한작지(Korean J. Crop Sci.), 51(S): 297~303(2006)

Chemical Variability of Leaf Cuticular Waxes According to Leaf Position in Tea Tree

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ABSTRACT Cuticular waxes on tea (*Camellia sinensis* L.) leaves consisted mainly of alkanes, fatty acids, primary alcohols, triterpenes, and a group of unknown compounds, dominated by primary alcohols and triterpenes. Tea tree accessions used in this study were M-1, M-2, Sakimidori, and Yabukita. For all accessions, the alkane, fatty acid, and primary alcohol constituents consisted of a homologues series, and the major constituents of primary alcohol class were the C28 and C30 homologues. Triterpenes consisted of friedelin, β-amyrin, and three unidentified ones and friedelin was the most abundant. Leaf area and the total amounts of cuticular waxes per leaf increased with lower leaf position from the apical bud in Yabukita variety. With different leaf position, total wax amount per unit leaf area on the youngest leaves of P1 (the uppermost leaf position) showed the largest amount (12.80 μg/cm^2), and on mature leaves of P2 to P6 ranged from 7.08 to 7.77 μg/cm^2, and then on the oldest leaves of P7 (the lowest leaf position) remained at an increased level (17.53 μg/cm^2). During leaf development (lower leaf position), the amount of primary alcohols decreased from P1 to P6 and increased at P7, whereas that of triterpenes increased from P1 to P7. The percentage of each wax class in the total wax amount occurred a decrease in primary alcohol and an increase in triterpene, with leaf age.

Keywords : cuticular wax, chemical variability, leaf position, tea tree, *Camellia sinensis* L.

Tea tree (*Camellia sinensis* L.) is an important beverage crop of the world and is also known for its medicinal properties such as antioxidant effect and cancer chemo-prevention (Gramza & Korczak, 2005; Nagle *et al.*, 2006). It is a woody, perennial, evergreen plant grown below latitude 35°N and has many kinds of local varieties and tea-manufacturing processes (Wilson & Cllifford, 1992).

Leaf cuticular waxes cover essentially all aerial plant surfaces and form a protective barrier between a plant and its environment. These waxes play an important role in plant resistance to a variety of biotic and abiotic stresses such as those caused by fungal pathogens, phytophagous insects, freezing temperatures, and drought (Jenks & Ashworth, 1999; Goodwin & Jenks, 2004; Shepherd & Griffiths, 2006). Frequently, tea tree cultivated in Korea has suffered from freezing and/or frost injury during the winter season and as bud break begins at early spring, and it occurred a decrease of tea yield and quality. Yabukita, a tea variety introduced from Japan, with less freezing tolerance and local varieties have been cultivated in many tea farms in the southern area of Korea.

Cuticular waxes have been studied in many plants such as rice (Kwon & Chung, 1992), faba bean (Griffiths *et al.*, 1999), sesame (Kim *et al.*, 2006), pea (Kolattukudy, 1970), potato (Szafranek & Synak, 2006), wheat (Uddin & Marshall, 1988), castor bean (Vermeer *et al.*, 2003), apple (Belding *et al.*, 1998), and sorghum (Jenks *et al.*, 1992). The composition and structure of cuticular wax has been associated with tolerances to frost damage and winter desiccation on some plants including willow (Hietala *et al.*, 1997), spruce (Esch & Mengel, 1998). Olyslaegers *et al.* (2002) reported that cuticle thickness was not a good indicator of atmospheric stress tolerance because genetic differences between clones were confounded by the clonal response of wax production to stress in tea plant. However, there is a lack of information available on the chemical variability of leaf cuticular waxes among tea tree accessions and leaf position, except for the studies on morphological leaf surface of tea plant by Mukherjee *et al.* (2000)

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and the difference of chemical components such as total nitrogen, tannin, and caffeine in different position of tea leaves (Park, 1997).

