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Regional and Varietal Variation of 1-Deoxynojirimycin (DNJ) Content in the Mulberry Leaves

Iksoo Kim*, Jin Won Kim, Heui Sam Lee, Nam Kyu Ha[1] and Kang Sun Ryu

Department of Sericulture & Entomology, National Institute of Agricultural Science and Technology, RDA, Suwon 441-100, Korea.

[1]Gyeong Nam Agricultural Research and Extension Services, Jinju 660-985, Korea.

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This study was carried out to find out whether there exists any content variation in 1-deoxynojirimycin (DNJ) among regional and varietal mulberry leaves. HPLC analysis was performed for 22 samples collected from several localities in Korea and Tsushima Island, Japan. The highest content was observed in the Cheongilppong sample collected at Youngchun City, Kyungsangbuk Province (1,882.7 μg/g DW), whereas lowest content was observed in the Cheongilppong sample collected at Jinju City, Kyungsangnam Province (395.65 μg/g DW). In the comparison of regional samples of Kaeryangppong variety, DNJ content was highest in the order of Wonju City (1,460.1) > Boun-gun (999.85) > Hweongsung-gun (939.12) > Cheongju City (688.78). In case of Cheongilppong, DNJ content was highest in the order of Youngchun City (1.882.8) > Sanchung-gun (1,125.3) > Sunchon City (649.17) > Byunsan-myon (441.54) > Jinju City (395.65). Average content of regional samples was higher in Kaeryangppong (1,021.7 μg/g DW) than that of Cheongilppong (898.87 μg/g DW). Average DNJ content of the wild mulberry leaves collected from Cheju Island and Tsushima Island (1,012.9 μg/g DW) was high enough to reach to that of the regional Kaeryangppong samples. In the comparison among varietal samples collected in Suwon, the highest DNJ content was observed in YK209 (768.16 μg/g DW), amongst overall low DNJ content of the varietal samples. Considering a few pieces of information from our data, DNJ content appears not to be influenced solely by annual temperature of the planted areas.

Key word : Mulberry leaves, 1-Deoxynojirimycin (DNJ), Secondary metabolite, *Morus*

Introduction

DNJ (1-deoxynojirimycin), which is an alkaloid belonging to polyhydroxylated piperidine family, exists abundantly in mulberry leaves and its roots (Asano *et al.*, 1994). This substance has been known to be one of the major blood glucose lowering substances among a diverse mulberry extract and a powerful competitive inhibitor for α-glucosidase (Asano *et al.*, 1995; Chen *et al.*, 1995; Kimura *et al.*, 1995; Lee *et al.*, 1998). IC_{50} values of DNJ against maltase, sucrose, and isomaltase in rat intestinal brush border membrane are 0.36, 0.21, and 0.30 μM, respectively (Hughes and Rudge, 1994; Asano, 2000). In addition, a recent study reported the silkworm-derived DNJ to have a strong repressive effect of blood glucose in the experiment animal (Lee *et al.*, 2001).

In contrast to an abundant scientific research on the repressive effect of DNJ on α-glucosidase activity, studies on its role within plant organism are scarce to quote. Judging from its inhibitory role against glucosidase activity in a diverse organism, it would be possible to speculate that DNJ may play a role as a repellant against several kinds of predators (*e.g.*, insects and birds) (Dethier, 1954; Kite *et al.*, 1991; Asano *et al.*, 1994). Excluding a few facts known and speculation, no study has ever been made on the above-mentioned effect of the mulberry leaf-derived DNJ, and the pattern and mechanism for its bio-synthesis.

One of the general purposes of the secondary metabolites in plant is a defense against the plantss enemies. On

*To whom correspondence should be addressed.
Department of Sericulture & Entomology, National Institute of Agricultural Science and Technology, RDA, Suwon 441-100, Korea. Tel: +82-31-290-8463; Fax: +82-31-295-2176; E-mail: iksookim@hanmail.net

this basis, if bio-synthesis of DNJ may be correlated with the defense mechanism of mulberry tree against non-specific herbivore, change in DNJ content can be thought to be closely related with proliferation of germs and/or insects populations, which have the potential to attack mulberry tree. This, in turn, can be influenced heavily on the weather and soil composition of the planted areas of the host plants. Thus, it would be reasonable to expect the content variation of DNJ, depending on seasons and varieties.

In this study, first of all, we studied whether variation of DNJ content exists among regional mulberry leaves or not. Furthermore, we compared DNJ content among the varietal samples planted at the Department of Sericulture & Entomology in Suwon to utilize the basic information for the subsequent study to investigate the variables of DNJ content in the mulberry trees.

