymene	1259
24.6	93	Terpinolene	1275
25.0	57	(*E*)-2-Penten-1-ol	1284
25.0	71	3-Methyl-2-buten-1-ol	1285
25.8	83	2-Heptenal	1305
26.1	43	Sulcatone	1315
26.2	56	1-Hexanol	1318
27.3	67	(*E*)-3-Hexen-1-ol	1351
28.2	87	2-Butoxy-ethanol	1375
28.2	82	Cyclohexanol	1377
28.4	81	2,4-Hexadienal	1380
28.5	91	1,3,8-*p*-Menthatriene	1383
29.0	150	Perillene	1397
29.7	117	*p*-Cymenene	1416
30.9	59	Linalool oxide	1447

Table 3. (Continued)

Retention Time (min)	Quantitation ion (m/z)	Component Name	Kovats Retention Index [1]
31.4	105	α -Cubebene	1460
32.9	77	Benzaldehyde	1496
33.1	119	α -Copaene	1500
33.6	95	Camphor	1510
34.3	81	β -Bourbonene	1524
35.7	81	Pinocarvone	1551
36.0	149	Methyl thymyl ether	1556
36.3	95	*L*-Bornyl acetate	1561
36.7	71	Terpinen-4-ol	1568
37.1	161	Longifolene	1577
37.5	105	Methyl benzoate	1584
38.0	161	β -Cubebene	1592
38.3	91	Caryophyllene	1597
38.8	79	Myrtenal	1606
38.9	161	Aromadendrene	1609
39.6	92	Pinocarveol	1620
40.6	69	*cis*-β -Farnesene	1638
40.7	109	*cis*-Verbenol	1640
41.3	95	Cryptone	1650
41.7	59	α -Terpineol	1656
42.2	95	Borneol	1665
42.3	93	α -Humulene	1666
43.0	161	γ -Muurolene	1678
43.5	135	Verbenone	1685
43.8	68	Linalool oxide1	1691
44.3	161	Germacrene-D	1699
44.8	105	α -Muurolene	1708
45.0	105	β -Selinene	1712
46.4	161	δ -Cadinene	1741
46.7	161	γ -Cadinene	1748
47.0	79	Myrtenol	1752
48.3	105	α -Cadinene	1778
49.3	135	*p*-Cymen-8-ol	1798
57.1	79	Caryophyllene oxide	1973
59.5	96	Humulene oxide	2027
62.5	91	Spathulenol	2091

[1]Kovats Retention Index : Retention indices were determined using n-alkane C7 to C30 as an external standard

3.2. VOCs analysis by HS-SPME-GC/MS

3.2.1. Preview of VOCs emitted from pine needles

HS-SPME-GC/MS analysis is a powerful tool for analyzing the metabolomic patterns of essential oils (Goncalves *et al*., 2012). Table 3, 4, and 5 show the qualitative and semi-quantitative (quantitation was performed using an internal normalization method) composition of the VOCs emitted from the needles in the present study. Each component was identified based on its mass spectrum using a NIST library search program (NIST MS Search 2.0 g). Interestingly, the VOCs content of the senescent and litter needles was comparable to that of intact needles, but the chemical composition of the VOCs was slightly different.

Both non-terpenic aliphatic compounds and terpenic compounds were found in all needles (Table 4 and 5). The most abundant chemical group consists of terpene compounds: monoterpenes and sesquiterpenes. The major volatile compounds emitted by intact needles were an alkene (2-hexenal), monoterpenes (α-pinene, β-myrcene, β-phellandrene, myrcene, 2-β-pinene, *d*-*L*-limonene, *L*-bornyl acetate, and camphene), and sesquiterpenes (caryophyllene and germacrene-D). These results are similar to those of Lee *et al.* (2005)

Table 4. Relative level of non-terpenic VOCs from red pine needles analyzed by HS-SPME-GC/MS