This study examined the amount and chemical composition of leaf cuticular waxes for four accessions of tea tree, and determined the changes that occurred in leaf cuticular waxes with different leaf position in Yabukita.

MATERIALS AND METHODS

Plant materials

Tea tree accessions used in this study were M-1 and M-2 collected from Moodeung Mountain, Jeonnam, and Sakimidori and Yabukita introduced from Japan. M-1 and M-2 were superior clones that had been selected based on the freezing tolerance during the severe winter season of 2005. Yabukita was used to determine the changes of leaf cuticular waxes with different leaf position. All plants have been cultivated in the experimental field or greenhouse at Mokpo, and were not pruned after the winter period.

Leaf sampling

A total of 10 leaves, 3rd to 5th leaves from the apical bud, were collected from each plant of four accessions on June 29, 2006. To examine the alteration of cuticular wax constituents by leaf position, the 8 to 11 leaves per each leaf position of five shoots were excised from two plants of Yabukita grown at the field on August 9, 2006. All sampled leaves were used immediately for cuticular wax analysis. Leaf position was expressed as P1 to P7 according to the node order attaching leaf to shoot. P1 was 1st to 3rd leaf from top (apical bud) and was not fully expanded, small leaves, which excluded an apical bud. P2 indicates 4th to 5th, P3 does 6th to 7th, P4 does 8th to 9th, P5 does 10th to 11th, P6 does 12th to 13th, and P7 does 14th to 16th leaves from top (apical bud). Leaves after P2 were fully expanded. Shoots used in this study had, on an average, 16 nodes by shoot extension, including bract and old leaves. Bract leaf had developed before the bud-break begins, and old leaf was probably two-years old one.

Wax analysis and leaf area measurement

Wax of tea leaves was extracted by dipping uncut tea leaves for 10 sec into a 500 mL beaker with approximately 20 mL chloroform ($CHCl_3$). Extracts contained waxes from both abaxial and adaxial leaf surfaces. The $CHCl_3$ extracts were evaporated to dryness under a nitrogen stream and the dried residue was prepared for gas chromatography (GC). Wax extracts were derivatized using a 50 μL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for 20 min at 100℃, and surplus BSTFA was evaporated under nitrogen, the sample was re-dissolved in the 1 mL of $CHCl_3$ for wax anlaysis using 6890N GC (Agilent Technologies Co., USA) equipped with a flame ionization detector (FID) and Agilent 7683 automatic liquid sampler. The GC column was a 12 m, 0.2 mm HP-1 capillary column and the carrier gas was nitrogen. The injector and FID detector temperatures were set at 300℃ and 320℃, respectively. The oven temperature of GC was programmed with an initial temperature of 80℃ and increased at 15℃ /min to 260℃, where the temperature remained unchanged for 10 min. The temperature was then increased at 5℃/min to 320℃, where the temperature was held for 15 min. Quantification was based on FID peak areas and hexadecane as internal standard was added to the original extract. Five standards such as C27, C29, C31, C33, and C35 alkanes were used as the external standards, and all other peaks for primary alcohols, triterpenes, and unknown constituents were calculated by the average conversion for external standards at comparable concentration. Each wax extract represented all extracts of leaves from tea accession and different leaf position bulked together before injection into the GC.

To identify the molecular structure of each wax constituent represented in each GC peak, further analysis was performed using a GC equipped with an Agilent 5973N mass spectrometer (GC/MS) to produce electron ionization (EI) mass spectra of each peak. The individual components were separated using a HP-5MS capillary column. The oven temperature of GC/MS was programmed with an initial temperature of 80℃ and held for 2 min, and increased at 15℃/min to 200℃, where the temperature

remained unchanged for 2 min. The temperature was then increased at 3℃/min to 320℃, where the temperature was held for 15 min.

The total amount of unknown constituents was calculated from the cumulative peak areas for all unidentified peaks. Total wax amount per unit leaf area was calculated as the total of all wax constituents including unknown peaks not identified by GC, and expressed as micrograms per total leaf area (μg/cm^2). After wax extraction, leaves were pressed and their surface areas were measured using a LI-3000 Area Meter (LI-COR, Inc., Lincoln, NE, USA). Leaf areas were calculated as the total of both abaxial and adaxial surfaces.