Materials and Methods

Sample collection

Twenty-two mulberry leaf samples were collected from May 29 to June 20, 2000 in the several localities in Korea and Tsushima Island, Japan. Collection information of the samples is listed in Table 1. To avoid "sampling error" and exaggeration of the data by paucity of sample into the whole locality and variety, mulberry leaves were collected from many trees as possible. For example, to obtain 20 g mulberry leaves were taken from at least 20 trees in most cases. The collected leaves were deposited on ice while transportation to the Department of Sericulture & Entomology and preserved in 70°C until alkaloid extraction was performed.

The original experimental scheme was about to compare DNJ content of Cheongilppong leaves collected over a wide geographic range in Korea. However, the variety, which was once most widely cultivated, is not any more available in many regions of Korea and, resultantly, an extensive comparison of a single variety is not possible any more. Thus, we mainly collected Kaeryangppong and Cheongilppong together within limited regions and used them for regional comparison. Although we also collected a few more varieties (*e.g.*, YK209), these were excluded at this time for simplicity. According to a recent study about seasonal mulberry samples, DNJ content was reported to increase steadily after opening of the first leaf in May, culminate in July and August, and decrease until falling (Kim *et al.*, unpublished). On the basis of this observation, we compared those regional samples collected within minimum time period. Thus, the regional samples were used those collected in two days (from May 29 to 30) for Kaeryangppong, three days (June 7 to 9) for Cheongilppong, and two days (June 19 and 20) for varietal samples collected in Suwon, respectively. However, the wild mulberry leaves were used those collected in 13 days (June 2 to 14) from Cheju Island and Tsushima Island, Japan, due to difficulty in travel schedule.

Analysis of DNJ content

Lyophilized mulberry leaves (0.1 g) were extracted with 10 ml of distilled water at 60°C for 1 hr, followed by centrifugation, and these steps were repeated three times. The supernatants were pooled, diluted to 100 ml with distilled water and reacted with 9-fluorenylmethyl chloroformate (Fluka, Switzerland) by means of the modified method of Cole *et al.* (1988). The reaction mixture was syringe-filtered (nylon, 0.45 μm) and injected to HPLC. Mobile phase was acetonitrile-0.1% of aqueous acetic acid (1 : 1, v/v) and flowed isocratically for 13 min through Phenomenex Luna C18 (2) column (5 μm ODS; 4.60× 250 mm I. D.). DNJ was detected as a FMOC derivative by fluorescence detector (excitation 264 nm and emission 314 nm).

Statistical analysis

Using SAS program, Duncan's multiple range test ($p<0.05$) was performed to test if any significant difference exists in the DNJ content among regional samples of Kaeryangppong, Cheongilppong, and wild mulberry leaves, and among mulberry varieties collected in Suwon, respectively.

Results and Discussion

Water content

Water content ranged from 67.50% to 80.40% among 22 samples (Table 1). The minimum water content was observed in the Cheongilppong sample collected in suwon (Sample Number 23; 67.50%), whereas maximum content was observed also in the Cheongilppong collected in Sanchung-gun, Kyungsangnam Province (SN 13; 80.40%). Mean water content of 22 samples was 74.26%.

Variation of DNJ content in the regional samples of Kaeryangppong and Cheongilppong varieties

DNJ content of the four Kaeryangppong samples is shown in Table 2. The Wonju City sample (SN 3) showed highest content (1,460.1 μg/g DW) and the estimate was statistically significant ($p<0.05$). At the same time, the estimate is the second largest one among 22 samples analyzed in this study. Table 3 shows DNJ content of the five regional samples of Cheongilppong. The highest estimate was observed in the Youngchun City sample (SN 11; 1,882.7

Table 1. Collection information of the mulberry leaf samples, average temperature, and water content

