WOOD RESEARCH
65 (3): 2020
415-422
doi.org/10.37763/wr.1336-4561/65.3.415422

CHARACTERIZATION OF NEW MUTANT *EUCOMMIA ULMOIDES* CONSTITUENTS IN THE DISCOLORATION DURING GROWING

Mengpei Liu
Zhengzhou University of Light Industry
China

Mengpei Liu, Qingxin Du, Panfeng Liu, Jun Qing, Hongyan Du, Lu Wang
Chinese Academy of Forestry
China

(Received May 2019)

ABSTRACT

A new mutant *E. ulmoides* with red xylem is found, and this red color will gradually metabolize over time. Comparisons of chemical properties and metabolites of xylem between the mutant and wild type were analyzed in this study in order to discover the cause of the red mutation. The results showed that the acid-insoluble lignin content of mutant type was about 13.83% higher than that of wild type, but the crude protein of wild type was almost 2 times of mutant type. Meanwhile, 6 most important amino acids and amino acid derivatives were detected, which had significant correlation with crude protein. Additionally, the contents of organic acids, polyphenols and alkaloids in the mutant type were 243%, 316% and 281% of those in the wild type, respectively, while the contents of flavonoids and phenolamines contents were 78.8% and 27.3% of those in the wild type, respectively. These results will provide an important reference for understanding the wood color variation during growing.

KEYWORDS: *Eucommia ulmoides*, mutant xylem, chemical properties, metabolites.

INTRODUCTION

Eucommia ulmoides Oliv. is the only member of Eucommiaceae, existed in the Eocene and therefore is considered a 'living fossil plant' (Sun et al. 2013, Call and Dilcher 1997). It is a traditional and valuable medicinal plant and high-quality natural rubber in China, which accounts for about 99% of the world's total resources (Feng et al. 2016, Nakazawa et al. 2013, Nakazawa et al. 2009, Du 1996). Its bark, as a Chinese medicine, has been used for over 2000

years with the effect of decreasing blood pressure and cholesterol, antibacterial and enhancing the body's nonspecific immune function (Kwan et al. 2003, Tomoda et al. 1990). *E. ulmoides* is also called a 'hard rubber tree' because of the abundant quantities of trans-polyisoprene rubber in its bark, leaf and seed (Wang et al. 2018, Chen et al. 2012). Departments of non-timber forestry research and development center of Chinese academy of forestry, has the world's largest *E. ulmoides* resources with more than 1800, which planted in Xinxiang, Henan Province, China (N35°18´13.71´, E113°55´15.05"). A red-xylem discolored *E. ulmoides* mutant is found at this base. Meanwhile, the grafted varieties of this red-xylem of *E. ulmoides* have the same performance in different places. Studies on wood discoloration are mostly focused on wood drying or processing, but rare in the growth process (Sahin et al. 2011, De Moura et al. 2013, Preklet et al. 2019). Therefore, the color variation of *E. ulmoides* xylem during growing is a very rare phenomenon, which deserves further study.

The extent of the color change of wood depends on specific wood constituents (Rowe 2012). Changes in wood chemical composition are a major cause of wood discoloration, such as lignin and cellulose. Additionally, metabolomics has been defined as the analysis of all metabolites in an organism and simultaneous measurement of all metabolites in a given biological system (Chen et al. 2012, Rowe 1989, Dixon and Strack 2003), and LC-MS has been regarded as a promising metabolomics tool for metabolic profiling of metabolites (Paupiere et al. 2017, Loskutov et al. 2017, Zhang et al. 2016, Lin et al. 2014). Therefore, the different of the new mutant and wild type of *E. ulmoides* was conducted through comparison of chemical properties and metabolites of xylem in this study in order to discover the cause of the red mutation, and the results will provide a reference for wood color variation during growing.

MATERIALS AND METHODS

Materials

The xylem of new mutant and wild type of *E. ulmoides* was collected in August, 2016 from *Eucommia ulmoides* cultivation base, Xinxiang, Henan province, China. The morphological characters between the mutant and wild type of *E. ulmoides* were showed in Fig. 1.

Fig. 1: The morphological characters of the mutant and wild type of Eucommia ulmoides.

Chemical and primary metabolites analysis

Acid-insoluble lignin, α-cellulose and brown cellulose were detected using equipment (NAI-CQW-6, Shanghai Na Ai Precision Instrument Co., Ltd.) in Zhejiang Academy of Forestry

Sciences, according to the China national standards (CNS) GB/T2677.8-1994, GB/T 744-2004 and GB/T2677.10-1995, resp. Near infrared reflectance spectroscopy method was chosen using Near infrared spectrometer (NIRQuest 256-2.1, 900-2050 nm, Boson Technology Co., Ltd.) according to CNS GB/T18868-2002 to detect crude proteins.

