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A comparative analysis of characteristics and antioxidant capacity of Korean mulberries for efficient seedling cultivation

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Abstract

Mulberry exhibits unique characteristics and functionalities across various components, including the roots, branches, leaves, and fruits. However, despite numerous studies on mulberry, research on this plant at the seedling stage is insufficient. Therefore, this study aimed to compare the suitability for seedling cultivation and antioxidant effects of four Korean mulberry cultivars, namely, Daesim, Suhong, Simgang, and Cheongsu. In terms of seed weight, germination rate, and growth rate, Daesim was the most suitable cultivar for seedling production. Polyphenol and flavonoid content analysis showed that all cultivars, except for Cheongsu, showed the highest phenolic content at the 2-week seedling stage. Similarly, antioxidant assays using 2,2'-azino-di-3-ethylbenzthiazoline sulfonic acid (ABTS) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals revealed that the antioxidant effect of all cultivars, except for Cheongsu, increased with cultivation at 2- 4- and 6-week. However, mulberry seedlings had a slower reaction rate against DPPH radical removal than mulberry leaves. In addition, ABTS radical scavenging activity showed a different correlation with polyphenol content. This phenomenon may be due to the different polyphenol compositions between mulberry leaves and seedlings. The results of this study suggest that mulberry seedlings exhibit different bioactivities from mulberry leaves, and component analysis is required in further research.

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Introduction

The mulberry is a well-established staple in silkworm nutrition and has a long history paralleling that of silkworm breeding. Over time, mulberry have undergone extensive development to optimize conditions for the growth of silkworms. Various mulberry varieties have evolved with different growth conditions depending on seasonal and temperature variations. In addition to improving the nutritional quality of mulberry leaves, concerted focus has been placed on elevating the qualitative attributes of mulberry fruits. Mulberry have long been recognized as useful trees, containing phytochemicals with beneficial properties for

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humans, depending on their parts. Notably, mulberry fruits are distinguished by their high antioxidant function due to their significant polyphenol and flavonoid content (Ren *et al.*, 2022; Lee *et al*., 2021). These fruits demonstrate efficacy in ameliorating various complications caused by fatty liver (Qiao *et al.*, 2023), inflammatory responses (Tomczyk *et al.*, 2019), and type 2 diabetes (Yao *et al.*, 2024). Recently, they have been reported to alleviate cognitive deficits induced by streptozotocin (Sood *et al.*, 2023A, 2023B). Mulberry root bark is a longstanding herbal remedy with roots in Asian traditional medicine (Yamatake *et al.*, 1976; Rahul *et al*., 2022). Rich in flavonoids and phytochemicals, it imparts various beneficial effects on humans (Choi *et al.*, 2020). Furthermore, studies have highlighted its antiviral, anti-inflammatory, cardioprotective, diuretic, antidepressant, and analgesic effects (Cao *et al.*, 2018; Chen *et al.*, 2013; Eo *et al.*, 2014; Li *et al.*, 2017; Lim *et al.*, 2015; Wu *et al.*, 2020; Zheng *et al.*, 2017). Previous research has also confirmed its efficacy in suppressing obesity by inhibiting digestive enzyme activity (Wu *et al.*, 2015). Mulberry twigs, which have been widely used in traditional Chinese medicine (Sakai and Otsuka, 1967), exhibit antioxidant effects, relieve inflammation, and demonstrate various other biological activities (Kim *et al.*, 2021; Qu *et al.*, 2021; Shi *et al.*, 2023). Notably, they have been demonstrated to confer hepatoprotective and hepatoregenerative effects (Chen *et al.*, 2014; Novo and Parola, 2008). Mulberry leaves are well known for their high content of flavonoids and phytochemicals, renowned for their excellent anti-inflammatory and antioxidant abilities (Gryn-Rynko *et al.*, 2016; Katsube *et al.*, 2006; Kim and Jang, 2011; Lin *et al.*, 2022). Recent research endeavors have been directed toward investigating mulberry leaves as potential interventions for mitigating diabetes, high blood pressure, cardiovascular diseases, and metabolic diseases (Jung *et al.*, 2019). Furthermore, mulberry leaf extract has been identified as an effective geoenvironmental factor for managing malaria (Maji *et al.*, 2023). The mulberry is a plant replete with benefits for humans, exhibiting advantageous properties in almost every part, from roots to leaves, twigs, and fruits. However, the procurement of medicinal mulberry parts is currently constrained to old mulberry. This limitation has spurred active exploration of functional substances in mulberry seedlings, as opposed to grown mulberry. A previous study has substantiated the accumulation of various physiologically active substances in mulberry seedlings depending on the plant parts (Li *et al.*, 2022). However, a considerable body of unknowns remain regarding mulberry seedlings.