Sample number	Variety	Date of collection	Collection locality	Average temperature (°C)*	Water content (%)
1	Kaeryangppong	2000. 5. 29	Cheongju Technical Institute of Sericulture, **Cheongju City**, Chungcheongbuk Province	12.5	75.80
2	Kaeryangppong	2000. 5. 29	Sericulture-specialized village, Euram-lee, **Boungun**, Chungcheongbuk Province	11.4	74.26
3	Kaeryangppong	2000. 5. 30	Kosan-lee, Hojeo-myon, **Wonju City**, Kangwon Province	11.6	77.82
4	Kaeryangppong	2000. 5. 30	Youngyoungpo-lee, **Hweongsung-gun**, Kangwon Province	10.6	73.16
6	Wild mulberry	2000. 6. 2	**Manjeki**, Mitsushima Town, Shimo Agata County, Tsushima, Japan	≥15	76.51
7	Wild mulberry	2000. 6. 4	**Zuzujaki (tree 1)**, Shimo Agata County, Tsushima, Japan	≥15	72.80
8	Wild mulberry	2000. 6. 4	**Zuzujaki (tree 2)**, Shimo Agata County, Tsushima, Japan	≥15	73.00
9	Wild mulberry	2000. 6. 4	**Zuzujaki (tree 3)**, Shimo Agata County, Tsushima, Japan	≥15	68.38
11	Cheongilppong	2000. 6. 7	Wansan-dong, **Youngchun City**, Kyungsangbuk Province	13.4	72.33
13	Cheongilppong	2000. 6. 8	Sanchung Sericultural Cooperative Association, **Sanchung-gun**, Kyungsangnam Province	12.5	80.40
14	Cheongilppong	2000. 6. 8	Kyungnam Agricultural Research and Extension services, Chojeon-dong, **Jinju City**, Kyungsangnam Province	13.0	73.19
16	Cheongilppong	2000. 6. 9	Kumsan-lee, Nakan-myon, **Sunchon City**, Chollanam Province	12.4	73.64
17	Cheongilppong	2000. 6. 9	Yuyu-dong, Mapo-lee, **Byunsan-myon**, Cholla buk Province	12.0	80.15
18	Wild mulberry	2000. 6. 14	**Ara-dong**, Cheju City, Cheju Province	15.0	72.98
19	Wild mulberry	2000. 6. 14	**Hanla Mt. (tree 1)**, Sungpanak, Cheju Cicy, Cheju Province	15.0	75.40
20	Wild mulberry	2000. 6. 14	**Hanla Mt. (tree 2)**, Sungpanak, Cheju Cicy, Cheju Province	15.0	78.87
21	Wild mulberry	2000. 6. 14	**Bongke-dong**, Cheju City, Cheju Province	15.0	78.87
22	Kaeryangppong	2000. 6. 19	Dept. of Sericulture and Entomology, Seodun-dong, NIAST, **Suwon City**, Kyunggi Province	12.1	68.03
23	Cheongilppong	2000. 6. 19	Dept. of Sericulture and Entomology, Seodun-dong, NIAST, **Suwon City**, Kyunggi Province	12.1	67.50
24	Shinkwangppong	2000. 6. 19	Dept. of Sericulture and Entomology, Seodun-dong, NIAST, **Suwon City**, Kyunggi Province	12.1	75.40
25	Yongcheonppong	2000. 6. 19	Dept. of Sericulture and Entomology, Seodun-dong, NIAST, **Suwon City**, Kyunggi Province	12.1	67.57
26	YK209	2000. 6. 20	Dept. of Sericulture and Entomology, Seodun-dong, NIAST, **Suwon City**, Kyunggi Province	12.1	69.48

Bold-faced characters were used to abbreviate locality names in the text.

*Average temperature was obtained with monthly temperature from May 1999 to April 2000.

Table 2. Comparison of DNJ content in the mulberry leaves among regional samples of Kaeryangppong variety

Sample number	Variety	Date of collection	Collection locality	Average temperature (°C)	*Mean DNJ content (μg/g DW)**
1	Kaeryangppong	2000. 5. 29	Cheongju Technical Institute of Sericulture, **Cheongju City**, Chungcheongbuk Province	12.5	688.78[a]
2	Kaeryangppong	2000. 5. 29	Sericulture-specialized village, Euram-lee, **Boun-gun**, Chungcheongbuk Province	11.4	999.85[b]
3	Kaeryangppong	2000. 5. 30	Kosan-lee, Hojeo-myon, **Wonju City**, Kang-won Province	11.6	1,460.1[c]
4	Kaeryangppong	2000. 5. 30	Youngyoungpo-lee, **Hweongsung-gun**, Kang-won Province	10.6	939.12[d]
Mean					1,021.7

*Average temperature was obtained with monthly temperature from May 1999 to April 2000.
** Means with the same superscripts are not significantly different. Statistical significance was tested at the level of $p<0.05$.