Chemical Class	Compounds	Intact	Senescent	Litter
Alkane	1-Hexanol	16.6 ± 23.7[1]	14.6 ± 18.2	4.0 ± 2.6
	2-Hexanol	n. d[2]	2.9 ± 0.4	0.8 ± 0.1
	4-Methyl-2-pentanol	n. d	1.8 ± 0.7	1.6 ± 1.6
	4-Methyl-2-pentanone	n. d	1.2 ± 1.2	2.8 ± 2.6
	2-Methyl-3-pentanone	n. d	0.9 ± 0.7	1.6 ± 1.0
	Cyclohexanol	1.0 ± 1.9	0.9 ± 0.8	1.1 ± 0.9
	2-Butoxy-ethanol	n. d	2.4 ± 0.4	1.5 ± 1.1
	Methyl hexanoate	1.7 ± 1.6	4.4 ± 4.9	4.6 ± 4.9
Alkene	1-Penten-3-ol	12.5 ± 5.0	0.8 ± 0.5	0.6 ± 0.4
	2,4-Hexadienal	1.8 ± 1.3	0.6 ± 0.7	n. d
	2-Methyl-2-butenoate	n. d	0.5 ± 0.4	1.2 ± 0.6
	2-Heptenal	1.2 ± 0.7	1.9 ± 0.4	0.2 ± 0.1
	(*E*)-2-Hexenal	203.8 ± 108.8	2.6 ± 0.5	n. d
	(*Z*)-2-Penten-1-ol	8.2 ± 4.4	0.3 ± 0.1	n. d
	Isoprenol	0.3 ± 0.2	2.0 ± 2.1	3.3 ± 3.3
	Sulcatone	0.6 ± 0.1	2.0 ± 0.6	3.0 ± 0.7
	Cyclohexene oxide	4.6 ± 0.5	0.6 ± 0.1	0.8 ± 0.1
	(*Z*)-3-Hexen-1-ol	25.5 ± 20.0	17.9 ± 19.8	1.9 ± 2.1
Heterocyclics Furan	2-Ethyl-furan	3.7 ± 2.5	0.4 ± 0.3	0.2 ± 0.1
Aromatic	Benzaldehyde	2.6 ± 1.8	21.1 ± 21.1	23.3 ± 12.7
	Methyl benzoate	7.8 ± 6.1	25.2 ± 11.5	19.2 ± 16.6

[1] Compound amount was estimated based on the percent ratio of peak area of identified compound to internal standard peak area. Each amount was calculated based on extracted ion chromatograms from scanning data.
[2] n. d: not detected

Table 5. Relative ratio level of terpenic VOCs from red pine needles analyzed by HS-SPME-GC/MS

Chemical Class	Compounds	Intact	Senescent	Litter
Monoterpenes				
Furan	Perillene	0.3 ± 0.1[1]	0.4 ± 0.1	0.4 ± 0.2
Bornane	Borneol	21.4 ± 12.7	76.8 ± 87.6	127.1 ± 115.6
	Camphor	0.7 ± 0.7	44.8 ± 59.1	53.4 ± 31.4
	L-Bornyl acetate	191.8 ± 71.1	50.1 ± 31.4	13.8 ± 8.3
Isocamphane	Camphene	179.3 ± 60.7	234.2 ± 37.4	214.4 ± 62.9
Myrcane	Linalool oxide (1)	1.2 ± 0.1	0.4 ± 0.4	1.5 ± 1.9
	β -Myrcene	1,170.6 ± 906.1	1,505.1 ± 595.9	833.4 ± 176.1
	Linalool oxide (2)	n. d[2]	6.3 ± 3.6	9.8 ± 5.9
Pinane	2-β -Pinene	386.1 ± 216.6	574.3 ± 219.0	445.9 ± 194.7
	cis-Verbenol	0.9 ± 1.1	1.1 ± 1.0	3.5 ± 3.2
	Myrtenal	n. d	1.2 ± 0.3	5.6 ± 4.5
	Myrtenol	0.5 ± 0.3	4.8 ± 4.5	5.6 ± 2.1
	Pinocarveol	n. d	2.4 ± 1.0	5.7 ± 2.9
	Pinocarvone	0.4 ± 0.2	1.0 ± 0.7	4.9 ± 3.7
	Verbenone	0.3 ± 0.1	3.0 ± 3.0	4 ± 3.7
	α -Pinene	2,277.2 ± 945.7	3,153.4 ± 560.3	2,759.2 ± 421.8
p-Menthane	1,3,8-*p*-Menthatriene	0.8 ± 0.4	2.5 ± 0.4	2.1 ± 0.7
	Cryptone	2.2 ± 1.4	2.1 ± 0.3	1.5 ± 1.1
	d-Limonene	248.6 ± 92.7	344.2 ± 54.7	299.8 ± 57.9
	l-Phellandrene	31.6 ± 17.1	26.7 ± 6.8	88.2 ± 129.0
	Methyl thymyl ether	98.5 ± 83.3	90 ± 23.3	57.9 ± 21.8
	p-Cymen-8-ol	2.2 ± 1.6	10.1 ± 7.1	13.5 ± 9.7
	p-Cymene	9.5 ± 3.7	31.1 ± 5.0	86.6 ± 47.5
	p-Cymenene	3.5 ± 2.6	19.7 ± 13.2	35.2 ± 24.0
	Terpinen-4-ol	2.0 ± 2.0	1.8 ± 1.8	2.0 ± 1.4
	Terpinolene	308.8 ± 187.9	531.2 ± 105.0	305.2 ± 85.6
	α -Terpinene	7.5 ± 3.1	5.7 ± 1.3	3.7 ± 1.1
	α -Terpineol	1.0 ± 1.0	5.1 ± 2.5	5.6 ± 4.5
	β -Phellandrene	1,918.8 ± 974.3	1,837.4 ± 312.9	1,210.2 ± 240.1
Thujane	2,4(10)-Thujadiene	0.7 ± 0.6	1.4 ± 1.1	3.3 ± 3.5
	Sabinene	45.5 ± 29.5	33.5 ± 24.4	10.1 ± 7.3
Sesquiterpenes				
Aromadendrane	Aromadendrene	4.3 ± 2.0	4.5 ± 2.1	5.0 ± 1.5
	Spathulenol	0.4 ± 0.2	2.6 ± 1.6	2.3 ± 1.0
Bicyclogermacrane	Bicyclogermacrene	31.7 ± 15.1	6.8 ± 5.8	4.0 ± 1.3
Bourbonane	β -Bourbonene	5.9 ± 5.2	10.0 ± 4.9	14.3 ± 3.2