The Rural Development Administration (RDA) in Korea is dedicated to the preservation and management of over 600 types of mulberry resources. Based on this, the RDA annually executes a breeding program aimed at refining specific quality traits, thereby perpetually augmenting the array of Korean mulberry resources. Over 10 varieties of mulberry are preserved in Korea, and research on mulberry is robust. However, studies on the growth, metabolism, and functional effects of Korean mulberry varieties at the seedling stage are lacking. Therefore, in this study, a comparative and analytical assessment of the antioxidant capacity and initial growth rate of foure representative mulberry seedlings in Korea was conducted to increase the industrial value of Korea's representative mulberry.

Materials and methods

Preparation of mulberry seeds and seedlings

In 2023, mulberry fruits of Cheongsu, Simgang, Suhong, and Daesim varieties were harvested in Wanju, Korea. The fruits were subjected to a process of crushing and washing with water, repeated 5–10 times, to separate the seeds. The precipitated seeds were then separated and air-dried at 25°C for 24 h. The prepared seeds were stored in dark conditions at 4°C until further experimentation. Subsequently, pots were filled with soil, and 1 g of seeds from each variety was sown. Watering was administered five times weekly, and seedlings were harvested at 2-, 4-, and 6-week. The harvested seedlings were given five successive washings to remove adhering soil.

Seed contents and weight measurement

The seed contents (%, *w/w* of fruit) was expressed as the seed weight relative to the weight of raw mulberry fruit. The weight of seeds (g/ 100 seeds) was determined by seeds precipitated in water and measuring the weight per 100 seeds. Each experiment was repeated thrice.

Germination rates of mulberry seeds

A petri dish was lined with autoclave filter paper, and 15 mL of distilled water was added. Seeds were sown, the dish was covered with a lid, and germination was conducted at 25°C. The germination rate was calculated by counting the number of germinated individuals on day 7. Each germination assay was repeated thrice, using 20 seeds per variety.

Mulberry seedling extraction

After adding 5 mL of 70% methanol to 0.1 g of the sample, the mixture was stirred and subjected to extraction at room temperature for 2 h. The resulting supernatant was separated and concentrated using SpeedVac (Labogen, Denmark). The extract was re-dissolved in dimethyl sulfoxide (Sigma-Aldrich, MO, USA) to achieve a final concentration of 10 mg/mL for subsequent experiments.

Total polyphenol content measurement

A sample or standard was dispensed in 10 μL aliquots into a 96 well-plate, followed by the addition of 200 μL of 2% Na_2CO_3 (Sigma-Aldrich). After 3 min, 10 μL of 50% Folin-Ciocalteu's reagent (Sigma-Aldrich) was added, mixed thoroughly, and allowed to react for 10 min. Total polyphenol content was determined by measuring absorbance at 750 nm (Multiskan GO, Thermo, USA), with 0.1% gallic acid (Sigma-Aldrich) serving as the standard. The measured values were converted to mg of gallic acid equivalent (GE)/g of extract.

Total flavonoid content measurement

After dispensing 20 μL of the sample or standard into a 96-well plate, 100 μL of distilled water (DW) and 6 μL of 5% $NaNO_2$ (Sigma-Aldrich) were added per well. After 5 min, 12 μL of 10% $AlCl_3 \cdot 6H_2O$ was introduced into each well. After 5 min, 40 μL of a 1 M NaOH (Sigma-Aldrich) solution was dispensed, and absorbance was measured at 510 nm after 10 min. Catechin (0.1%) (Sigma-Aldrich) was used as the standard, and the measured values were converted to mg of catechin equivalent (CE)/g of extract.