Chemicals identified as cuticular wax constituents on tea leaves were abbreviated, respectively, as follows : *n*-pentacosane, *n*-heptacosane, *n*-nonacosane, and *n*-hentriacosane as C25, C27, C29, and C31 alkanes; 1-hexacosanol, 1-octacosanol, 1-triacontanol, and 1-dotriacontanol as C26, C28, C30, and C32 primary alcohols; tetracosanoic, hexacosanoic, octacosanoic, and triacontanoic acids as C24, C26, C28, and C30 fatty acids.

RESULTS AND DISCUSSION

Composition of leaf cuticular waxes of tea tree

Cuticular waxes on tea leaves consisted mainly of alkanes, fatty acids, primary alcohols, triterpenes, and a group of unknown compounds (Table 1). The average total wax amount was 19.29±6.32 μg/cm^2 for all four tea accessions. Total wax amount was the sum of alkanes, fatty acids, primary alcohols, triterpenes, and unknown constituents. The average amounts for each wax class were 0.81 μg/cm^2 for alkanes, 2.31 μg/cm^2 for fatty acids, 8.20 μg/cm^2 for primary alcohols, 6.46 μg/cm^2 for triterpenes, and 1.51 μg/cm^2 for the unknowns, showing the most abundant amount in primary alcohol class. Caffeine was included in leaf cuticular wax extracts of tea and its average amount was 0.48 μg/cm^2. Though Athayde *et al.* (2000) have reported the presence of caffeine in epicuticular wax of *Ilex paraguariensis*, called yerba mate, it could be extracted from the inner tissues in leaves in addition to epicuticular wax, presumably due to both of hydrophilic and lipophilic character of caffeine (Baumann *et al.*, 1995).

Alkane constituents on tea leaves consisted of a homologues series dominated by odd numbers of carbon atoms from the C25, C27, C29, and C31 homologues, containing 0.01, 0.05, 0.07, and 0.16 μg/cm^2, respectively, as averaged across all 4 accessions (Fig. 1). Fatty acids and primary alcohols also consisted of a homologues series, with even chain-length constituents predominating. Fatty acid homologues were the C24, C26, C28, and C30 chain constituents, containing 0.04, 0.59, 1.12, and 0.54 μg/cm^2, and the primary alcohol constituents were the C26, C28, C30, and C32 homologues, containing 0.34, 1.95, 2.30, and 0.74 μg/cm^2, respectively, as averaged across all 4 accessions. Triterpenes consisted of friedelin (T1), β-amyrin (T5), and three unidentified ones. Two unidentified peaks (T3 and T4) on GC/MS chromatogram had the base peak at m/z 219 and the fragmentation patterns were similar to each other. One unidentified compound (T2) showed the base peak at m/z 137 on GC/MS chromatogram and the fragmentation pattern was similar to canophyllal, a friedelane triterpene. The amount of friedelin and β-amyrin on tea leaves was 3.10 and 1.11 μg/cm^2, and three unidentified ones (T2, T3, and T4) contained 1.51, 0.35, and 0.39 μg/cm^2, respectively, when expressed as an average across all accessions. Friedelin was the most

Table 1. Amounts of leaf wax constituents of four accessions in tea tree.

Accession	Alkanes	Fatty acids	1-Alcohols	Triterpenes	Unknowns	Total wax
M-1	0.96	1.88	5.76	3.57	0.81	12.97
M-2	1.74	2.34	8.10	10.23	2.85	25.27
Yabukita	0.30	3.12	11.78	8.06	1.76	25.02
Sakimidori	0.23	1.91	7.14	3.98	0.63	13.90

Values were expressed as micrograms per total leaf area (μg/cm^2); Total wax was the sum of alkanes, fatty acid, 1-alcohol, triterpene, and unknown constituents.