The freeze-dried xylem was crushed using a mixer mill (MM 400, Retsch) with a zirconia bead for 1.5 min at 30 Hz. 100 mg powder was weighted and extracted overnight at 4°C with 1.0 mL 70 % aqueous methanol. Following centrifugation at 10,000 g for 10 min, the extracts were absorbed (CNWBOND Carbon-GCB SPE Cartridge, 250 mg, 3 mL; ANPEL, Shanghai, China) and filtrated (SCAA-104, 0.22 μm pore size; ANPEL, Shanghai, China) before LC-MS analysis.

The sample extracts were analyzed using an LC-ESI-MS/MS system (HPLC, Shim-pack UFLC SHIMADZU CBM30A system; MS, Applied Biosystems 4500 Q TRAP). The analytical conditions were as follows, HPLC: column, Waters ACQUITY UPLC HSS T3 C18 (1.8 μm, 2.1 mm*100 mm); solvent system, water (0.04% acetic acid), acetonitrile (0.04% acetic acid); gradient program, 100:0 V/V at 0 min, 5:95 V/V at 11.0 min, 5:95 V/V at 12.0 min, 95:5 V/V at 12.1 min, 95:5 V/V at 15.0 min; flow rate, 0.40 mL·min^{-1}; temperature, 40 °C; injection volume: 5 μL. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (Q TRAP)-MS.

Statistical analyses

All analyses of chemical properties were done using three replicates. Univariate analyses of variance (ANOVA) and least significant differences (LSD) were performed using JMP Pro 12 (SAS Institute, Cary, NC, USA). Biomarker selection was done by partial least squares discriminant analysis (PLS-DA) in MetaboAnalyst 3.5 and heat map was done by MetaboAnalyst 3.5.

RESULTS

Chemical properties

The wood composition of the mutant *E. ulmoides* with wild type as control was showed in Tab. 1. Acid-insoluble lignin, α-cellulose and brown cellulose were detected in this study.

Tab. 1: Acid-insoluble lignin, α-cellulose and brown cellulose of the mutant and wild type of Eucommia ulmoides (%).

	Acid-insoluble lignin	α-cellulose	Brown cellulose
Mutant 1	28.82±0.93^{a}[1]	81.43±0.65^{bc}	77.96±0.78^{c}
Mutant 2	29.56±0.25^{a}	82.56±0.27^{ab}	77.84±0.45^{c}
Mutant 3	27.52±0.03^{b}	81.01±0.71^{c}	78.47±0.53^{bc}
Average	28.63	81.67	78.09
Wild 1	24.63±0.07^{d}	80.67±0.22^{c}	79.91±0.33^{a}
Wild 2	26.06±0.56^{c}	83.32±0.58^{a}	79.42±0.57^{ab}
Wild 3	24.75±0.05^{d}	81.87±0.57^{abc}	78.74±0.15^{abc}
Average	25.15	81.95	79.36

[1]Data are expressed as mean ± SD (n = 3). Means were separated by LSD $p \leq 0.05$.

The results indicated that there were no significant difference in α-cellulose and brown cellulose between the mutant and wild type of *E. ulmoides*. However, significant variation

existed in acid-insoluble lignin of mutant and wild type, with the average 28.63% and 25.15%, respectively. The acid-insoluble lignin content of mutant was 13.83% higher than that of wild type. Discoloration in the cell wall was influenced by photochemical reactions leading to the degradation of wood constituents, mainly lignin (Ozgenc et al. 2012, Li et al. 2017). Lignin contains many chromophores and has an aromatic structure that absorbs sunlight, especially in the UV region. Approximately 80-95% of UV light incident on wood surface is absorbed by lignin, and it is therefore easily decomposed by photo-oxidative processes. UV light interacts with lignin to initiate discoloration and deterioration (Li et al. 2015, Hayoz et al. 2003). However, fiber-rich cellulose with a higher resistance against ultraviolet light degradation remains in the cell wall without significant change (Rowell and Barbour 1989, Feist and Hon 1984).