Measurement of ABTS radical scavenging capacity

A 2,2'-azino-di-3-ethylbenzthiazoline sulfonic acid (ABTS) (Sigma-Aldrich) test solution was prepared by dissolving 7.4 mM ABTS and 2.6 mM potassium persulfate (Sigma-Aldrich) in DW. The resulting mixture was allowed to react in the dark for 1 d and then diluted to achieve an absorbance value of 1.0 at 734 nm and used for subsequent experiments. A sample or standard diluted 5-fold in a 96-well plate was dispensed in 10 μL aliquots, followed by the addition of 200 μL of the ABTS test solution. After 30 min, absorbance was measured at 734 nm. Trolox (Sigma-Aldrich) was used as the standard, and the radical scavenging activity value was converted to mg trolox equivalent (TE)/g of extract.

Measurement of DPPH radical scavenging capacity

A 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich) test solution was prepared by dissolving DPPH in ethanol to achieve a concentration of 0.2 mM. The sample or standard was dispensed in 10 μL aliquots into a 96-well plate, followed by the addition of 200 μL of the DPPH test solution. After allowing it to react for 30 min, 1 h, 3 h, and 5 h, absorbance was measured at 520 nm. Trolox (Sigma-Aldrich) was used as the standard, and the radical scavenging activity value was converted to mg TE/g of extract.

Statistical analysis

Statistical analysis was performed using a one-way analysis of variance using SPSS Statistics 23 (IBM, USA), with the significance of means determined using Duncan's multiple range test ($P < 0.05$).

Results and Discussion

Comparison between the seeds of Korean mulberry varieties

Mulberry seed content, seed weight, and seed germination rate were compared to select suitable seedling cultivation varieties. Mulberry are distributed worldwide, with fruit form and quality varying greatly depending on the variety (Awasthi *et al*., 2004). Korean mulberry produce fruits with various characteristics for each variety. Given the variability in fruit characteristics, it is expected that the condition and quality of the seeds contained in the fruits would differ accordingly. Since mulberry trees are mostly managed by farmers in a vegetative propagation, information on the quality of mulberry seeds is insufficient. In this study, early growth rate of Korean mulberries was compared for efficient seedling cultivation. Seeds capable of normal germination were collected from the fruits of representative Korean mulberry varieties, namely Daesim, Suhong, Simgang, and Cheongsu. The seeds were collected from ripe fruits, and the ratio of seed weight to raw fruit weight (based on fresh weight) was determined. Simgang exhibited the highest ratio of seed to fruit weight, followed by Suhong, Cheongsu, and Daesim (Fig. 1A). The weight of 100 seeds was compared across the different mulberry varieties. Daesim, which had the lowest seed-to-fruit ratio, exhibited the heaviest seed weight, followed by