Table 5. (Continued)

Chemical Class	Compound Name	Intact	Senescent	Litter
Cadinane	α -Cadinene	5.3 ± 2.5	3.6 ± 1.5	3.8 ± 1.1
	γ -Cadinene	32.1 ± 13.9	34.7 ± 7.0	45 ± 8.3
	δ -Cadinene	49 ± 20.4	49.4 ± 8.9	59.7 ± 13.1
Caryophyllane	Caryophyllene	196.6 ± 67.5	246.9 ± 46.3	213.5 ± 24.1
	Caryophyllene oxide	0.8 ± 0.3	7.0 ± 2.2	12.8 ± 5.8
Copaane	α -Copaene	13.2 ± 5.8	16.2 ± 2.8	18.1 ± 2.2
Cubebane	α -Cubebene	8.0 ± 3.0	8.4 ± 1.9	9.3 ± 1.0
	β -Cubebene	6.7 ± 3.1	5.8 ± 2.0	6.8 ± 1.1
Eudesmane	α -Selinene	5.4 ± 2.5	9.0 ± 3.1	9.9 ± 3.1
	β -Selinene	6.2 ± 2.9	10.2 ± 3.7	12.1 ± 3.8
Farnesane	*cis*-β -Farnesene	4.5 ± 3.0	8.8 ± 6.9	6.8 ± 3.0
Germacrane	Germacrene-D	122.8 ± 61.4	54.4 ± 37.3	35.6 ± 30.5
Humulane	Humulene oxide	n. d	1.1 ± 0.4	1.9 ± 1.0
	α -Humulene	89.8 ± 34.4	107.4 ± 19.3	101.7 ± 17.6
Longifolane	Longifolene	14.7 ± 6.3	21.5 ± 8.6	21.9 ± 6.3
Muurolane	α -Muurolene	16.0 ± 7.4	12.9 ± 2.6	16.5 ± 3.2
	γ -Muurolene	24.0 ± 10.4	22.1 ± 5.8	28.8 ± 2.8
Hemiterpeneste	Isopentanol	0.8 ± 0.1	7.0 ± 1.8	9.0 ± 10.2
	2-Methyl-1-propanol	0.2 ± 0.1	0.4 ± 0.3	0.5 ± 0.2
	3-Methyl-2-buten-1-ol	0.4 ± 0.6	7.0 ± 2.9	4.7 ± 7.8

[1] Compound amount was estimated based on the percent ratio of peak area of identified compound to internal standard peak area. Each amount was calculated based on extracted ion chromatograms from scanning data.
[2] n. d: not detected

and Yu *et al*. (2004).

The VOCs composition emitted from senescent and litter needles was similar to that of intact needles, but the emissions of germacrene-D, 2-hexenal, and *L*-bornyl acetate were much lower in the senescent and litter needles. Several alkanes, such as a C6 compound of 2-hexanol, C5 compounds of 4-methyl-2-pentanol, 4-methyl-2-pentanone, and 2-methyl-3-pentanone, and a C4 compound of 2-butoxyethanol, were detected only in the senescent and litter needles, in addition to terpenic compounds, such as linalool oxide, myrtenal, pinocarveol, *p*-cymene, *p*-cymenen, caryophyllene oxide, and humulene oxide. According to Isidorov *et al*. (2003), oxidized or modified forms of terpenes were increased or found only in the litter needles of the Scots pine, including toluene, *p*-cymene, verbenene, *p*-cymenen, *p*-cymene-8-ol, anisaldehyde, and linalool acetate as minor compounds. The authors suggested that most of these compounds are secondary products of microorganism metabolism involving dehydration and oxidation of precursor terpenic compounds in intact needles. Our results are similar to those of Isidorov *et al.* (2003), but the experimental evidence on this topic is insufficient and further study is needed.

