Quantitative determination of 1-deoxynojirimycin in 146 varieties of mulberry fruit

Zhenjiang Wang\textsuperscript{a,b}, Fanwei Dai\textsuperscript{a,b}, Cuiming Tang\textsuperscript{a,b}, Gengsheng Xiao\textsuperscript{a}, Zhiyi Li\textsuperscript{a}, and Guoqing Luo\textsuperscript{a}

\textsuperscript{a}Sericultural & Agri-Food Research Institute Guangdong Academy of Agricultural Sciences, Guangzhou, China; \textsuperscript{b}Key Laboratory of Urban Agriculture in South China, Ministry of Agriculture and Rural Affairs, Guangzhou, China

ABSTRACT
1-deoxynojirimycin (DNJ) has attracted much attention due to its high biological activity, which have favorable anti-diabetic, anti-tumor, and anti-virus effects. In order to explore the effects of maturity, variety, and growth area on the DNJ concentration of mulberry fruit, we examined 146 varieties in this study. First, to explore the influence of maturity, we determined the concentration of DNJ from \textit{M. atropurpurea} Roxb. and \textit{M. wittiorum} var. \textit{mawa} Koidz at different stages of maturity using High Performance Liquid Chromatography (HPLC). On this basis, we next determined the DNJ concentration from 146 mulberry fruits to investigate the effect of variety and growth area, also by HPLC. Our results showed that DNJ existed mainly in the young tissues of mulberry fruit (MS-I). As the mulberry fruit matured, the concentration of DNJ decreased. The DNJ concentrations of 0.005 ~ 0.138 mg/g of fruit weight (FW) accounted for 97.28% of the 146 mulberry fruit varieties. The variety had a significant effect on the DNJ concentration of mulberry fruit, but plant origin did not. Among the varieties, \textit{M. atropurpurea} Roxb planted in Guangdong had up to 0.1716 ± 0 mg/g FW. This work provides new strategies for the study of DNJ and advances our basic knowledge about DNJ in mulberry fruits.

Introduction
1-deoxynojirimycin (DNJ) is a polyhydroxypiperidine alkaloid found in the mulberry plant, as well as in other plants and microorganisms. Its structure is similar to α-1,4-glucose, and it is an effective component for the treatment of type 2 diabetes mellitus.\textsuperscript{[1]} In addition, DNJ has strong anti-tumor and anti-viral activity.\textsuperscript{[2–8]} DNJ is primarily obtained from two sources. One is from mulberry and some genera plants,\textsuperscript{[9]} including \textit{Commelina communis}, \textit{Hyacinthus orientalis}, and \textit{Adenophora triphylla} var. \textit{japonica}.\textsuperscript{[10–12]} The other is from microbial synthesis, including \textit{Streptomyces lavendulae}, \textit{Escherichia coli},\textsuperscript{[13]} and \textit{bacillus}\textsuperscript{[14,15]} Among these, the mulberry tree has the highest concentrations of DNJ, but that the concentration differs significantly across mulberry tree varieties, seasons of growing, and the part of tree.\textsuperscript{[16–18]}

Fruccus Mori, also known as mulberry fruit, mulberry jujube, etc., contains essential amino acids, proteins, vitamins, and various mineral elements. It is rich in various active components such as brass, polyphenols, polysaccharides, and volatile oils.\textsuperscript{[19]} Additionally, it possesses anti-oxidant, anti-tumor, hypoglycemic, immune regulatory and other functional characteristics.\textsuperscript{[20,21]} These properties lend it a certain nutritional and medicinal function leading to its wide use in both the food and medical fields. The flowering period of the mulberry is generally 4 to 5 months, the fruiting period is 6 to 7 months, and the harvesting period is usually from April to June. During mulberry growth, the color change is
divided into five stages, namely the green fruit period (MS-I & MS-II), the color-changing fruit period (MS-III), the pink fruit period (MS-IV), the red fruit period (MS-V), and the black fruit period (MS-VI). The levels of nutrients and active components change dramatically throughout these periods. At present, DNJ research focuses mainly on non-edible parts, such as mulberry leaves and mulberry branches, with less research on the edible parts, such as mulberry fruit.