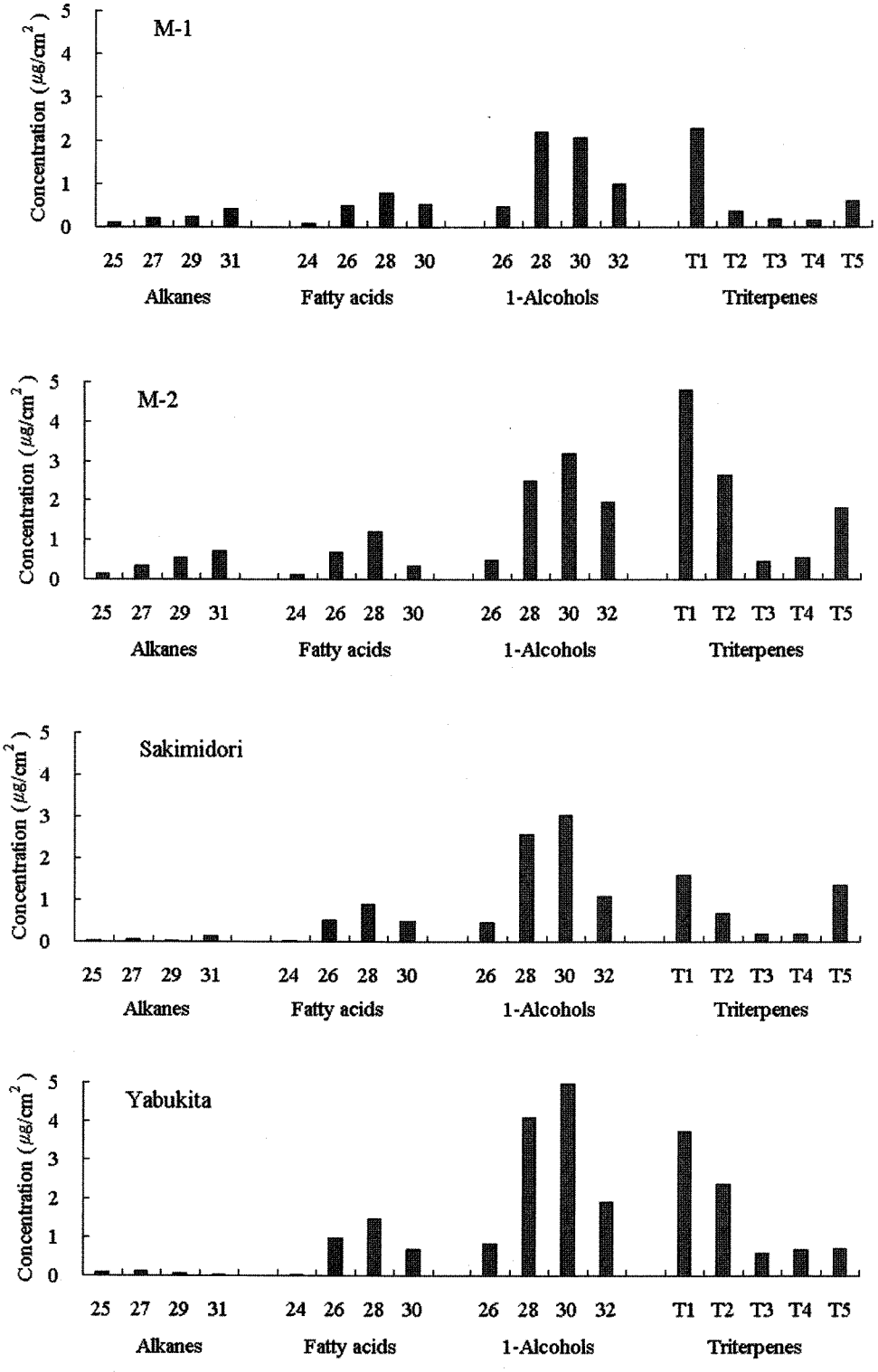

Fig. 1. Individual leaf wax constituents for the alkane, fatty acid, primary alcohol classes, and triterpenes in leaves of four accessions of tea tree. Chemical classes and the chain lengths of individual wax constituents are labeled on the horizontal axis. T1, friedelin; T2, T3, and T4, unidentified triterpenes; T5, β-amyrin.

