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Physiological and psychological effects of olfactory stimulation with D-Limonene

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Key words: heart rate, heart rate variability, limonene, physiological relaxation, semantic differential method.

Abstract: Although D-Limonene can be considered an important component of nature-based stimuli, the physiological effects of olfactory stimulation with D-Limonene have not been completely clarified by scientific studies. The physiological and psychological effects of olfactory stimulation with D-Limonene were studied measuring heart rate variability (HRV), heart rate, and subjective evaluation using a modified semantic differential method; thirteen Japanese female university students (mean age±SD, 21.5±1.0 years) participated in the study. A concentration of 60 µL of D-Limonene was used as olfactory stimulant and room air as control. Subjects were exposed for 90 s while sitting with eyes closed. During D-Limonene inhalation: (1) the high-frequency (HF) value of HRV, a marker of parasympathetic nervous activity that is enhanced in relaxing situations, was significantly higher; (2) the heart rate was significantly lower; and (3) subjects reported feeling significantly more comfortable during D-Limonene administration than control. The results obtained clearly indicate that olfactory stimulation with D-Limonene induced physiological and psychological relaxation, providing important scientific evidence of the health benefits of D-Limonene.

1. Introduction

In the modern age, people are forced to lead busy lives and are exposed to a state of stress (Lederbogen *et al*., 2011). Thus, measures to prevent and relieve this stress state are urgently needed.

Recently, forest therapy has emerged as a method to address stress states, and much data on the physiological and psychological relaxing effects of forest environments have been accumulated. Previous studies have reported that viewing forest scenery or walking in forests can: increase parasympathetic nervous activity, which is enhanced in relaxing situations and suppresses sympathetic nervous activity which is increased in stress states (Tsunetsugu *et al*., 2007; Park *et al*., 2008; Lee *et al*., 2009; Park *et al*., 2009; Park *et al*., 2010; Lee *et al*., 2011; Park *et al*., 2012; Tsunetsugu *et al*., 2013; Lee *et al*., 2014); decrease cerebral blood flow in the prefrontal cortex (Park *et al*., 2007); and decrease salivary cortisol concentration of stress hormone (Tsunetsugu *et al*., 2007; Park *et al*., 2007; Park *et al*., 2008; Lee *et al*., 2009; Park *et al*., 2010). In addition, visiting a forest enhanced natural killer-cell activity and improved immune function (Li *et al*., 2007; Li *et al*., 2008 a, b, c) and the effect lasted 30 days (Li *et al*., 2008 b). In subjective evaluations, it was reported that people feel more "comfortable," "soothed," and "natural" when experiencing a forest environment (Park *et al*., 2007; Tsunetsugu *et al*., 2007; Park *et al*., 2008; Lee *et al*., 2009; Park *et al*., 2009; Lee *et al*., 2011; Park *et al*., 2011; Tsunetsugu *et al*., 2013; Lee *et al*., 2014), and that the "tension-anxiety," "depression," "anger-hostility," "fatigue," "confusion," and "vigor" of the mood state profile (McNair and Lorr, 1964; McNair *et al*., 1992; Yokoyama, 2005) improved (Li *et al*., 2008 a, b, c; Park *et al*., 2010; Lee *et al*., 2011; Park *et al*., 2011; Tsunetsugu *et al*., 2013; Lee *et al*., 2014). Unfortunately, many people living in cities find it difficult to access forest environments. Thus, much attention has been focused on nature-based stimuli, such as walking in an urban park (Song *et al*., 2013), viewing rooftop forests (Matsunaga *et al*., 2011), the presence of plants, including dracaena (Igarashi *et al*., 2014) or roses (Ikei *et al*., 2014), and physical contact with wood (Sakuragawa *et al*., 2008), and the relaxing effects of these stimuli have been reported.

Nature-based stimuli are intuitively perceived through the five senses. Of these five senses, the physiological effects of olfactory stimulation have been characterized

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in detail. Miyazaki *et al*. (1992) conducted a pioneering study which revealed that olfactory stimulation with *Chamaecyparis taiwanensis* essential oil significantly decreased blood pressure. Furthermore, inhalation of rose oil odor was shown to suppress sympathetic nervous activity and decrease adrenaline concentration (Haze *et al*., 2002). Lavender oil has been shown to induce deep sleep (Goel *et al*., 2005) and improve concentration (Sakamoto *et al*., 2005).

However, evidence-based research using the indices of autonomic nervous activity to clarify the effect of components of these essential oils is lacking.

The essential oil components of *Cryptomeria japonica* and *Pinus densiflora*, representative forest trees, have been reported (Cimanga *et al*., 2002; Hong *et al*., 2004; Cheng *et al*., 2009). These oils are composed of various volatile organic compounds, including D-Limonene, α-Pinene, β-Pinene. D-Limonene is the main component of citrus peel oil (Bernhard, 1960; Attaway *et al*., 1968; Shaw, 1979; Chiralts *et al*., 2002; Yoo *et al*., 2004).

The purpose of the present study was to investigate the physiological effect of olfactory stimulation with D-Limonene on autonomic nervous activity by measuring its effect on heart rate variability (HRV) (Camm *et al*., 1996; Kobayashi *et al*., 1999) and the heart rate.

2. Materials and Methods

Subjects

Thirteen Japanese female university students (age range, 21.5±1.0 years; mean±SD) participated in the study. Before beginning the experiment, a full explanation about the research aim, the experimental procedure, and all measured indices was provided. Informed consent was obtained from all subjects. This study was conducted in accordance with the regulations of the Ethics Committee of the Center for Environment, Health, and Field Sciences, Chiba University, Japan.

Study protocol

Physiological and psychological measurements were carried out in a chamber with an artificial climate maintained at 25°C with 50% relative humidity and 230-lux illumination. D-Limonene (>95.0% purity, Tokyo Chemical Industry Co., Ltd., Japan) was used as an olfactory stimulant, and room air was used as a control. A total of 60 μL D-Limonene was injected into a 24-L odor bag (polyethylene terephthalate film heat seal bag; NS-KOEN Co., Ltd., Kyoto, Japan) and the odors were presented to each subject by means of a device fitted on the chest and situated approximately 10 cm under the nose (Fig. 1). The flow rate of the odor was set at 3 L/min. Subjective sensitivity to the odor was determined in a preliminary investigation. The subjects were exposed to the odor for 90 s while sitting with their eyes closed. The order of presentation of D-Limonene and control was counterbalanced.