Table 3. Comparison of DNJ content in the mulberry leaves among regional samples of Cheongilppong variety

Sample number	Variety	Date of collection	Collection locality	Average temperature (°C)	*Mean DNJ content (μg/g DW)**
11	Cheongilppong	2000. 6. 7	Wansan-dong, **Youngchun City**, Kyung-sangbuk Province	13.4	1,882.7[a]
13	Cheongilppong	2000. 6. 8	Sanchung Sericultural Cooperative Association, **Sanchung-gun**, Kyungsangnam Province	12.5	1,125.3[b]
14	Cheongilppong	2000. 6. 8	Kyungnam Agricultural Research and Extension services, Chojeon-dong, **Jinju City**, Kyungsangnam Province	13.0	395.65[c]
16	Cheongilppong	2000. 6. 9	Kumsan-lee, Nakan-myon, **Sunchon City**, Chollanam Province	12.4	649.17[d]
17	Cheongilppong	2000. 6. 9	Yuyu-dong, Mapo-lee, **Byunsan-myon,** Chollabuk Province	12.0	441.54[c]
Mean					898.87

*Average temperature was obtained with monthly temperature from May 1999 to April 2000.
** Means with the same superscripts are not significantly different. Statistical significance was tested at the level of $p<0.05$.

μg/g DW). The estimate was several fold higher than that of the Jinju City (SN 14; 395.65 μg/g DW) and Byunsan-myon (SN 17; 441.54 μg/g DW) and was statistically significant at the level of $p<0.05$.

It is noteworthy that the collection locality of the Youngchun City is highest in the mean annual temperature among the mainland localities (excluding the southern-most Cheju Island and Tsushima Island, Japan) where mulberry leaves were collected (13.4°C) (Table 1). If we tentatively regard that DNJ is produced as a defense mechanism against insect attack as many other secondary metabolites do, it would be reasonable to assume that more DNJ would be produced in the place where higher annual temperature is maintained. This is because attack of mulberry leaves by more diverse insect populations may generate more stress to the mulberry. In this regard, the highest DNJ content in the Youngchun City sample appears to agree with the above assumption. Furthermore, Kim *et al.* (unpublished) showed that DNJ content was significantly high in the July and August mulberry leaves. On the other hand, the mean DNJ content of the regional samples of Kaeryangppong was higher (1,021.70 μg/g DW) than that of Cheongilppong samples (898.87 μg/g DW), but mean annual temperature was reverse (12.7°C in Cheongilppong- and 11.5°C in Kaeryangppong-planted areas, respectively). Thus, this result alone appears not to support that DNJ content may not be influenced solely by the annual temperature of the planted areas. For more

Table 4. Comparison of DNJ content in the mulberry leaves among mulberry varieties

Sample number	Variety	Date of collection	Collection locality	Average temperature (°C)	*Mean DNJ content (μg/g DW)**
22	Kaeryangppong	2000. 6. 19	Dept. of Sericulture and Entomology, Seodun-dong, NIAST, **Suwon City**, Kyunggi Province	12.1	524.15[a]
23	Cheongilppong	2000. 6. 19	Dept. of Sericulture and Entomology, Seodun-dong, NIAST, **Suwon City**, Kyunggi Province	12.1	652.10[b]
24	Shinkwangppong	2000. 6. 19	Dept. of Sericulture and Entomology, Seodun-dong, NIAST, **Suwon City**, Kyunggi Province	12.1	542.66[a]
25	Yongcheonppong	2000. 6. 19	Dept. of Sericulture and Entomology, Seodun-dong, NIAST, **Suwon City**, Kyunggi Province	12.1	407.10[c]
26	YK209	2000. 6. 20	Dept. of Sericulture and Entomology, Seodun-dong, NIAST, **Suwon City**, Kyunggi Province	12.1	768.16[d]
Mean					578.83

*Average temperature was obtained with monthly temperature from May 1999 to April 2000.
** Means with the same superscripts are not significantly different. Statistical significance was tested at the level of $p<0.05$.

decisive conclusion samples taken from several times over a longer period and an experiment in a controlled condition will be required (*e.g.*, content variation depending on temperature change).