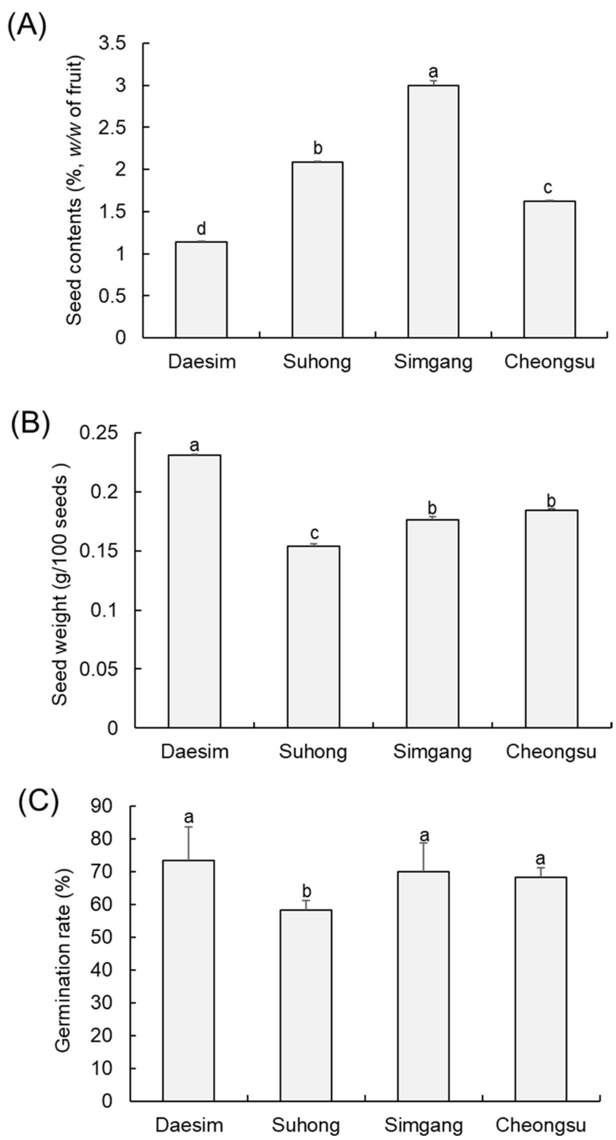

Fig. 1. Comparison of seed characteristics and germination rates according to the mulberry variety. (A) The seed content in fruits of four mulberry varieties (Daesim, Suhong, Simgang, and Cheongsu) was measured by weight of seed per fruit. (B) Weight of 100 seeds was measured from four mulberry varieties (Daesim, Suhong, Simgang, and Cheongsu). (C) Germination rates of four mulberry examined using four varieties (Daesim, Suhong, Simgang, and Cheongsu). Germination rate was measured on day 7 of incubation. Different letters indicate significant differences ($P < 0.05$).

Cheongsu, Simgang, and Suhong (Fig. 1B). Simgang had the heaviest seed weight within the fruit; however, Daesim had the heaviest individual seeds among the test varieties. Simgang has a large number of seeds per unit of fruit. In contrast, Daesim had low seed content but individual seed weight was highest. Germination rate was confirmed to evaluate Korean varieties suitable for mulberry seedling cultivation. Germination rates were determined by incubating the seeds on wet paper for 7 d without breaking dormancy. The germination rate was approximately 70%, with no significant differences observed among Daesim, Simgang, and Cheongsu (Fig. 1C). Among the four mulberry varieties, only Suhong exhibited a germination rate below 60%. Therefore, for seedling production purposes, Daesim, Simgang, and Cheongsu would be more appropriate than Suhong. Before confirming seedling functionality, the initial growth performance of mulberry seedlings was measured based on variety. Seedlings of Daesim, Suhong, Simgang, and Cheongsu were cultivated in the soil for 6-week, and their total length was measured from the roots (Fig. 2). The average length of seedling was 6.6, 11.8, and 15.7 cm at 2-, 4-, and 6-week. Suhong exhibited slower growth at the beginning of 2-week, which increased at 4-week, but ultimately demonstrated smaller length and lower growth performance compared to the other varieties at 6-week. Consequently, Suhong was suggested to not only have a weaker seedling germination rate but also a more diminished seedling growth performance. Ovarall, considering the mulberry germination rate and seedling length, Daesim is considered a suitable variety for seedling production.

Korean mulberry seedling polyphenol and flavonoid content

In this study, we focused on identifying the antioxidant capacity as a pivotal functional attribute of mulberry seedlings across four different mulberry varieties. The amounts of polyphenols and flavonoids in the seedlings from each mulberry variety were analyzed at 2-, 4-, and 6-week (Fig. 3). The polyphenol content in the seedlings of the four mulberry varieties generally exhibited a decreasing trend as they progressed to 6-week (Fig. 3A). However, Cheongsu seedlings exhibited elevated polyphenol levels at 4-week, followed by a subsequent decline. The flavonoid content in the four mulberry seedlings displayed a similar pattern to the polyphenol content (Fig. 3B). The flavonoid content of Daesim and Suhong was highest at 2-week and gradually decreased in the 4- and 6-week. The phenolic content in Simgang peaked at 2-week and then declined to similar levels at 4- and 6-week. Cheongsu had the highest polyphenol and flavonoid contents at 4-week. Based on these results, it can be inferred that the four Korean mulberry varieties generally accumulated the highest concentrations of polyphenols and flavonoids in their early seedling stage, specifically at