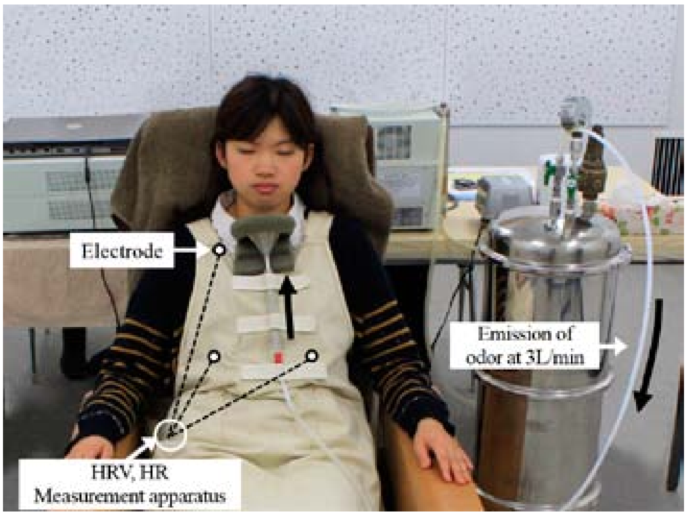

Fig. 1 - Olfactory stimulation setup.

Heart rate variability and heart rate

HRV was measured as the periods between consecutive R waves (R-R intervals) in an electrocardiogram recorded with a portable electrocardiograph (Activtracer AC-301A, GMS, Japan). In this study, two major spectral components of HRV, the low-frequency (LF; 0.04–0.15 Hz) band and the high-frequency (HF; 0.15-0.40 Hz) band were obtained by the maximum-entropy method (MemCalc/Win, GMS, Japan). The HF power was considered to reflect parasympathetic nervous activity, and the LF/HF power ratio was considered to reflect the sympathetic nervous activity (Camm *et al*., 1996; Kobayashi *et al*., 1999). Heart rate was also investigated using R-R interval data.

Semantic differential method

The subjects provided a subjective evaluation of the emotional impact of the odors according to a modified semantic differential (SD) method (Osgood *et al*., 1957). This method allowed the subject to assess a pair of adjectives, such as "comfortable-uncomfortable," using a 13-point scale. The SD method was performed after administration of each odor.

Statistical analysis

All statistical analyses were performed using Statistical Package for Social Sciences software version 20.0 (IBM Corp., Armonk, NY, USA). A paired t-test was used to compare differences in the physiological responses over the 90 s of exposure to D-Limonene and air. Wilcoxon signed-rank test was applied to analyze differences in psychological response between D-Limonene and air. A one-sided test was used in this study. In all cases, the significance level was set at $P < 0.05$.

3. Results

The results of the HRV data after exposure to D-Limonene and control were compared, and a significant difference was found in the HF value, which is a marker of parasympathetic nervous activity, as shown in Figure 2. The HF value increased 26.4% during D-Limonene administration (827.2±191.3 ms^2; mean±SE) compared with control (654.4 ±163.6 ms^2), indicating that parasympathetic nervous activity was significantly higher during D-Limonene administration (P<0.05). However, no significant difference was found in the LF/HF power ratio for the two stimuli.

Figure 3 shows a comparison of the heart rate during the administration of D-Limonene and control. Heart rate decreased during D-Limonene administration (72.8±2.3 bpm) compared with control (74.1±2.5 bpm), and this difference was significant (P<0.05).

Figure 4 shows the results for a "comfortable" feeling according to the subjective evaluation. Subjects reported significantly more comfortable ratings during D-Limonene administration than control (P<0.01).

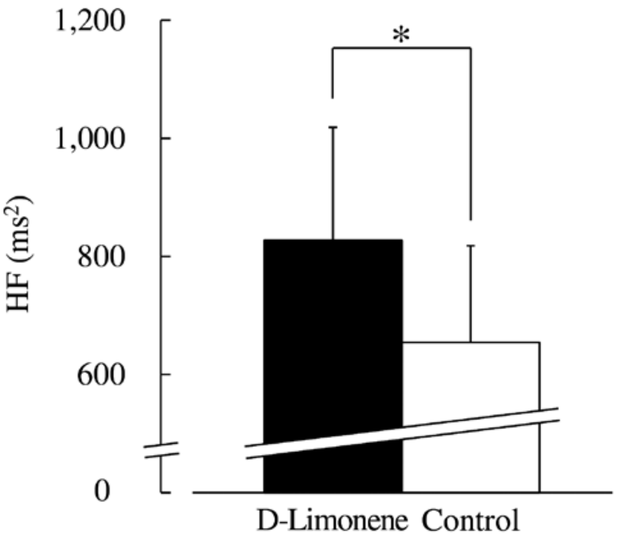

Fig. 2 - Comparison of high-frequency power levels of heart rate variability during olfactory stimulation with D-Limonene or control (air). Data are expressed as mean ± SE; n = 13. *P < 0.05 by paired t-test.

4. Discussion and Conclusions

D-Limonene is one of the most common volatile organic compounds in nature (Sun, 2007). It is a major component of various citrus oils, such as lemon, orange, grapefruit, and lime (Attaway *et al*., 1968; Bernhard, 1960; Chiralts *et al*., 2002; Shaw, 1979; Yoo *et al*., 2004), as well as essential oils from coniferous trees, such as *Pinus densiflora*, *Pinus koraiensis*, *Chamaecyparis obtusa*, and *Cryptomeria japonica* (Cimanga *et al*., 2002; Hong *et al*., 2004; Cheng *et al*., 2009). In addition, because of its citrus fragrance, D-Limonene is commonly added to perfumes, soaps, and cosmetics (Bakkali *et al*., 2008).

Although D-Limonene is an important component of nature-based stimuli, the physiological effect of olfactory stimulation with D-Limonene has not been completely clarified. Previously, Tsunetsugu *et al*. (2012) investigated the physiological effect of olfactory simulation with D-Limonene on blood pressure and showed that olfactory simulation with a concentration of 10 μL D-Limonene decreases subjects' systolic blood pressure. However, to our knowledge, no previous study has examined the physiological effect of olfactory stimulation with D-Limonene on HRV and heart rate.