abundant in the triterpenes, followed by T2 and β-amyrin in decreasing abundance. Friedelin was a friedelane triterpene reported for the first time in tea, and has been also reported to be the major component of grapefruit epicuticular wax (Nordby & McDonald, 1994). The relative chain length distributions of the alkane, fatty acid, and primary alcohol classes for four accessions appeared very similar, whereas the relative amounts of triterpene constituents varied slightly more with accession.

Changes of leaf area and wax amount per leaf with different leaf position

Leaf area increased with lower leaf position, increasing from P1 (5.9 cm^2/leaf) to P5 (40.2 cm^2/leaf) and reducing to P7 (18.7 cm^2/leaf) (Fig. 2). Because the leaf position is corresponding to leaf age, P1 (the uppermost position) is the youngest, not fully expanded leaves, and P7 (the last and the lowest position) is the oldest leaf after shoot extension.

The total amounts of cuticular waxes per leaf continuously increased as the leaf position increased from P1 (135.1 μg/leaf) to P7 (331.1 μg/leaf) due to mainly increased individual leaf area. Wax amount on P7 leaves was similar to those (339.4 μg/leaf) of old leaves, while that on bract leaf showed 152.4 μg/leaf, with small leaf size (5.0 cm^2/leaf).

Changes of cuticular wax constituents with different leaf position

Total wax amount per unit leaf area on the youngest, the smallest, and the uppermost leaves of P1 showed the largest amount of 12.80 μg/cm^2, while the wax coverage on mature, fully expanded leaves of P2 to P6 ranged from 7.08 to 7.77 μg/cm^2 (Fig. 3). It was decreased considerably as the leaf area expanded and the new shoot extended. The wax amount of the lowest, the oldest leaves of P7 in this study remained at an increased level (17.53 μg/cm^2). The results (Fig. 2 and 3) were almost the same to that in the epicuticular waxes of apple leaves (Bringe *et al.*, 2006). They reported that during ontogenetic development of apple leaves, the leaf area increased and wax mass per unit area tended to decrease.

Amounts of alkanes and fatty acids remained almost constant during leaf development (increasing leaf position), varying from 0.02 to 0.36 μg/cm^2 and from 1.29 to 2.53 μg/cm^2, respectively. The amount of primary alcohols decreased from P1 (9.92 μg/cm^2) to P6 (1.73 μg/cm^2) and increased to 5.96 μg/cm^2 (P7) during leaf development, while that of triterpenes increased from P1 (0.10 μg/cm^2) to P7 (6.58 μg/cm^2). It demonstrated that, in the leaf cuticular waxes of tea tree dominated by triterpenes and primary alcohols, the wax constituents such as triterpenes

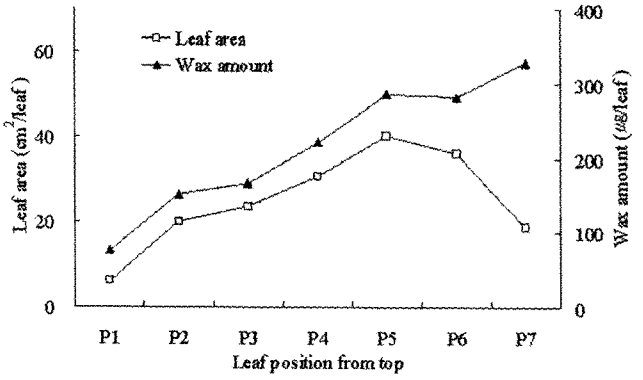

Fig. 2. Changes of leaf area and wax amount per individual leaf with different leaf position on new shoot of tea tree cv. Yabukita. Leaf position abbreviations labeled on the horizontal axis are explained in the text. P1 did not contain the terminal sprout (bud).