Variation of DNJ content among the mulberry varieties collected in Suwon

DNJ content of the five mulberry varietal samples collected in Suwon is presented in Table 4. The mean DNJ content was 578.83 μg/g DW and the highest estimate was obtained from YK209 (768.16 μg/g DW), whereas the lowest one from Youngcheonppong (407.10 μg/g DW). These estimates were overall very low compared with other samples. For example, the mean estimate of the varietal samples (578.83 μg/g DW) is only the half of regionally collected Kaeryangppong samples (1,021.70 μg/g DW) and far less than Cheongilppong samples (898.87 μg/g DW). Because Suwon samples were collected at least one or two weeks later than Kaeryangppong or Cheongilppong, respectively, the low DNJ content in the Suwon varieties appears not be influenced by low temperature in Suwon. In fact, the mean annual temperature in Suwon is somewhat higher (12.1°C) than that of an averaged temperature of the Kaeryangppong-planted localities (11.5°C), although DNJ content was reverse. Thus, the mean annual temperature appears not to be the main factors influencing DNJ content. In stead, several environmental factors coupled with the temperature are more likely to influence directly and/or indirectly to the DNJ content in the mulberry leaves. Although the varietal samples were obtained from the plants with similar environmental conditions (*e.g.*, region, cultivation condition, and collection time), it would not be desirable to further infer on the factor caused DNJ content variation among varieties. This is mainly because the samples were subjected to HPLC analysis using mulberry leaves obtained at one seasonal time. For accuracy, a long-term collection of the varietal samples will be required. This may be true because Kim *et al.* (unpublished) has proposed seasonal variation of DNJ content in the Cheongilppong planted in Suwon.

Variation of DNJ content in the regional samples of wild mulberry

Table 5 shows DNJ content of each four wild mulberry leaves collected from Cheju Island and Tsushima Island, Japan, respectively. The highest estimate was observed from the Zuzujaki (tree 2) sample (SN 8; 1,435.4 μg/g DW) and this estimate is the third highest value followed by the Youngchun City sample (SN 11) and Wonju City sample (SN 3). The mean DNJ content of the wild mulberry leaves was 1,012.9 μg/g DW and this estimate is almost similar to the mean estimate of regional Kaeryangppong samples (1,021.70 μg/g DW), suggesting that the wild mulberry leaves also contain abundant DNJ. The result is consistent with the observation that the wild mulberry syncarp collected from Hweongsung, Kangwon Province contained 2~3-fold more GABA (γ-aminobutyric acid), which exerts several medicinal effects, than the cultivated ones (Kim *et al.*, 1999). Again, if we consider that DNJ is a defense substance in mulberry, the more DNJ content throughout defense mechanism would be expected in the wild mulberry than cultivated ones that are protected by insect pesticides and human care. Although limited, the calcium content in the wild mulberry leaves collected from Cheju Island and Tsushima Island, Japan

Table 5. Comparison of DNJ content in the mulberry leaves among wild mulberry

Sample number	Variety	Date of collection	Collection locality	Average temperature (°C)	*Mean DNJ content (μg/g DW)**
6	Wild mulberry	2000. 6. 2	**Manjeki**, Mitsushima Town, Shimo Agata County, Tsushima, Japan	≥15	858.45[a]
7	Wild mulberry	2000. 6. 4	**Zuzujaki (tree 1)**, Shimo Agata County, Tsushima, Japan	≥15	1,199.9[b]
8	Wild mulberry	2000. 6. 4	**Zuzujaki (tree 2)**, Shimo Agata County, Tsushima, Japan	≥15	1,435.4[c]
9	Wild mulberry	2000. 6. 4	**Zuzujaki (tree 3)**, Shimo Agata County, Tsushima, Japan	≥15	768.68[d]
18	Wild mulberry	2000. 6. 14	**Ara-dong**, Cheju City, Cheju Province	15.0	1,115.8[be]
19	Wild mulberry	2000. 6. 14	**Hanla Mt. (tree 1)**, Sungpanak, Cheju Cicy, Cheju Province	15.0	1.029.7[e]
20	Wild mulberry	2000. 6. 14	**Hanla Mt. (tree 2)**, Sungpanak, Cheju Cicy, Cheju Province	15.0	663.39[f]
21	Wild mulberry	2000. 6. 14	**Bongke-dong**, Cheju City, Cheju Province	15.0	1,031.5[e]
Mean					1,012.9

*Average temperature was obtained with monthly temperature from May 1999 to April 2000.
** Means with the same superscripts are not significantly different. Statistical significance was tested at the level of $p<0.05$.

also was much higher than that of cultivated ones, although the estimate was not statistically significant (Kim *et al.*, 2001).

However, because the wild samples were collected from the areas with highest mean annual temperature (≥ 15°C) among collection localities, our result is not clear whether the higher DNJ content was stemmed from "wildness" or high annual temperature. Thus, more detailed experiment on the temperature effect will be required.

In summary, although our data suggest that there exist DNJ content variation by regions and varieties, a long-term examination of the samples that showed higher DNJ content will be required for a decisive conclusion. In addition, a series of test with a few limited factors in a controlled facility will be useful to obtain the result whether temperature and/or particular environmental factors play a significant role for the variation of DNJ content. Finally, the high DNJ content in the wild mulberry leaves is noteworthy, so more extensive sampling will be required for the utilization of DNJ from wild mulberry.

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