Crude protein, amino acid and amino acid derivative

The crude protein in mutant type was 1.46-1.90%, which was significantly lower than that in wild type with 3.32-3.44%. It meant the crude protein of wild type was almost 2 times of that in mutant type. Because the red color of xylem of the mutant type would gradually metabolize over time, then amino acid metabolites between the mutant and wild type was analyzed. 50 amino acids and amino acid derivatives were detected in this study as well as the correlation with crude protein. It showed that 15 most important discriminatory biomarkers were detected between the mutant and wild type of *E. ulmoides* (Crude protein, Xanthurenic acid O-hexoside, L-Phenylalanine, L-Asparagine, L-Alanine, Phenylalanine, Oxitriptan, L-Proline, D-3-Methylhistidine, D-Ala-d-ala, N-Acetyl-L-leucine, N-Hydroxy-L-tryptophan, L-Serine, Serotonin and L-Isoleucine) (VIP value>1.1) (Fig. 2a).

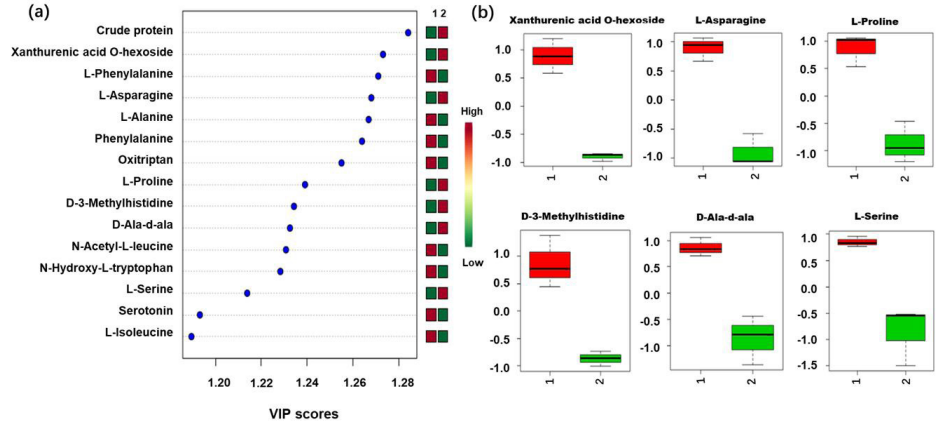

Fig. 2: Amino acid and amino acid derivative analysis between the mutant and wild type of Eucommia ulmoides in VIP scores: (a) the top 15 selected biomarkers based on variable importance in projections (VIP value>1.1), (b) 6 selected biomarkers positively correlated with crude protein. The number 1 and 2 indicates the mutant type and wild type, resp.

There was a significant positive correlation between crude protein and D-Ala-d-ala, L-Serine, with correlation coefficient 0.89 and 0.87, respectively ($p<0.05$). Meanwhile, extremely significant positive correlation also existed between crude protein and Xanthurenic acid O-hexoside, L-Proline, D-3-Methylhistidine, L-Asparagine, with correlation coefficient

0.98, 0.98, 0.96 and 0.95, respectively ($p<0.01$) (Fig. 2b). These 6 components may have some correlation with the decrease of crude protein in mutant type of *E. ulmoides*.

Flavonoid, polyphenol, organic acid and other metabolites

Except amino acid and amino acid derivative, other metabolites were also detected between mutant and wild type of *E. ulmoides*, including flavonoid, polyphenol, organic acid, alkaloid, phenolamine and terpenoid (Fig. 3).