Therefore, we set out to explore changes in the DNJ concentrations during the process of mulberry fruit maturity. In this study, we measured the DNJ concentrations in *M. atropurpurea* Roxb. and *M. wittiorum* var. *mawa* Koidz via high-performance liquid chromatography (HPLC) during the six different mature stage (MS) types based on their fruit color and sizes, namely the green fruit period (MS-I and MS-II), the color-changing fruit period (MS-III), the pink fruit period (MS-IV), the red fruit period (MS-IV), and the black fruit period (MS-VI). In order to compare the different DNJ concentrations in different varieties of mulberry, we measured the DNJ amount in 9 genera of mulberry and 146 varieties of mulberry fruits by HPLC. Taken together, these findings provided the theoretical basis for the functional development of mulberry fruits and has the potential to expand their application in the food and drug fields.

**Materials and methods**

**Materials**

For DNJ determination, we selected 146 Mulberry fruit varieties of nine Morus species, collected from provinces in China and 6 other countries and regions in Asia and South America. Of these varieties, 129 were collected from China, 7 from Thailand, 3 from Vietnam, 3 from China Taiwan Region, 1 from Argentina, and 1 from Korea. All mulberry fruit varieties tested in this study were preserved in the South China Branch of the National Mulberry Germplasm Resources Bank, and were grown under the same conditions in Guangzhou, Guangdong Province. Approximately 200 g of mature disease-free mulberries from each cultivar were harvested in May 2015–2016. *M. atropurpurea* Roxb. (Yueshenda10) and *M. wittiorum* var. *mawa* Koidz (Changguosang) are two typical cultivars widely grown in China. The fruits of these two varieties were classified into six different MS types based on their fruit color and sizes. The fruits at MS-I and MS-II were green, and at these two stages the fruits were one and three weeks after flowering. At MS-III, the fruits were white green (turning stage), approximately four weeks after flowering. MS-IV was the pink phase, MS-V was the red to reddish black stage, and MS-VI was the black stage, as the fruits were fully matured. The collected fruits were immediately frozen in a box with liquid nitrogen, and then transported to the laboratory for preservation at −80°C until use.

**DNJ extraction**

The DNJ extraction method was performed according to the procedure described by Kim et al. [22] and Wei et al. [23] with slight modification. Briefly, 25 g of mulberry fruit of each variety were placed in liquid nitrogen and ground using a mortar before extraction with 250 mL of 0.05 mol/L HCl solution in a 30°C water bath for 10 min. The extract was then centrifuged at 10000 × g for 5 min at 20°C (CT15RE, Hitachi, JAPAN), and the supernatant was collected. The precipitate was extracted once again, according to the above procedure, and the combined supernatant was diluted to 500 mL with distilled water.

**DNJ derivation**

The DNJ derivation method also referred to Kim et al. [22] and Wei et al. [23], with slight modification. We mixed 100 μL of DNJ extract, 100 μL of 0.4 mol/L potassium borate buffer (pH 8.5), and 200 μL of 9-fluorenylmethyl chloroformate (FMOC-Cl: Dissolved in acetonitrile) in a 20°C water bath for
20 min, before adding 100 μL of 1 mol/L glycine to neutralize the excess FMOC-Cl for termination of the reaction. Afterward, we added 100 μL of 1% acetic acid stable DNJ derivative, and diluted with 400 μL of distilled water. Finally, the dilution was filtered through a 0.22 μm syringe filter to determine the DNJ concentration.

**DNJ determination**

To determine the concentration of DNJ, 20 μL of the derivatized mulberry samples were injected into a six-way valve in an HPLC system (LC-10AVP Plus, SHIMADZU, JAPAN). A Penomenex C18 (250 mm × 4.6 mm, 5 μm) column at 25°C was used to separate the DNJ derivatives, and then the concentrations were determined at 254 nm via a SPD-10 AVP Plus UV-Vis dual-wavelength detector. The mobile phase was methanol − 0.5% glacial acetic acid (45:55, v/v) at the flow rate of 1.0 mL/min for 60 min.