The present study shows that olfactory stimulation with D-Limonene induced (1) a significant increase in parasympathetic nervous activities, (2) a significant decrease in the

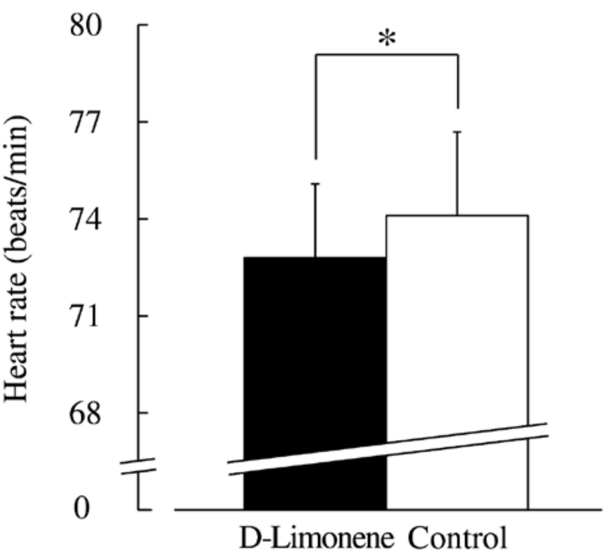

Fig. 3 - Comparison of the heart rate during olfactory stimulation with D-Limonene or control (air). Data are expressed as mean ± SE; n = 13. *P < 0.05 by paired t-test.

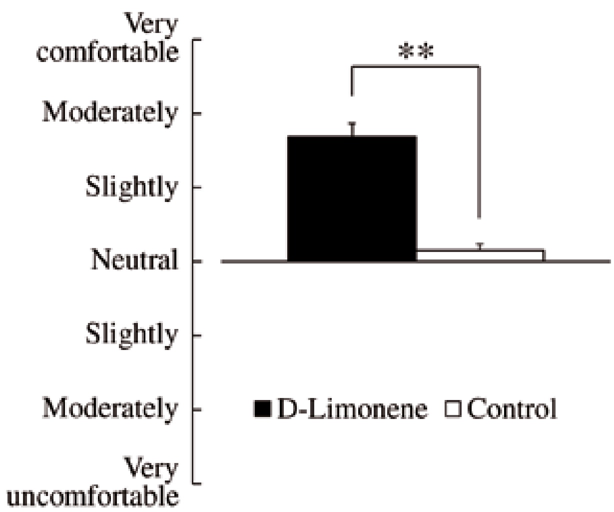

Fig. 4 - Subjective evaluation of "comfortable" measured by a modified semantic differential questionnaire after olfactory stimulation with D-Limonene or control (air). Data are expressed as mean ± SE; n = 13. **P < 0.01 by Wilcoxon signed-rank test.

heart rate, and (3) a significant increase in a "comfortable" feeling. These results agree with previous studies of other nature-based stimuli (Tsunetsugu *et al*., 2007; Park *et al*., 2008; Park *et al*., 2009; Park *et al*., 2010; Lee *et al*., 2011; Park *et al*., 2012; Song *et al*., 2013; Tsunetsugu *et al*., 2013; Ikei *et al*., 2014, Lee *et al*., 2014). Park *et al*. (2012) showed that the HF value of HRV was significantly increased while viewing scenery of forests using the results of field experiments at 35 forests in Japan. Ikei *et al*. (2014) reported that the HF component was significantly increased by viewing roses. Song *et al*. (2013) revealed that parasympathetic nervous activity was enhanced and the heart rate was significantly lower after walking in an urban park than walking in a city area. Our results support the hypothesis that olfactory stimulation with D-Limonene has a relaxation effect that is similar to other nature-based stimuli.

In conclusion, our results clearly indicate that olfactory simulation with D-Limonene induced physiological and psychological relaxation. And these finding provide important scientific evidence on the health benefits of D-Limonene exposure.

As all the participants in this study were healthy females in their twenties, further studies are needed to ascertain the effect in diverse groups, including males and different age groups. In addition, it is necessary to examine the effect using multiple indices, such as prefrontal cortex activity, stress hormones, and others.

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Characterization of chloroplast *matK* sequences of *Citrus tachibana* and *Citrus depressa*, two indigenous species in Japan

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Key words: cpDNA, genetic resources, Ryukyu islands, shiikuwasha, tachibana.

Abstract: *Citrus tachibana*, *C. nippokoreana*, and *C. depressa* are indigenous mandarin species in Japan. We deduced their phylogenetic relationships from nucleotide sequences of the chloroplast *matK* gene. The results indicate that *C. tachibana*, *C. nippokoreana*, and *C. depressa* accessions can be classified into two types: type A, all sixteen *C. tachibana* and six *C. depressa*; type B, eleven *C. depressa* and one *C. nippokoreana*. Both type A and type B accessions of *C. depressa* were found on the Okinawa Islands, whereas only type B accessions of *C. depressa* were found on the Sakishima and Amami Islands. This cpDNA divergence seemed to indicate a polyphyletic origin of *C. depressa*. The *matK* genes of type A were found only in *C. tachibana* and some *C. depressa*. From these results, both species probably possess a characteristic chloroplast genome among various *Citrus* species.

1. Introduction

Citrus is one of the most important fruit crops in Japan and also worldwide. Various accessions of *Citrus* species are adapted to the southwest of Japan, and although they are cultivated in this region, almost all of them are non-native, that is, they were introduced from abroad, arose as chance seedlings, were selected from bud sports, and were bred by artificial pollination. Only two species, *Citrus tachibana* (Makino) Tanaka (Tachibana) and *Citrus depressa* Hayata (Shiikuwasha) were present in Japan before recorded history.

C. tachibana mainly grows indigenously on the Pacific side of the southwest of Japan's main islands (Kyushu, Shikoku, and Honshu). *C. tachibana* was recorded in "Kojiki", the oldest chronicle in Japan dating from the early 8th century. Its indigenous trees were also found on the Ryukyu Islands (islands including the Okinawa Islands, Sakishima Islands, and Amami Islands, which were ruled by Japan from the 17th to19th centuries) and Taiwan (Tanaka, 1931; Lin and Chen, 2006; Inafuku-Teramoto *et al.*, 2010). *C. depressa* is indigenous to both the Ryukyu Islands and Taiwan (Tanaka, 1936; Lin and Chen, 2006). Compared to *C. tachibana*, *C. depressa* is considered to be adapted to a warmer climate; the former is usually used as an ornamental for gardens and its fruit is inedible. On the other hand, fruit of *C. depressa* is in much demand as an ingredient for food and drinks, to garnish dishes similar to a lemon or lime, to make juice and jam, and as an additive to soy sauce and distilled spirits. Recently, this fruit has attracted attention because it contains high levels of polymethoxyflavonoids, one of the most important health-promoting components of citrus (Inafuku-Teramoto *et al.*, 2010).