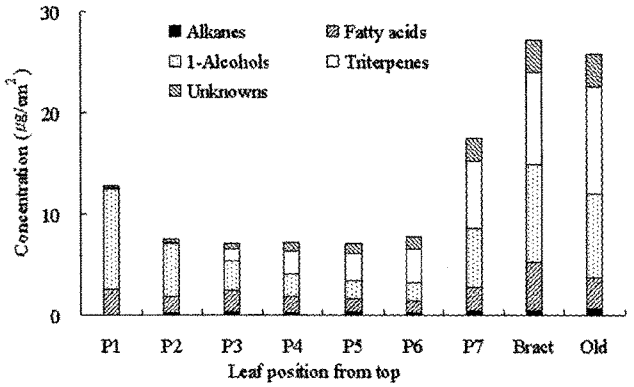

Fig. 3. Changes of wax classes on leaves with different leaf position on new shoot of tea tree cv. Yabukita. Leaf position abbreviations labeled on the horizontal axis are explained in the text. P1 did not contain the terminal sprout (bud); Bract and Old indicate bract leaf and old leaf before new shoot extension.

with high molecular weight increased during the leaf development (lower leaf position), being comparable to a decrease of primary alcohols. However, there were different results that with leaf age, the proportion and absolute amounts of triterpenes decreased whereas that of wax alcohols increased in apple leaf (Bringe *et al.*, 2006), and the wax composition of lettuce and common plantain only slightly changed with leaf developmental stage (Bakker *et al.*, 1998).

The relative chain length distributions of the alkane, fatty acid, and primary alcohol classes for all leaf position (P1-P7) appeared very similar and friedelin among the triterpenes was the most abundant for all leaf position, as a result of Fig. 1.

The change of the percentage of each wax class with different leaf position appeared similar to that of wax amount, showing a decrease of the primary alcohol percentage from 77.5% of P1 to 22.3% of P6 and an increase of triterpene percentage from 0.8% of P1 to 42.2% of P6 (though there were the reverse changes as 34.0% and 37.5% of P7 in primary alcohol and triterpene class, respectively) in the total wax amount. Total wax amounts (30.22 and 26.13 $\mu g/cm^2$, respectively) per unit leaf area of bract and old leaves were more than that of P7 with the largest amount (17.70 $\mu g/cm^2$) among all leaf position. Of the total wax amount on tea plants, the percentages for each wax class in the oldest P7 leaves were 2.1% alkanes, 13.4% fatty acids, 34.0% primary alcohols, 37.5% triterpene, and 13.0% unknown constituents, whereas the corresponding percentages for these classes in the old leaf (two-years old) were 2.5, 11.8, 32.5, 40.7, and 12.5%, respectively, revealing a similar percentages in wax classes.

These results showed that the wax amount (Fig. 2) and its composition (Fig. 3) on P7 leaves sampled on August 29, 2006, was very similar to that of the old leaves, showing that cuticular waxes on tea leaves were deposited differently, with leaf age or leaf position.

This is the first report of cuticular wax composition on tea tree plant (*Camellia sinensis* L.) and reveals the changes in leaf cuticular wax amount and composition during the leaf development and the new shoot extension (corresponding to leaf position). Further studies are needed to investigate the role of genotype in tea tree wax synthesis in different growth stages, and their response to environmental stresses such as freezing and frost injury during the winter season and/or early spring. Such studies are also required to elucidate the role of cuticular wax induction in tea tolerance to biotic stresses, and to use the leaf wax characteristics as a selection criterion for breeding new tea variety tolerant to stressful conditions, especially associated with freezing tolerance, although we have no knowledge of the relationship between cuticular wax and freezing tolerance in tea plant.

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