3.2.2. Multivariate analysis

As shown in Table 4 and Table 5, major compounds with higher ratio values are more important than minor

compounds with lower ratio values. However, the more compounds in a sample, the more complex and difficult it becomes to determine which compounds differ between levels of senescence. Therefore, we used a clustering and correlation statistical technique to determine which compounds among the VOCs of needles are most important for identifying senescence, and then to investigate changes in VOCs during senescence or after shedding.

In order to improve the quality of the data by reducing inconsistency and noise, pre-processing algorithms were applied to the raw data. To minimize data skewness, the log transformation (log x) was applied to the raw data in Table 4 and Table 5, and then Pareto scaling was applied to eliminate the unfavorable effects among compounds present in substantially different amounts.

PCA model of VOCs from pine needles

PCA was applied to the pre-processed data to investigate the correlation structure.

On the PCA-derived score plot (Fig. 1A), intact needles were located far from senescent and litter needles, indicating that intact needles are different based on principal component (PC) 1. The first two PCs were able to explain 56.7% of the total variance of the variable set, and there was no outlier in this model. However, there was overlap between senescent and litter needles; therefore, it was not possible to differentiate these two variables on this PC1 and PC2 plane model. While chlorophyll content variation was high among samples due to the ecological environment (Shan *et al.*, 1996), seasonal variation (Gond *et al.*, 1999; Deligoz *et al.*, 2018), soil type, etc., the Ca/Cb ratio has lower deviation in the same sampling part and was significantly different between samples. Therefore, the Ca/Cb ratio was used to define classes in a PCA-derived score plot (Fig. 1B) with three classes (Ca/Cb ratio values: Class 1, 0.5−0.95; Class 2, 0.95−1.35; Class 3, 1.35−1.80). The senescent and litter needles were more easily differentiated based on Ca/Cb ratio; Y31, Y32, and Y33 were close to the litter needles (Class 1) due to their low Ca content (Table 2). Thus, it is thought that the Ca/Cb ratio value is an ideal discriminant variable for intact and litter needles on the PC1 plane of a PCA-derived score plot.

OPLS-DA model of VOCs from pine needles

The OPLS-DA model was applied to the data set after reprocessing according to Ca/Cb ratio classes in order to investigate variability in chemical compounds to discriminate between senescence levels in needles.

The resulting score plot (Fig. 2A) shows that classi-

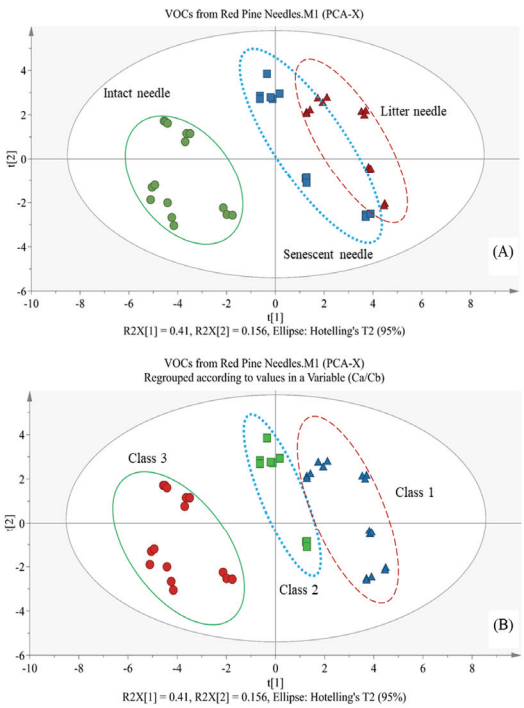

Fig. 1. PCA score plot of the volatile compounds of intact (green round), senescent (blue square), and litter (red triangle) needles according to sample (A). The Ca/Cb ratio was used to define classes in a PCA-derived score plot (B) with three classes (Class 1: Ca/Cb ratio values 0.5−0.95; Class 2: Ca/Cb ratio values 0.95−1.35; Class 3: Ca/Cb ratio values 1.35−1.80).

fication is possible based on Ca/Cb ratio classes. The plot (Fig. 2B) strongly indicates that the original model is valid; all predicted Q2-values are lower than the original points and the regression line of the Q2-points intersects the vertical axis below zero. Also, all R2-values to the left are lower than the original points to the right, which is another indication of the validity of the original model.