We have investigated the phylogenetic relationships of *Citrus* and its relatives through the analysis of genes encoded in chloroplast DNA (cpDNA) (Tshering *et al.*, 2010, 2013). In our recent study (Tshering *et al.*, 2013) in which various *Citrus* accessions were used as materials, we found that *C. tachibana* and *C. depressa* possess a characteristic cpDNA genome based on the sequences of the chloroplast *matK* genes, which encode a maturase involved in splicing type II introns from RNA transcripts (Hilu and Liang, 1997; Hilu *et al.*, 2003; Olmstead and Palmer, 1994). There are many accessions in both species, and intraspecies diversity is found within each species (Hirai *et al.*, 1990; Yamamoto *et al.*, 1998; Kinjo, 2007; Inafuku-Teramoto *et al.*, 2010; Yamamoto *et al.*, 2011).

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However, a limited number of accessions were used in our previous study (Tshering *et al*., 2013).

Therefore, for the present work, we analyzed the *matK* gene sequences of a number of *C. tachibana* and *C. depressa* plants grown in various regions in Japan to reveal their characteristic profiles of the cpDNA genome. *C. nippokoreana* (Korai Tachibana), a *C. tachibana* relative indigenous to Hagi City, Yamaguchi Prefecture, Japan, and Cheju Island, Korea (Kimura and Taninaka, 1995), was also investigated.

2. Materials and Methods

Plant materials

Sixteen *C. tachibana*, one *C. nippokoreana*, 17 *C. depressa*, and 13 control accessions were used in this study. The sources of the materials are shown in Table 1 and Figure 1.

PCR amplification and DNA sequencing

Genomic DNA was extracted from leaves using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). By using this genomic DNA as a template, the *matK* gene was amplified by PCR using proofreading PrimeSTAR GXL DNA Polymerase (TAKARA BIO, Ohtsu, Shiga, Japan). The primers used for PCR amplification of the *matK* gene were matK1F (5′-ACCGTATCGCACTATGTATC-3′) and matK1R (5′-GAACTAGTCGGATGGAGTAG-3′). The amplified DNA fragments were purified using the NucleoSpin Gel and PCR Clean-up Kit (MACHEREY-NAGEL, Düren, Germany). The primers used for sequencing of the *matK* gene were matK1F, matK2F (5′-ACGGTTCTTTCTCCACGAGT-3′), matK3F (5′-GGTCCGATTTCTCTGATTCT-3′), matK1R, matK2R (5′-AGAATCAGAGAAATCGGACC-3′), and matK3R (5′-ACTCGTGGAGAAAGAACCGT-3′). The purified DNA fragments were sequenced in both directions in an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems) with a BigDye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems) as described previously (Platt *et al*., 2007). Sequence data were submitted to DDBJ/GenBank/EBI and were assigned accession numbers ranging from AB839905 to AB839932. The sequences of the accessions from No. 29 to No. 34 were deposited in our previous study (Tshering *et al*., 2013).

Fig. 1 - Collection sites of *Citrus tachibana*, *C. nippokoreana*, and *C. depressa* in the present study.

Phylogenetic analyses

The neighbor-joining (NJ) and maximum likelihood (ML) methods from the MEGA (version 5.2.1) program (Tamura *et al*., 2011) were used to create phylogenetic trees. The reliability of each branch was tested by bootstrap analysis with 1,000 replications.

3. Results and Discussion

We constructed multiple sequence alignments of 1,630-bp fragments containing the *matK* gene from different *Citrus* accessions. Each sequence contained a 1,530-bp protein-coding sequence and 100 bp of the 3′ UTR. One exception is the *matK* gene of trifoliate orange (*Poncirus trifoliata*), which has a 6 bp insertion at the 3′ UTR. Of these, 23 bases were variable and six bases were phylogenetically informative.

We created phylogenetic trees using the NJ and ML methods. The topologies of the different trees were identical (data not shown). Therefore, we present here only the ML tree (Fig. 2). *C. tachibana*, *C. depressa*, and *C. nippokoreana* accessions were classified into two types as follows:

Type A: all 16 *C. tachibana* and six *C. depressa* [Shiikuwasha-Okinawa#1 (No. 17), Shiikuwasha-Okinawa#3 (No. 19), Shiikuwasha-Okinawa#6 (No. 22), and Shiikuwasha-Oku (No. 26), Kabishi (No. 29), and Fusubuta (No. 31)].

Type B: Eleven *C. depressa* [Shiikuwasha-Taketomi (Nohara) (No. 11), Shiikuwasha-Taketomi (Takana) (No. 12), Shiikuwasha-Iriomote (No. 13), Shiikuwasha-Iriomote (Katoura) (No. 14), Shiikuwasha-Kohama (Ufudake) (No. 15), Shiikuwasha-Kohama (Omori) (No. 16), Ishikunibu (No. 28), Mikanguwa (No. 30), Kaachi (No. 32), Shiikunin (No. 33), and Shiikurubu (No. 34)] and one *C. nippokoreana*.

None of the control accessions belonged to type A, whereas all seven control mandarin accessions belonged to type B. The other control accessions were clearly distinguished from type A and type B. This finding is consistent with the results of our previous study (Tshering *et al*., 2013).

All 16 *C. tachibana* accessions carried an identical *matK* sequence. Previous studies (Hirai *et al.*, 1990; Yamamoto and Tominaga, 2003) reported that *C. tachibana* was genetically differentiated from *Citrus* species originating from all

Table 1 - *Citrus tachibana*, *C. nipponkoreana*, and *C. depressa* accessions used in the present study