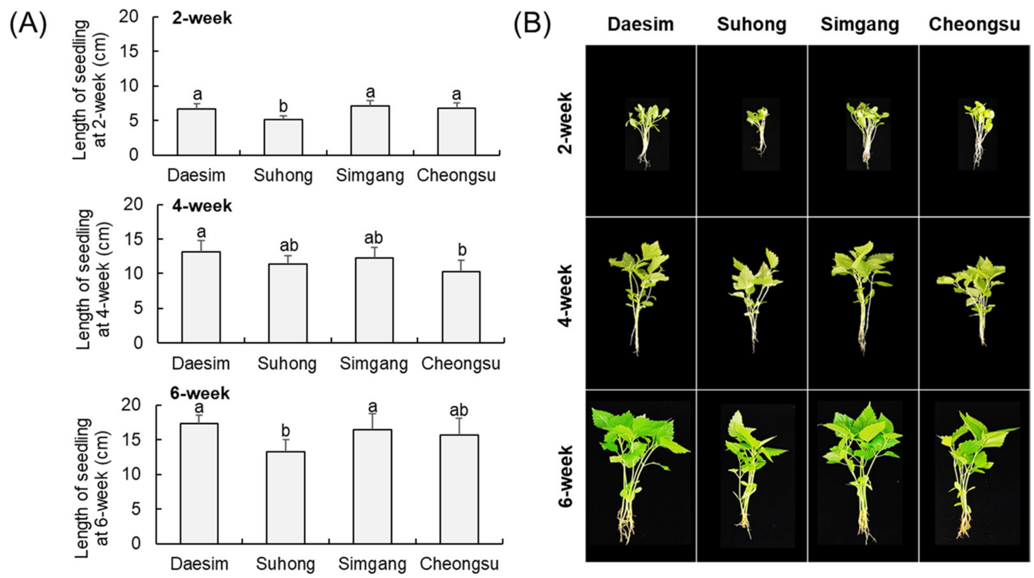

Fig. 2. Differences in the early growth rate of four Korean mulberry varieties. (A) Full length of seedlings were measured in each variety at 2-, 4-, and 6-week. (B) Early growth performances of four Korean mulberry seedlings were compared. The images show the seedling at each time. The average and error values were calculated by measuring the length of 15 seedlings in each group. Different letters indicate significant differences (P < 0.05)

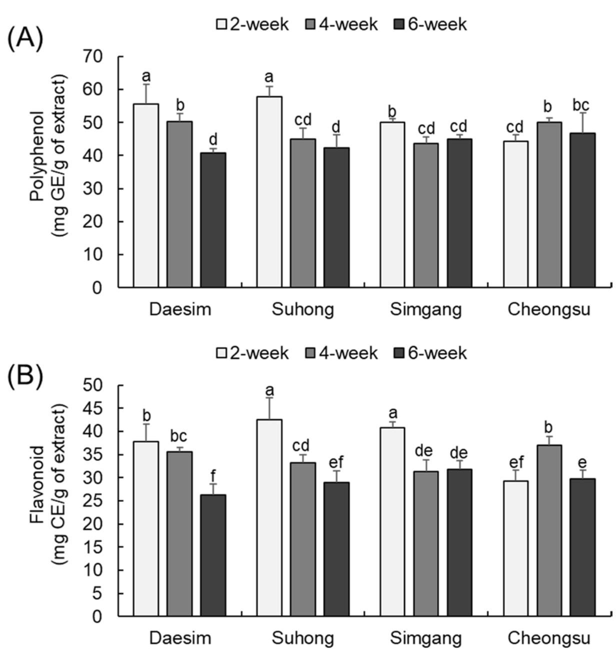

Fig. 3. Polyphenol and flavonoid contents according to the growth period of four Korean mulberry seedlings. (A) Polyphenol contents according to the growth period of four Korean mulberry seedlings were analyzed. (B) Flavonoid contents according to the growth period of four Korean mulberry seedlings were quantified. Each measurement was performed in triplicate. Different letters indicate significant differences (P < 0.05).