The variable importance for the projection (VIP) plot (Fig. 3) summarizes the importance of the variables both to explain variables (compounds) and to correlate to sample character in a statistical projected plane. The non-terpenic compounds (*E*)-2-hexenal, 1-penten-3-ol, 4-methyl-2-pentanone, 2-ethyl furan, (*Z*)-3-hexen-1-ol, and 2-penten-1-ol, and terpenic compounds of camphor, *L*-bornyl acetate, myrtenol and caryophyllene oxide were importantly found (VIP value > 1.0) in the VIP plot, and deviated more among needles than the terpenic compounds. Most strong (VIP value > 1.5) VIPs were camphor and 1-penten-3-ol, without 2-hexenal due to its large deviation, and were thought to have the critical role in the overall discrimination in the OPLS-DA-derived score plot, especially to explain the differences between intact and litter samples. And figure 2C shows the distribution of VOCs related to sample classification; their distribution deviated among needle samples. The contribution list (Table 6) from the bi-plot (Fig. 2C) shows the difference between each point (scaled and centered as the workset) and the average of the model, which indicates the characteristics of each class and which compounds are discriminants. For the intact needles (Class 1), 1-penten-3-ol and 2-ethyl furan, and *L*-bornyl acetate were the strong (score > 1.5) contributors to the dissimilarity of this class to the others, while camphor, pinocarvone, and myrtenol were for

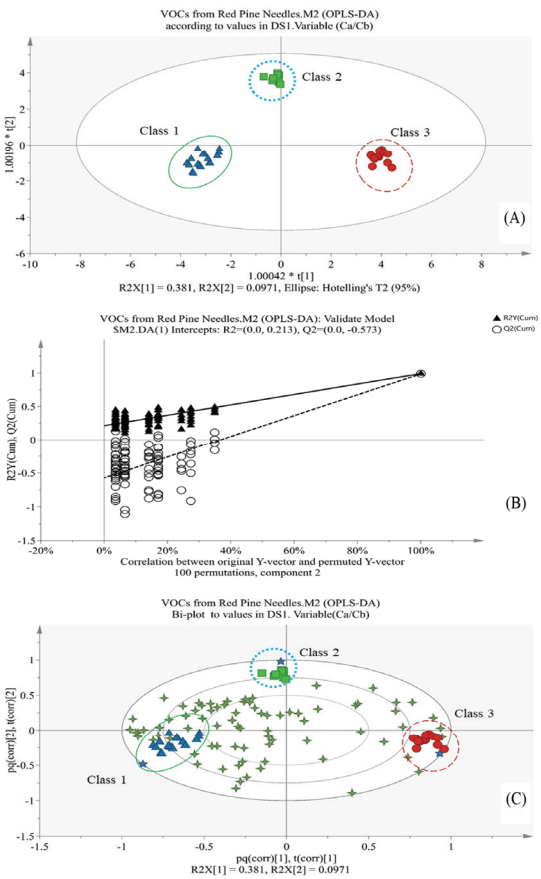

Fig. 2. OPLS-DA analysis based on Ca/Cb ratio classes (Class 1: Ca/Cb ratio values 0.5-0.95; Class 2: Ca/Cb ratio values 0.95−1.35; Class 3: Ca/Cb ratio values 1.35−1.80).
(A): OPLS-DA derived score plot,
(B): OPLS-DA permutation plot,
(C): OPLS-DA bi-plot.

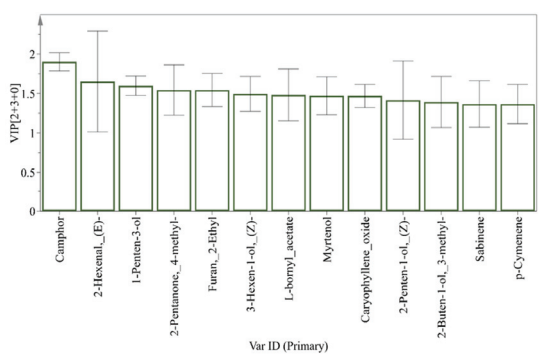

Fig. 3. The major volatile metabolites identified in red pine needles according to variables importance plot (VIP > 1.0) for OPLS-DA derived score plot.

Class 3 (litter needles), and 2-heptanal, β-myrcene, 3-methyl-2-buten-1-ol, and spathulenol were for of Class 2 (senescent needles) in the OPLS-DA-derived score plot plane. 1-Penten-3-ol and camphor were most strong contributors for intact (Class 1) and litter (Class 3) needle, respectively, which result had a same trend with the result by VIP values evaluation.