No.	Accession	Latin name	Origin	Note
1	Tachibana-Dazaifu (uchi)	*Citrus tachibana* (Makino) Tanaka	Fukuoka, Kyushu	Planted tree
2	Tachibana-Dazaifu (soto)	*C. tachibana* (Makino) Tanaka	Fukuoka, Kyushu	Planted tree
3	Tachibana-Heian Jingu	*C. tachibana* (Makino) Tanaka	Kyoto, Honshu	Planted tree
4	Tachibana-Iwashimizu Hachimangu	*C. tachibana* (Makino) Tanaka	Kyoto, Honshu	Planted tree
5	Tachibana-Kitano Tenmangu	*C. tachibana* (Makino) Tanaka	Kyoto, Honshu	Planted tree
6	Tachibana-Toshijima (Mie)	*C. tachibana* (Makino) Tanaka	Mie, Honshu	Native tree
7	Tachibana-Matsuoyama (Kochi)	*C. tachibana* (Makino) Tanaka	Kochi, Shikoku	Native tree
8	Tachibana-Nangoku (Kochi)	*C. tachibana* (Makino) Tanaka	Kochi, Shikoku	Planted tree
9	Korai Tachibana	*C. nippokoreana* Tanaka	Kochi, Shikoku	Planted tree
10	Tachibana-Ishigakijima	*C. tachibana* (Makino) Tanaka	Ishigaki-jima, Sakishima	Native tree
11	Shiikuwasha-Taketomi (Nohara)	*C. depressa* Hayata	Taketomi-jima, Sakishima	Native tree
12	Shiikuwasha-Taketomi (Takana)	*C. depressa* Hayata	Taketomi-jima, Sakishima	Native tree
13	Shiikuwasha-Iriomote	*C. depressa* Hayata	Iriomote-jima, Sakishima	Native tree
14	Shiikuwasha-Iriomote (Katoura)	*C. depressa* Hayata	Iriomote-jima, Sakishima	Native tree
15	Shiikuwasha-Kohama (Ufudake)	*C. depressa* Hayata	Kohama-jima, Sakishima	Native tree
16	Shiikuwasha-Kohama (Omori)	*C. depressa* Hayata	Kohama-jima, Sakishima	Native tree
17	Shiikuwasha-Okinawa#1	*C. depressa* Hayata	Okinawa-honto	Native tree
18	Tanibuta-Okinawa#2	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
19	Shiikuwasha-Okinawa#3	*C. depressa* Hayata	Okinawa-honto	Native tree
20	Tanibuta-Okinawa#4	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
21	Tanibuta-Okinawa#5	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
22	Shiikuwasha-Okinawa#6	*C. depressa* Hayata	Okinawa-honto	Native tree
23	Tanibuta-Okinawa#7	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
24	Tanibuta-Okinawa#8	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
25	Garagara	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
26	Shiikuwasha-Oku	*C. depressa* Hayata	Okinawa-honto	Native tree
27	Tanibuta	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
28	Ishikunibu	*C. depressa* Hayata	Okinawa-honto	Native tree
29	Kabishi	*C. depressa* Hayata	Okinawa-honto	Native tree
30	Mikanguwa	*C. depressa* Hayata	Okinawa-honto	Native tree
31	Fusubuta	*C. depressa* Hayata	Okinawa-honto	Native tree
32	Kaachi	*C. depressa* Hayata	Okinawa-honto	Native tree
33	Shiikunin	*C. depressa* Hayata	Tokuno-shima, Amami	Native tree
34	Shiikuribu	*C. depressa* Hayata	Okinoerabu-jima, Amami	Native tree
Control accessions				
	Satsuma mandarin 'Aoshima'	*C. unshiu* Marcow.		
	Ponkan 'Yoshida Ponkan'	*C. reticulata* Blanco		
	Mediterranean mandarin	*C. deliciosa* Ten.		
	Dancy	*C. tangerina* hort. ex Tanaka		
	Kinokuni 'Hirakishu'	*C. kinokuni* hort. ex Tanaka		
	Sunki	*C. sunki* (Hayata) hort. ex Tanaka		
	Cleopatra	*C. reshni* hort. ex Tanaka		
	Yuzu 'Yamane'	*C. junos* Siebold ex Tanaka		
	Sweet orange 'Fukuhara'	*C. sinensis* (L.) Osbeck		
	Lemon 'Eureka'	*C. limon* (L.) Burm. f.		
	Pummelo 'Mato Buntan'	*C. maxima* (Burm.) Merr.		
	Citron 'Maru Busshukan'	*C. medica* L.		
	Trifoliate orange 'Standard'	*Poncirus trifoliata* (L.) Raf.		

other countries except Japan. The present study also confirmed that the *matK* sequence of *C. tachibana* was not identical to those of studied accessions originating from all other countries except Japan. However, we found that the *matK* sequence of *C. tachibana* was identical to those of some investigated *C. depressa* accessions that are indigenous to the Ryukyu Islands, Japan. This suggests that *C. tachibana* has been isolated from the mandarins elsewhere, and evolved in Japan in unique ways. We found no diversity within species. However, further study considering more accessions is needed since the materials used here did not cover the entire area where *C. tachibana* grows. The *matK* sequence of *C. nippokoreana* was not identical to that of *C. tachibana*, indicting genetic differentiation between the two species. Because it is considered that *C. nippokoreana* is related to *C. tachibana* (the Japanese name "Korai Tachibana" means "Tachibana from Korea"), this finding is interesting.

C. depressa accessions were divided into two types according to *matK* sequences. One was the same type as *C. tachibana* and the other was the same type as several mandarins such as *C. reticulata* and *C. sunki*. This result completely agrees with the results of our previous study (Tshering *et al*., 2013). Differentiation of the cpDNA genome in *C. depressa* was also reported by Urasaki *et al*. (2005) and Yamamoto *et al*. (2013), who analyzed the *trnL-trnF* and *trnF-trnVr* regions, respectively. These results strongly suggest a polyphyletic origin of *C. depressa*. This divergence of *matK* genes was found in *C. depressa* accessions grown on Okinawa-honto (the main island of Okinawa Islands) but not in those grown on the Sakishima and Amami Islands. *C. depressa* possessing *C. tachibana*-type cpDNA (A type) was found only on Okinawa-honto. Similar results were reported by Yamamoto *et al*. (2013) who studied *C. depressa* on Okinawa-honto and the Amami Islands. However, Urasaki *et al.* (2005) found that *C. depressa* accessions possessed *C. tachibana*-type cpDNA (*trnL-trnF* sequence) on the Sakishima Islands. Thus, further study using many *C. depressa* accessions grown on various islands is necessary to resolve the distribution of each type.