2-week. To facilitate the extraction of polyphenols and flavonoids from Daesim, Suhong, and Simgang, it is advisable to harvest the seedlings at 2-week. However, in the case of Cheongsu seedlings, an unusually high presence of polyphenols and flavonoids was observed at 4-week. This observation suggests that Cheongsu seedlings may accumulate specific substances at 4-week, unlike other varieties. This may be related to the slow growth rate of Cheongsu until 4-week (Fig. 2), and further research is needed to examine this phenomenon.

Differences in the antioxidant capacity of Korean mulberry seedlings

ABTS and DPPH radical scavenging activities were used to confirm the antioxidant capacity of the seedlings of each Korean mulberry variety. ABTS radical scavenging activity did not differ significantly based on seedling growth period or variety (Fig. 4A). Simgang showed highest ABTS radical scavenging activity at 6-week whereas Suhong has highest effect at 2-week. Subsequently, another experiment was conducted to verify antioxidant capacity through DPPH radical scavenging activity. However, the results of DPPH radical scavenging activity indicated a general decline in efficacy with progressing seedling growth periods, except for Cheongsu (Fig. 4B). Interestingly, this study revealed that the longer the enzyme reaction time, the higher the DPPH radical scavenging activity of the mulberry seedlings. Particularly, when DPPH was allowed to react for 5 h, the radical scavenging activity of the mulberry seedlings at

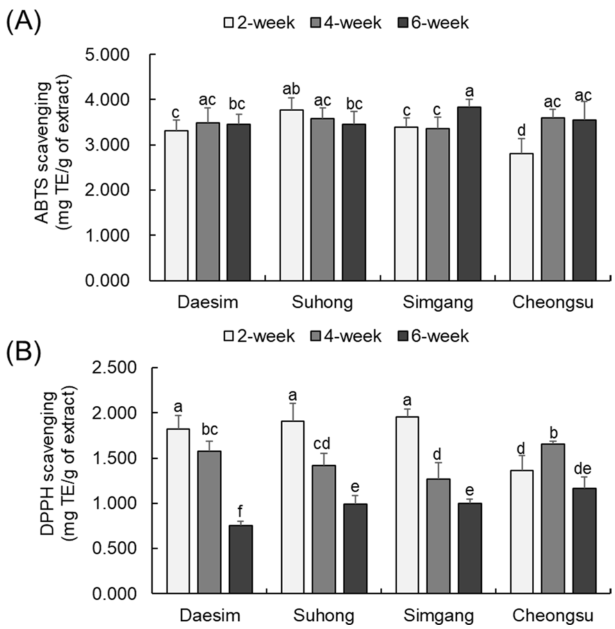

Fig. 4. Comparison of 2,2'-azino-di-3-ethylbenzthiazoline sulfonic acid (ABTS) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities according to the growth period of four mulberry seedlings. (A) ABTS radical scavenging activity of four mulberry seedlings were compared according to early growth stages. (B) DPPH radical scavenging activity of four mulberry seedlings were analyzed according to early growth stages. Each measurement was performed in triplicate. Different letters indicate significant differences ($P < 0.05$).