The content of the leaf aldehyde (*E*)-2-hexenal, an aroma-active compound of fresh red pine needles (Yu *et al*., 2004), was higher in intact needles and almost absent in litter needles (Table 4). The content of the leaf alcohol (*Z*)-3-hexen-1-ol followed a similar pattern to (*E*)-2-hexenal. The C6 volatile compounds (*Z*)-3-hexen-1-ol and (*E*)-2-hexenal are biosynthesized via the lipoxygenase pathway, which includes a secondary hydroxyperoxide lyase reaction from unsaturated fatty acids, such as linolenic acid and linoleic acid, and the formation of the C5 volatile compounds 1-penten-3-ol and 2-penten-1-ol from inhibited secondary hydroxyperoxide lyase (Shen *et al*., 2014). The levels of (*E*)-2-hexenal, 1-penten-3-ol, (*Z*)-3-hexen-1-ol, and 2-penten-1-ol were significantly higher in intact needles than in senescent and litter needles, and these compounds with high VIP values were characteristic non-terpenic compounds of intact needles. As for terpenic compounds, also shown in Table 4 and Table 5, the content of camphor, an oxidized form of monoterpene with a borane skeleton, was higher in senescent and litter needles than in intact needles, while the opposite was true of *L*-bornyl acetate, which features the same borane skeleton. Furthermore, the levels of *p*-cymenene and *p*-cymene, which are chemically stable compounds due to the presence of a benzene ring, were higher in litter than intact needles, while the content of labile bicyclogermacrene and germacrene-D were higher in intact needles. Oxidized or modified terpenes were contributors (Table 6) and had high VIP values (Fig. 3) in litter needles in the

Table 6. The major VOCs identified in red pine needles according to contribution plot (weight > 1.0) list of OPLS-DA derived score plot

Class according to Ca/Cb ratio	Compound Name	Chemical Class	Score Contrib (Group - Average), Weight=p1p2
Class 1	1-Penten-3-ol	alkene	2.50226
(Intact Needle)	2-Ethyl furan	heterocyclics	2.28275
	L-Bornyl acetate	monoterpene	1.90871
	(*Z*)-3-Hexen-1-ol	alkene	1.25681
	Bicyclogermacrene	sesquiterpene	1.09741
	Cyclohexene oxide	alkene	1.0649
	Germacrene-D	sesquiterpene	1.02957
Class 2	2-Heptenal	alkene	2.04603
(Senescent Needle)	β-Myrcene	monoterpene	1.68568
	3-Methyl-2-buten-1-ol	alkene	1.57739
	Spathulenol	sesquiterpene	1.38274
Class 3	Camphor	monoterpene	2.09959
(Litter Needle)	Pinocarvone	monoterpene	1.68045
	Myrtenol	monoterpene	1.46641
	Caryophyllene oxide	sesquiterpene	1.43508
	p-Cymenene	monoterpene	1.32866
	p-Cymene	monoterpene	1.32645

OPLS-DA model, and were characteristic compounds of senescent and litter needles.

Therefore, it was suggested that the oxidation reaction involving the lipoxygenase pathway occurred in the intact needles, to a lesser extent or not at all in the senescent and litter needles, and that the terpenic compounds were also degraded or modified during senescence or after shedding. Unfortunately, in this paper, there were no experimental data on the evidence about oxidation, degradation, and modification reactions of VOCs in the process of senescence and shedding.

Bivariate regression of important variables with Ca/Cb ratio

Variables of contribution list (Table 6) and VIP plot (Fig. 3), were further processed by a bivariate regression with Ca/Cb ratio with no data transformation, to find out which compound has more higher correlation with Ca/Cb ratio, meaning the dependence on degree of senescence or after shedding.

As shown in Table 7, with the exception of β-myrcene, all variable revealed significant ($p > 0.01$) correlation coefficients with a sign of positive or negative. The level of variables of positive correlation

Table 7. Bivariate correlation coefficients between Ca/Cb ratio and variables extracted from contributors (Table 6) and VIPs (Fig. 3)