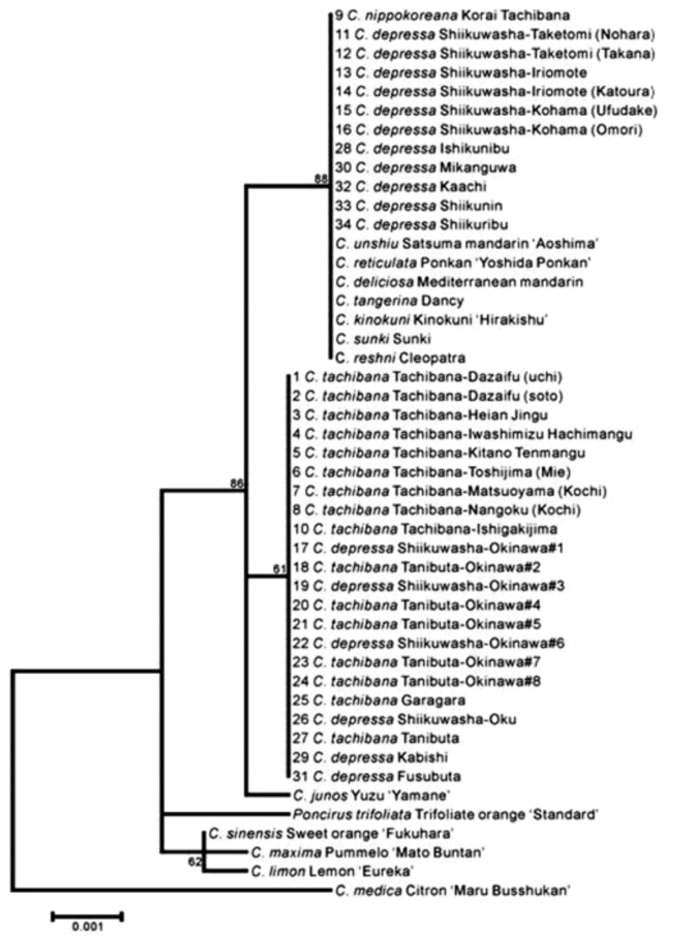

Fig. 2 - Maximum likelihood tree of the *matK* genes from *Citrus tachibana*, *C. nippokoreana*, and *C. depressa* and their control accessions. Numbers at the nodes indicate bootstrap values (% over 1000 replicates). The scale bar shows the number of substitutions per site.

There is a possibility that type A *C. depressa* is genetically closer to *C. tachibana* than type B. However, this hypothesis is not supported since the proportion of common bands from random amplified polymorphic DNA (RAPD) analysis between *C. depressa* of type A and *C. tachibana* was not so different from that of type B and *C. tachibana* (Yamamoto *et al*., 1998). Since the origin and/or relationship of *C. depressa* to *C. tachibana* cannot be elucidated only by cpDNA analysis, cpDNA analysis combined with nuclear genome analysis such as simple sequence repeat (SSR), sequence-related amplified polymorphism markers (SRAPs) (Barkley *et al*., 2006; Uzun *et al*., 2009), and restriction site-associated DNA sequences (RAD-seq) (Baird *et al*., 2008) is considered to be necessary. For this purpose, structural analysis (Barkley *et al*., 2006) seems to be informative.

The present work demonstrates the characteristic profiles of the chloroplast genome of *Citrus tachibana* and *Citrus depressa*, two indigenous species in Japan, using a number of accessions grown in various regions based on the results of *matK* sequencing. Furthermore, the divergence of the cpDNA genome of *C. depressa* seems to indicate a polyphyletic origin of this species. These findings are a contribution to progress in the study of the genetic resources in *Citrus* and related genera.

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Potential marker proteins for ozone-induced yield reduction in rice

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Key words: grain yield, ozone stress, protein markers, 60-kDa chaperonin.

Abstract: Three proteins - a 60-kDa chaperonin (CPN-60), chloroplastic ATP synthase, and enolase 1 - were evaluated as potential markers of ozone-induced yield responses in six rice (*Oryza sativa* L.) cultivars ('Kirara 397', 'Koshihikari', 'Nipponbare', 'Takanari', 'Kasalath', 'Suphanburi 90') under ozone stress in laboratory-scale tests. The levels of all three proteins decreased after ozone exposure in cultivars identified as ozone-sensitive while they increased or remained constant after ozone exposure in tolerant cultivars, although ATP synthase tended to decrease. Furthermore, the protein level and grain yield in each cultivar exposed to ozone were significantly positively correlated for all three proteins. Thus, CPN-60 and enolase 1 are potential markers for chronic ozone stress in rice.

1. Introduction

Ozone is a major gaseous pollutant in the troposphere and ozone concentrations have in recent years increased rapidly in developing Asian countries. Indeed, the emission of anthropogenic nitrogen oxides (ozone precursors) in Asia under a no-further-control scenario was predicted to increase by 350% between 1990 and 2020 (Aunan *et al*., 2000).

An elevated ozone concentration will reduce the growth and yield of crop plants including rice, the most important food crop in Asia (Kobayashi *et al*., 1995; Yonekura *et al*., 2005). Many researchers have described the mechanisms responsible for visible injury on plant leaves by acute ozone exposure (reviewed by Kangasjarvi *et al*., 2005). The primary mechanism is oxidative damage caused by an increase in levels of reactive oxygen species (ROS). However, the cause for yield reductions under chronic ozone stress remains unclear. In a previous report we described how ozone sensitivity in evaluated rice cultivars, in terms of visible injury (chlorotic or necrotic lesions), did not coincide with that indicated by the grain yield reduction (Sawada and Kohno, 2009). In addition, conventional evaluation of chronic ozone effects relies on measurements such as growth and yield reductions, which require large-scale studies (e.g. in a field or greenhouse) and long time periods (e.g. about six months). A rapid and small-scale method for early evaluation of chronic ozone effects, such as the use of molecular markers, would make it faster, easier, and less expensive to select ozone-tolerant cultivars.

Kubo *et al*. (2011) reported that during ozone stress, sakuranetin, a flavonone in the phytoalexin family, appears to serve as a molecular marker of the stress response. Sakuranetin contents in rice leaves exposed simultaneously to ozone and high temperature increased only in the three cultivars whose grain yield was unaffected by ozone stress. However, their experiment was performed under both elevated ozone and elevated temperature, making it difficult to determine the separate effect of each factor. Moreover, the ozone concentration was 150 nl l^{-1} (ppb), much higher than ambient ozone levels. Therefore, more practical markers are needed.

Proteomic studies are useful to reveal protein markers associated with various stress tolerance (reviewed by Kosova *et al*., 2011). In a previous study, we conducted differential proteome analysis using three rice cultivars that showed different levels of ozone sensitivity (indicated by the reduction in grain yield) when exposed to elevated ozone during the cultivation season in open-top chambers (Sawada *et al*., 2012). In these cultivars, we observed significant changes in the size of spot that contained three proteins: a 60-kDa chaperonin (CPN-60), chloroplastic ATP synthase, and enolase 1. The change in size of this spot was proportional to their ozone sensitivity, measured as the reduction in grain yield. These results suggest that these proteins are closely involved in the mechanisms that underlie the yield reduction

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that occurs under elevated ozone levels, and therefore they have potential as molecular markers that can predict the ozone-induced yield loss. To clarify the usefulness of these proteins for use in laboratory-scale tests, we investigated the levels of these candidate proteins in the seedlings of six rice cultivars under short-term ozone exposure in growth chambers, and tested for a significant correlation between the protein levels and relative grain yield.