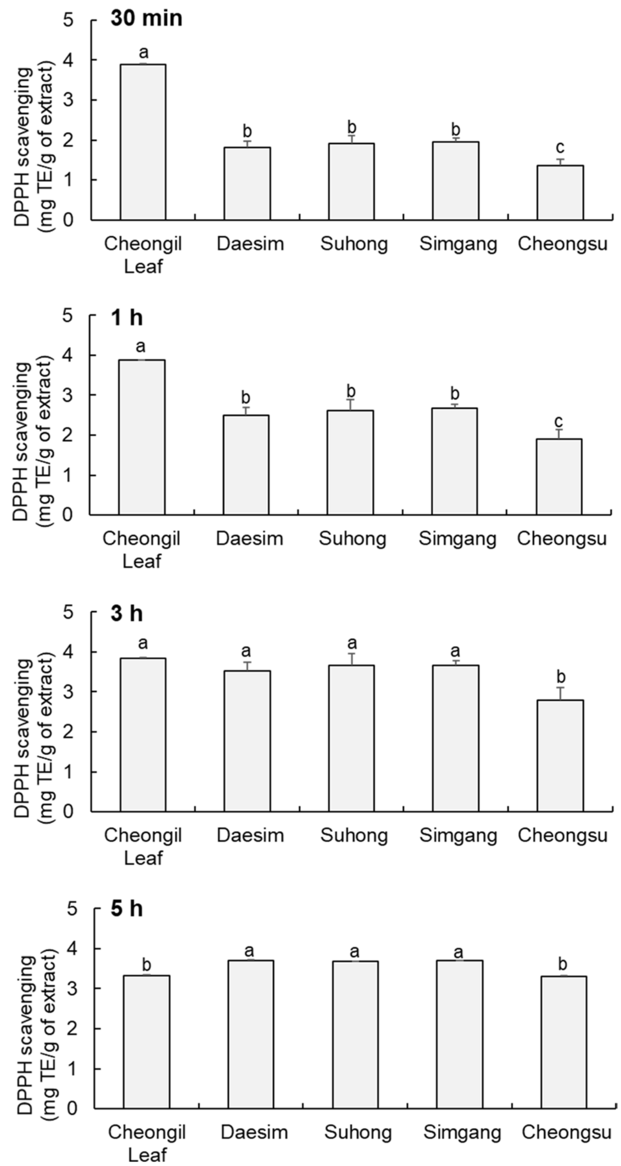

Fig. 5. Comparison of DPPH radical scavenging activities of four mulberry seedlings according to treatment time. Radical scavenging activities of 2-week-old mulberry seedlings were displayed depending on DPPH incubation time (0.5-5 h). The value of 3-year-old Cheong-il mulberry leaf was used as a control. Each measurement was performed in triplicate. Different letters indicate significant differences ($P < 0.05$).

2-week exceeded that of 3-year-old Cheong-il mulberry leaves (Fig. 5). Mulberry leaves possess high antioxidant capacity owing to their abundant flavonoid and polyphenol contents (Katsube *et al.*, 2006; Kim, 2005; Park *et al.*, 2008). This result suggests that mulberry seedlings are posited to contain substances with antioxidative functions distinct from those of adult mulberry leaves. The rate of DPPH radical removal is relative with chemical composition. In previous study (Fadda *et al.*, 2014), ascorbic acid shows a rapid DPPH radical scavenging effect, the reaction was completed within 3 minutes. Otherwise, butylated hydroxyl-toluene (BHT) took 5 h to complete the DPPH radical scavenging reaction. However, BHT is strong antioxidant because 1 mol of BHT reduces about 3 mol of DPPH radicals (Bondet *et al.*, 1997). Therefore, it is difficult to compare antioxidant effects based on reaction speed, we compared the radical scavenging activity until 5 h. In addition, mulberry seedlings showed different ATBS and DPPH radical scavenging activities. ABTS was dissolved in water, and DPPH was dissolved in ethanol. In general, ABTS has good reactivity with hydrophilic antioxidants, and DPPH has good reactivity with hydrophobic substances. It is assumed that in mulberry seedlings, there is a large difference in the contents of hydrophobic antioxidants by week but no significant difference in hydrophilic substances (Floegel *et al.*, 2011). Subsequent research endeavors should aim to delineate the specific components and enzymes uniquely produced in mulberry seedlings that contribute to their distinctive antioxidative functionality compared to that of mulberry leaves. Overall, these results highlight the potential of Korean mulberry seedlings to possess useful antioxidants, similar to other parts of the mulberry plants. With continued research in this trajectory, the economic impact of mulberry seedlings on the mulberry industry is projected to be significant.

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