Variables	Pearson Correlation	*p*-value (1-tail)
	Positive Correlation	
1-Penten-3-ol	0.848**	0.000
L-Bornyl acetate	0.832**	0.000
(*Z*)-2-Penten-1-ol	0.799**	0.000
(*E*)-2-Hexenal	0.791**	0.000
Bicyclogermacrene	0.781**	0.000
Cyclohexene oxide	0.632**	0.000
2-Ethyl furan	0.672**	0.000
Germacrene-D	0.631**	0.000
(*Z*)-3-Hexen-1-ol	0.550**	0.000
2-Heptenal	0.469**	0.001
β-Myrcene	0.147	0.177
	Negative Correlation	
Caryophyllene oxide	-0.791**	0.000
p-Cymene	-0.733**	0.000
Myrtenol	-0.684**	0.000
Spathulenol	-0.639**	0.000
p-Cymenene	-0.601**	0.000
Camphor	-0.547**	0.000
3-Methyl-2-buten-1-ol	-0.424**	0.003
4-Methyl-2-pentanone	-0.364**	0.009
Pinocarvone	-0.264*	0.046

* significant at 0.05 level
** significant at 0.01 level

coefficients increase dependently on Ca/Cb ratio increase, which reflects the closeness to being intact, while for negative correlation Ca/Cb ratio dependently decreases which reflects the closeness to being litter.

1-Penten-3-ol had the highest positive correlation coefficient, while caryophyllene oxide had the negative one. Interestingly, this result informs of importance of 1-penten-3-ol as an excellent discriminant of intact needle, in that this is a strong contributor for intact needle and also important VIP on OPLS-DA evaluation. However, camphor, having strong contribution to litter needle and the highest VIP value, revealed relatively low negative correlation coefficient but significant. This result was thought to be derived by very low content of intact needle and high content of senescent and litter needles (Table 5) because resultantly data points deviated largely from linear regression line.

From the additional bivariate regression analysis, several compounds of VOCs in red pine needle increase or decrease proportionally to degree of senescence of needle, represented by the Ca/Cb ratio. And it was thought that these compounds react very sensitively and sequentially to surrounding environment changes during senescence or after shedding and would be useful as indicators or makers for degree of sensescence or aging after shedding.

4. CONCLUSION

The present study successfully determined which VOCs in red pine needles are changed over the course of senescence or after shedding by statistical analyses and finally revealed similarities and dissimilarities among intact, senescent, and litter needles. The Ca/Cb ratio was a good indicator of whether needle was intact or not; the intact needle had higher average Ca/Cb ratio than the senescent and the litter needles. Whether needles were intact, senescent, or litter was well explained by multivariate statistical interpretation of VOCs using the PCA and the OPLS-DA models with Ca/Cb ratio values as a classification variable.

Further investigation on effects of variables (chemical components) to needle classification revealed several significant compounds. 1-Penten-3-ol and camphor were most strong contributors for intact (Class 1) and litter (Class 3) needle, respectively, which result had a same trend with the result by VIP values evaluation. The oxidation reaction involving the lipoxygenase pathway occurred in the intact needles, to a lesser extent or not at all in the senescent and litter needles, and that the terpenic compounds were also degraded or modified during senescence or after shedding. The content of compounds having high correlation coefficient would increase or decrease proportionally to degree of senescence of needle, represented by the Ca/Cb ratio, indicating that these compounds react very sensitively and sequentially to surrounding environment changes during senescence or shedding.

In addition, this study suggests that senescent and litter needles along with intact needles are also good bioresources of biogenic volatiles in the forest ecosystem and fragrance and flavor industry because major compounds of VOCs were also found between needles, but their chemical composition or content of several chemical compounds are markedly different. It was thought that further works on biological or physiological or environmental pathway should be conducted to elucidate or explain the deviation of VOCs in needle samples.

REFERENCES

Ahn, C., Park, M.J., Kim, J.W., Yang, J., Lee, S.S., Jeung, E.B. 2018. Cytotoxic evaluation of plant essential oils in human skin and lung cells. Journal of the Korean Wood Science and Technology 46(2):

166-177.

Bernal, F.A., Orduz-Díaz, L.L., Coy-Barrer, E.C. 2016. Application of PARAFAC and OPLS-DA analyses on HPLC fingerprints for the characterization of *Hibiscus sabdariffa* Calyxes. Quím. Nova 39(2): 160-166.

Deligoz, A., Bayar, E., Genc, M., Karatepe, Y., Kirdar, E., Cankara, F.G. 2018. Seasonal and needle age-related variations in the biochemical characteristics of *Pinus nigra* subsp. pallasiana (Lamb.) Holmboe. Journal of Forest Science 64(9): 379-386.

Finkelstein, R. 2013. Abscisic acid synthesis and response. The Arabidopsis Book / American Society of Plant Biologists. 11: e0166. doi:10.1199/tab.0166. PMC 3833200. PMID 24273463.

Goncalves, J., Figueira, J., Rodrigues, F., Camara, J.S. 2012. Headspace solid-phase microextraction combined with mass spectrometry as a powerful analytical tool for profiling the terpenoid metabolomic pattern of hop-essential oil derived from Saaz variety. Journal of Separation Science 35(17): 2282-2296.