2. Materials and Methods

Chronic ozone exposure

Six rice (*Oryza sativa* L.) cultivars were used in this study: 'Kirara 397', 'Koshihikari', 'Nipponbare' (*japonica* cultivars), 'Takanari' (a hybrid *indica* cultivar), 'Kasalath', 'Suphanburi 90' (*indica* cultivars). Seedlings (n = 40) of each cultivar were grown in seedling boxes for three weeks in a glasshouse under ambient atmospheric conditions, then transplanted into pots (at four plants per pot with a 0.05-m^2 surface area and a 0.015-m^3 volume) in open-top chambers (OTCs; 3.6 × 3.6 m) at an experimental field of the Akagi Testing Center of the CRIEPI (Maebashi, Japan) in the late spring of 2007, 2008, and 2009. Fertilizer was supplied at a rate of N-P_2O_5-K_2O=15-15-15 g m^{-2}. The OTC fumigation system has been described previously (Frei *et al*., 2011). Ozone was added in the chambers using a mass-flow controller combined with a PID controlling system to maintain the designated concentrations. Three ozone-level treatments were established, from transplanting of rice plants into the pots to harvest, for three years with a regular diurnal pattern: charcoal-filtered air (CF), ambient ozone (Ozone ×1), and twice ambient ozone (Ozone×2). Concentrations of ozone were continuously monitored in each chamber at 3-min intervals using a UV absorption ozone analyzer (ML9810, Monitor Labs, Englewood, CO, USA). Mean ozone concentration, air temperature and relative humidity in the different treatments are summarized in Table 1.

Measurement of the yield

The rice cultivars were harvested between September and November in 2007, 2008, and 2009. Harvesting of each cultivar was conducted when about 80% of the grains had turned yellow. After harvesting, grains were separated from the panicles and categorized into two groups (filled and unfilled grains) using an automatic seed-sorting machine (FV-459A, Fujiwara Seisakusho KK, Tokyo, Japan). The filled grains (rough rice) were weighed to determine the grain yield.

Short-term ozone exposure

Rice seedlings were grown in indoor growth chambers at 28/23°C (day/night), photosynthetic photon-flux density of 400 µmol m^{-2} s^{-1}, with a 12-h photoperiod, and a relative humidity of 60±5%. After two weeks, the 'Kirara 397', 'Koshihikari', and 'Takanari' seedlings were exposed to three levels of ozone (12 h/day) for three days in three individual replicates: CF, ambient ozone (40 ppb), and twice ambient ozone (80 ppb). Similarly, 'Nipponbare', 'Kasalath', and 'Suphanburi 90' seedlings were exposed to CF and 40 ppb of ozone. Ozone was generated with a silent electrical discharge in dry oxygen. The concentration of ozone in the chambers was monitored continuously during exposure with a UV absorption ozone detector (Model 1150, Dylec Inc., Tokyo, Japan). At the end of the exposure, we removed the third leaves, immediately froze them in liquid nitrogen, and stored them in -80°C until the immunoblot analysis was performed.

Immunoblot analysis

Leaves (100 mg) were homogenized in sodium dodecyl sulfate (SDS) buffer (10% (w/v) glycerol, 5% (v/v) β-mercaptoethanol, 2.3% (w/v) SDS, and 62.5 mM Tris-HCl, pH 6.8). Equal amounts of protein samples were separated using 15% SDS-polyacrylamide gel electrophoresis (PAGE). After the SDS-PAGE, the protein samples were transferred onto a polyvinylidene fluoride membrane or they were stained by Coomassie brilliant blue (CBB).

Table 1 - Ozone concentrations and environmental conditions in the open-top chambers during the cropping seasons of rice

		Ozone concentration (ppb)			Temperature (ºC)	Relative humidity (%)
		12 h mean	24 h mean	Mean daily Maximum	24 h mean	24 h mean
2007	CF	3.1	2.1	4.1	–	–
	Ozone x1	37.6	31.1	61.3	–	–
	Ozone x2	68.6	56.3	101.7	–	–
2008	CF	4.7	3.9	6.5	21.1	83.4
	Ozone x1	40.4	27.5	57.6	21.3	83.5
	Ozone x2	82.7	57.0	118.3	21.3	81.7
2009	CF	5.1	5.0	9.7	20.6	78.7
	Ozone x1	35.1	27.9	56.9	20.7	78.9
	Ozone x2	73.5	54.7	110.2	20.9	77.4

Measurements of environmental conditions and ozone concentrations were recorded at 3 and 10-minute interval throughout the experiment, respectively. Average values of the two replicate chambers per treatment are shown. 12 h means were calculated for the period from 6:00 to 17:59 hours. The temperature and relative humidity were not measured in 2007.

The blotted membrane was blocked for 1 h in TBS-T (20 mM Tris-HCl, pH 7.6, 150 mM NaCl and 0.1% v/v Tween-20) containing 5% (w/v) nonfat milk (Skim milk; Difco, Sparks, MD, USA). The membrane was subsequently incubated with the monoclonal antibody anti-heat-shock protein 60 (Acris Antibodies GmbH, Herford, Germany), with the polyclonal antibodies anti-ATP synthase β-subunit (AntiProt, Pullach i. Isartal, Germany), and anti-enolase (Aviva system biology, San Diego, CA, USA) at 1:5000 dilutions for 1 h at room temperature. As secondary antibodies, we used anti-mouse or anti-rabbit IgG with conjugated HRP (Bio-Rad Laboratories Inc., Hercules, CA, USA). After incubation for 1 h with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies, we detected the immunoblot signals using the ECL plus western blotting detection kit (GE Healthcare, Piscataway, NJ, USA) following the manufacturer's protocols and the results were visualized using an LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan). The relative intensities of the bands were calculated using PD-Quest software (version 8.0.1, Bio-Rad).

3. Results and Discussion

After chronic ozone exposure during three years of growing seasons (from 2007 to 2009) each cultivar showed a similar yield response to ozone in all years of the experiment. The grain yields of 'Kirara 397', 'Takanari' and 'Kasalath' decreased significantly by 15 to 36%, 10 to 21%, and 12 to 19%, respectively, under twice the ambient ozone level (about 80 ppb treatment, daily 12-h mean concentration), although the grain yields did not differ significantly from CF under ambient ozone level (about 40 ppb treatment), except for 'Kirara 397' and 'Takanari' in 2007 (Fig. 1, P <0.05). The grain yields of 'Koshihikari', 'Nipponbare', and 'Suphanburi 90' did not decrease significantly with ozone stress. On this basis, we defined 'Kirara 397', 'Takanari', and 'Kasalath' as ozone-sensitive cultivars, and 'Koshihikari', 'Nipponbare', and 'Suphanburi 90' as ozone-tolerant cultivars.