**Data processing**

The experimental data is expressed by $\bar{x} + s$, and the analysis of variance was performed using SPSS v16 statistical software.

**Results and discussion**

**DNJ concentrations during maturity**

The nutrients and active components of mulberry fruits changed along with maturity during the growth process. In order to exert the bioactivity of mulberry fruits and explore changes in nutrients and bioactive compositions with maturity, this study measured the DNJ concentrations in *M. atropurpurea* Roxb. and *M. wittiorum* var. *mawa* Koidz via HPLC during the green fruit period (MS-I & MS-II), the color-changing fruit period (MS-III), the pink fruit period (MS-IV), the red fruit period (MS-V), and the black fruit period (MS-VI). The results are shown in Figure 1.

The obtained results showed the highest DNJ concentrations in the first stage, MS-I, with values of 0.1425 mg/g FW and 0.1514 mg/g FW of, From Figure 1, *M. atropurpurea* Roxb. and *M. wittiorum* var. *mawa* Koidz, respectively. In the second stage, MS-II, the increased volume of mulberry fruits caused a significant decrease in the DNJ concentrations of both two mulberries to 55.09% and 30.58% of MS-I concentration, respectively. As the mulberries matured, the increase in volume and the accumulation of glucose limited the biosynthesis of DNJ, causing the DNJ concentrations of both mulberries further decreased in the MS-III, MS-IV, MS-V, and MS-VI stages. Among them, the order of DNJ concentration at each mature stage was MS-IV > MS-VI > MS-III > MS-V. At the MS-VI stage, the

![Figure 1. Effect of mulberry maturity on DNJ concentrations. *M. atropurpurea* Roxb. (Left, white bars); Right: *M. wittiorum* var. *mawa* Koidz (Right, gray bars).]
mulberry fruits had matured and the DNJ concentration had accumulated in vivo, which resulted in an increase in the DNJ concentration per unit volume relative to the MS-V stage, but less than that in the MS-I stage. In MS-VI, the DNJ concentration was 14.39% and 21.99% in M. atropurpurea Roxb. and in M. wittiorum var. mawa Koidz, respectively, compared to MS-I concentration. These results indicated that DNJ existed mainly in young tissues during the maturation of the mulberry, and that the concentration of DNJ decreased with increasing maturity. In addition, The concentration of DNJ in M. wittiorum var. mawa Koidz (Changguosang) is higher than M. atropurpurea Roxb. (Yueshenda10) (Supporting Information 1, S1).

**Change of DNJ concentration in 146 varieties of mulberry**

Different varieties, maturity, and growing areas, among other factors, will cause variation in the DNJ concentration in mulberry fruits. In order to comprehensively explore the differences in the DNJ concentration in different mulberry fruit varieties, we conducted this study to determine the concentration of DNJ in 146 varieties of mulberry fruits via HPLC, comparing the DNJ concentration in mulberries from different regions and of different varieties. Our results are shown in Figure 2, Table 1, and Table 2.

As can be seen in Figure 2, DNJ concentrations ranging from 0.005 ~ 0.138 mg/g FW accounted for 97.28% among the 146 mulberry fruit varieties. The greatest number of mulberry fruit varieties had DNJ concentrations ranging from 0.005–0.038 mg/g FW and 0.038–0.071 mg/g FW, which accounted for 40.82% and 36.05% of the total number of varieties, respectively. On the contrary, mulberry varieties with a high concentration of DNJ, were less numerous. There were twenty-three mulberry varieties in the DNJ concentration range of 0.071–0.104 mg/g FW, seven mulberry varieties in the DNJ concentration range of 0.104–0.138 mg/g FW, and one mulberry variety each in the DNJ concentration ranges of 0.138–0.171 mg/g FW and 0.171–0.204 mg/g FW.