Gond, V., Pury, D., Veroustraete, F., Ceulemans, R. 1999. Seasonal variations in leaf area index, leaf chlorophyll, and water content; scaling-up to estimate fAPAR and carbon balance in a multilayer, multispecies temperate forest. Tree Physiology 19(10): 673-679.

Isidorov, V.A., Smolewska, M., Purzynska-Pugacewicz, A., Tyszkiewicz, Z. 2010. Chemical composition of volatile and extractive compounds of pine and spruce leaf litter in the initial stages of decomposition. Biogeosciences 7: 2785-2794.

Isidorov, V.A., Vinogorova, V.T., Rafaowski, K. 2003. HS-SPME analysis of volatile organic compounds of coniferous needle litter. Atmospheric Environment 37(3): 4645–4650.

Jeong, M.J., Yang, J., Choi, W.S., Kim, J.W., Kim, S.J., Park, M.J. 2017. Chemical compositions and antioxidant activities of essential oil extracted from *Neolitsea aciculata* (Blume) Koidz leaves. Journal of the Korean Wood Science and Technology 45(1): 96-106.

Jo., G.G., Kim, J.H. 2010. Changes in terpenes of three kinds of pine needles during litter decomposition. Journal of Ecology and Field Biology 33(2): 175-186.

Kim, S.H., Lee, S.Y., Cho, S.M., Hong, C.Y., Park, S.Y., Park, M.J., Choi, I.G. (2017). Antioxidant activities of *Cryptomeria japonica* leaves extracts by extraction methods. Journal of the Korean Wood Science and Technology 45(5): 495-510.

Krammer, P. J., Kozlowski, T.T. 1979. Physiology of Wood Plants. Academic Press Inc, pp. 550-554.

Lee, J.G., Lee, C.G., Back, S., Jang, H.J., Kwag, J.J., Lee, G.H. 2005. Volatile compounds of pine needles from *Pinus densiflora* S. using solid phase microextraction-gas chromatography-mass spectrometry. Korean Journal of Food and Nutrition 18(4): 373-379.

Lichtenthaler, H.K., Wellburn, A.R. 1983. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochemical Society Transaction 11(5): 591-592.

Shan, Y., Izuta, T., Aoki, M., Totsuka, T. 1996. Effects of O_3 and soil acidification, alone and in combination, on growth, gas exchange rate and chlorophyll content of red pine seedlings. Water, Air, and Soil Pollution 97: 355-366.

Shen. J., Tieman, D., Jones, J.B., Taylor, M.G., Schmelz, E., Huffaker, A., Bies, D., Chen, K., Klee, H. J. 2014. A 13-lipoxygenase, TomloxC, is essential for synthesis of C5 flavour volatiles in tomato. Journal of Experimental Botany 65(2): 419-428.

Talyor, J.E., Whitelaw, C.A. 2001. Signals in abscission. New Phytologist 151(2): 323-339.

Thimann, K.V. 1985. The interaction of hormonal and environmental factors in leaf senescence. Biologia Plantarum 27(2): 83-91.

Venkatesan, T., Choi, Y.W., Kim, Y.K. 2020. Comparative evaluation of the impact of extraction solvent and time on the yield and antioxidant potential of *Pinus densiflora* needle and bark extracts. Wood Science and Technology 54: 587-598.

Wang, M., Schurgers, G., Hellen, H., Lagergren, F., Hoist, T. 2018. Biogenic volatile organic compound emissions from a boreal forest floor. Boreal Environment Research 23: 249-265.

Webster, B.D., Leopold, A.C. 1972. Stem abscission in *Phaseolus vulgaris* explants. Botanical Gazette 133(3): 292-298.

Wellburn, A.R. 1994. The spectral determination of chlorophyll a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. Journal of Plant Physiology 144(3): 307-313.

Yang, J., Choi, W.S., Kim, J.W., Lee, S.S., Park, M.J. 2019. Anti-inflammatory effect of essential oils extracted from wood of four coniferous tree species. Journal of the Korean Wood Science and Technology 47(6): 674-691.

Yu, E.J., Kim, T.H., Kim, K.H., Lee, H.J. 2004. Aroma-active compounds of *Pinus densiflora* (red pine) needles. Flavour and Fragrance Journal 19(6): 532-537.

Zhang, X., Wang, B., Liuc, Z. 2018. Coniferous litter extracts inhibit the litter decomposition of *Catalpa fargesii* Bur. and *Eucommia ulmoides* Oliver. Acta Oecologica 93: 7-13.