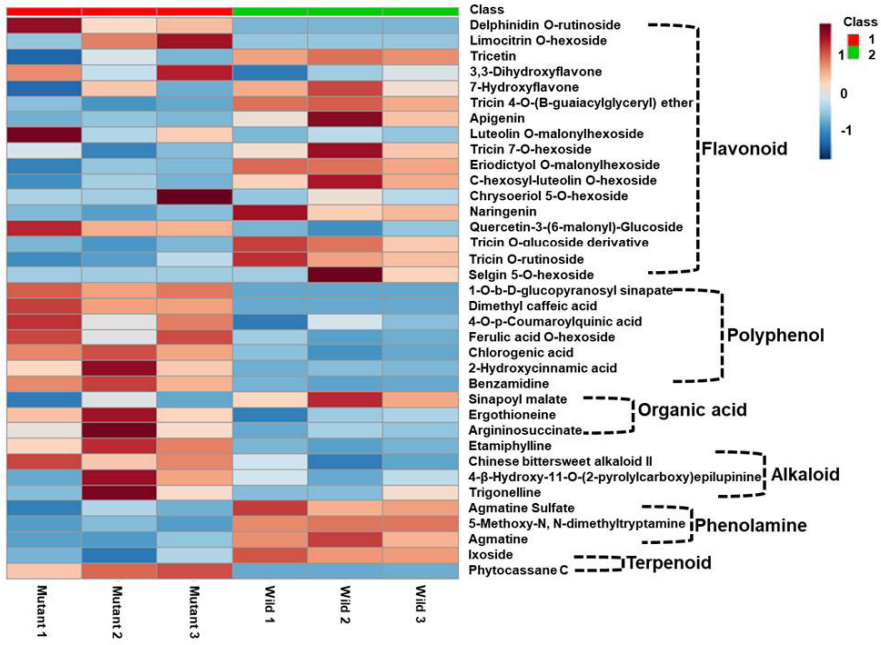

Fig. 3: Flavonoid, polyphenol, organic acid and other metabolites analysis between the mutant and wild type of Eucommia ulmoides in heat map. The number 1 and 2 indicate the mutant type and wild type, respectively.

It showed that 2 flavonoids were higher in mutant type, Delphinidin O-rutinoside and Quercetin-3-(6-malonyl)-Glucoside, respectively, but 10 flavonoids were higher in wild type. The contents of the mutant type flavonoids was 78.8% of that of the wild. Except Sinapoyl malate, the contents of organic acids, polyphenols and alkaloids in the mutant type were 243%, 316% and 281% of those in the wild type, respectively, while phenolamines contents were 27.3% of those in the wild type. With regard to terpenoids, Phytocassane C was higher in mutant type, whereas Ixoside was higher in wild type. Therefore, there were also significant differences in flavonoids, polyphenols, organic acids and other metabolites between the mutant and wild type of *E. ulmoides*.

DISCUSSION

There are many researches on the color change of wood in the process of post-harvest or drying. Wood discoloration is a complex phenomenon, mainly affected by heat, light, physiological and biochemical reactions, as well as from attack by microorganisms (Sandoval et al. 2010, Chang et al. 1999, Salca et al. 2015). However, there is no related research on the color change in the process of wood growth. The mutant *E. ulmoides* with red-xylem during growing is found, but this red color will gradually metabolize over time, which is a rare situation in wood discoloration. The chemical properties of xylem between the mutant and wild type of *E. ulmoides* showed that the acid-insoluble lignin content of mutant type was just about 13.83% higher than that of wild type. However, the crude protein of wild type was almost 2 times of that in mutant type, which had significant positive correlation with D-Ala-d-ala, L-Serine, Xanthurenic acid O-hexoside, L-Proline, D-3-Methylhistidine and L-Asparagine. Moreover, most of the polyphenols, organic acids and alkaloids were higher in mutant type, but flavonoids and phenolamines were higher in wild type.

E. ulmoides is rich in primary and secondary metabolites, such as amino acids, flavonoids and organic acids, which do not only play an important role in the growth and development, but also have the basis of medicinal value of *E. ulmoides*. The color change of the mutant with red-xylem maybe closely related to the variation of its metabolic components. These results provided an important reference for understanding the color variation in *E. ulmoides* during the growing. Further studies on amino acids, flavonoids and polyphenols related pathways can be carried out at the later stage, as well as further validation and analysis in combination with other genomes such as transcriptome.

CONCLUSIONS

(1) There was significant variation in acid-insoluble lignin between the mutant and wild type. The content of acid-insoluble lignin in mutant was 13.83% higher than that in wild type. (2) The crude protein in wild type was almost 2 times of that in mutant type, which had significant positive correlation with D-Ala-d-ala, L-Serine, Xanthurenic acid O-hexoside, L-Proline, D-3-Methylhistidine and L-Asparagine. (3) The contents of organic acids, polyphenols and alkaloids in the mutant type were 243%, 316% and 281% of those in the wild type, respectively, while the contents of flavonoids and phenolamines contents were 78.8% and 27.3% of those in the wild type, respectively.

ACKNOWLEDGEMENTS

The work was financially supported by the fundamental research funds for the central nonprofit research institution of the Chinese Academy of Forestry, Beijing, China (CAFYBB2016QA017).

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Mengpei Liu
Zhengzhou University of Light Industry
School of Food and Bioengineering
Zhengzhou, 450002
China

Qingxin Du, Panfeng Liu, Jun Qing, Hongyan Du, Lu Wang*
Chinese Academy of Forestry
Non-Timber Forest Research and Development Center
Zhengzhou, 450003
China
*Corresponding author: wanglu181716@163.com