The concentration of DNJ varied from 0.0057 ~ 0.1776 mg/g FW among the 146 mulberry fruit varieties (Table 1). The results of independent sample Kruskal-Wallis test showed that the DNJ concentrations among the mulberry fruit varieties were significantly different (p< .05) (S2). The mulberry variety with the highest concentration of DNJ was M. atropurpurea Roxb, and that with the lowest concentration of DNJ was M. alba L. The concentration of DNJ between the two mulberry varieties was significantly different, which could be used to investigate the mechanism of DNJ synthesis. As the variety with the highest DNJ concentration, M. atropurpurea Roxb could be used as a DNJ-enriched variety for breeding.

![Figure 2](image-url) The concentration distribution of DNJ in 146 mulberry varieties (in the mature period).
According to Table 1 and Figure 2, the concentrations of DNJ in the different mulberry varieties were significantly different. The concentration of DNJ in *M. atropurpurea* Roxb. was more than 31 times that of *M. alba* L., and there was an obvious variety difference. Table 2 depicts the 146 mulberry varieties that originated from 11 different provinces of China, Thailand, Vietnam, India, Argentina, and Korea. Among them, the mulberry from Shandong, China had the highest DNJ concentration, which was 132.00 ± 0.00 μg/g FW, while the mulberry from Argentina had the lowest DNJ concentration, which was 9.70 ± 0.00 μg/g FW. Kruskal-Wallis test results showed that there was no significant difference in the DNJ concentrations of mulberry fruits from different plant origins (Supporting Information, S3).
According to Tables 1 and Table 2, the DNJ concentrations of the mulberry fruits were more affected by inherent factors, such as mulberry fruit varieties. External environmental conditions, such as region, affected the DNJ concentration of mulberry fruit, but there was no significant difference. Therefore, choosing the optimal mulberry varieties and most suitable planting environment conditions, such as long daylight hours, mild environmental stress, and etc., could cultivate a mulberry with a higher DNJ concentration, which could increase the medicinal value of mulberry and expand its application in both food and medicine.

Based on above results, we have screened three germplasm with a DNJ concentration above 0.150 mg/g FW, and we have already assessed the genetic diversity of these high DNJ concentration germplasm for agronomic traits, and screening of agronomic traits. So, this study can screen excellent breeding material, which can provide breeding material for the innovative use of mulberry germplasm resources with high yield, good quality and high DNJ concentration and the selection of new varieties, so as to select and breed new varieties with high in this way, new health-care mulberry varieties will be selected for industrial application.

Conclusion

Our present work explored the effect of maturity, variety, and plant origin on the concentration of DNJ in mulberry fruits. We obtained the following conclusions: DNJ existed mainly in the MS-I stage of the mulberry fruit. As the mulberry fruit matured, the concentration of DNJ decreased. A higher concentration of DNJ was observed in the variety M. wittiorum var. mawa Koidz. Among the 146 mulberry fruit varieties, the DNJ concentration range was 0.005 ~ 0.138 mg/g FW, which accounted for 97.28% of the total number of varieties. Additionally, the variety of mulberry fruit, but not the plant origin, had a significant effect on the DNJ concentration. Of the varieties tested, M. atropurpurea Roxb. planted in Guangdong had DNJ concentrations of up to 0.1716 ± 0 mg/g FW. Mulberry germplasm resources are the material basis for the selection and breeding of new crop varieties and related research. This research had effectively identified of DNJ concentration of mulberry germplasm resources, which could provide scientific guidance for the selection of parents for subsequent cross breeding. In addition, this study had screened the germplasm with high DNJ concentration, which could be directly grown and extracted high DNJ from the mulberry fruits to develop health food and pharmaceutical products for the treatment of diabetes, tumor prevention, virus suppression and weight loss and body beauty.

Abbreviations

1-deoxynojirimycin (DNJ); FW (fruit weight); green fruit period (MS-I & MS-II); High Performance Liquid Chromatography (HPLC); M. atropurpurea Roxb. (Yueshenda10); M. wittiorum var. mawa Koidz (Changguosang); the black fruit period (MS-VI); the color-changing fruit period (MS-III); the pink fruit period (MS-IV); the red fruit period (MS-V)
Disclosure statement
The authors claim there is no conflict of interest among them.

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