To confirm whether CPN-60, ATP synthase, and enolase 1 can be used as markers for ozone-induced rice yield loss in laboratory-scale tests, we analyzed the levels of these proteins (Fig. 2A). Levels of CPN-60 decreased significantly after three days of exposure to 40 (the ambient concentration) and 80 ppb (twice the ambient concentration) of ozone in 'Kirara 397' and 80 ppb of ozone in 'Takanari' (Fig. 2B, P<0.05). Levels of ATP synthase and enolase 1 tended to decrease after ozone exposure, although not significantly (except for 'Takanari' exposed to 80 ppb of ozone), in both 'Kirara 397' and 'Takanari'. These cultivars also showed lower grain yield under ozone exposure (Fig. 1). In contrast, levels of CPN-60 and enolase 1 in 'Koshihikari' exposed to 40 ppb of ozone increased significantly and remained the same compared with the levels in CF (P <0.05). Moreover, enolase 1 production also remained constant in 'Koshihikari' at 80 ppb ozone exposure. The level of ATP synthase tended to decrease after ozone exposure in 'Koshihikari'. Because the levels of CPN-60 and enolase 1 decreased and increased at 40 ppb ozone exposure in ozone-sensitive and ozone-tolerant cultivars, respectively, 'Kasalath', 'Nipponbare', and 'Suphanburi 90' seedlings were exposed to CF and

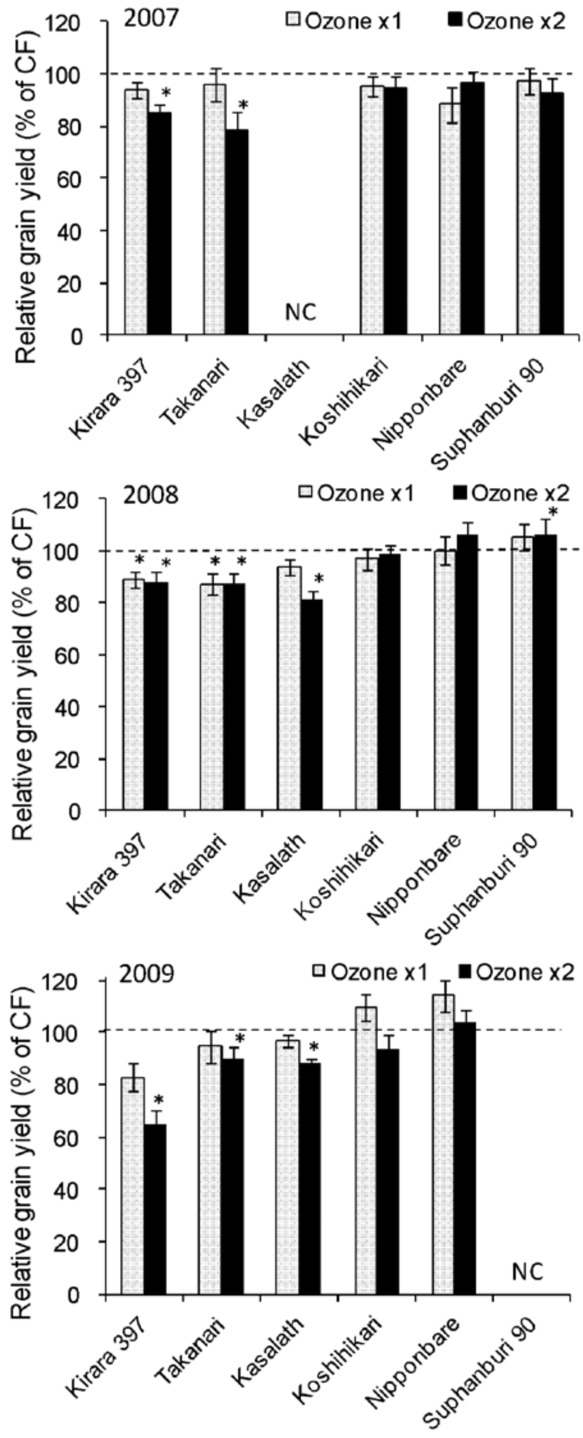
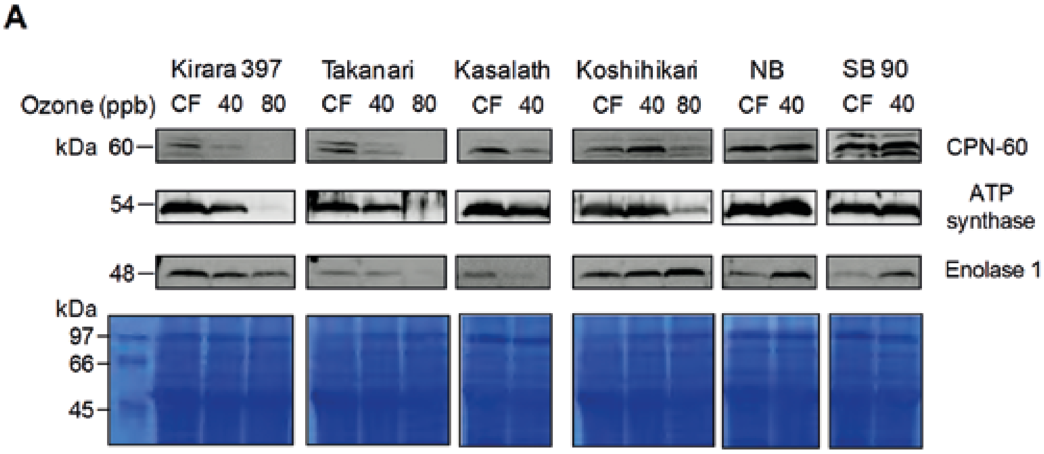


Fig. 1 - Effects of chronic ozone exposure on the grain yields of six rice cultivars in 2007, 2008, and 2009. Values are mean ± SE (n= 40). Asterisk indicates a significant difference compared with CF according to Dunnett's test (P<0.05). 'Kasalath' and 'Suphanburi 90' were not cultivated in 2007 and 2009, respectively, and yields are shown as "NC".



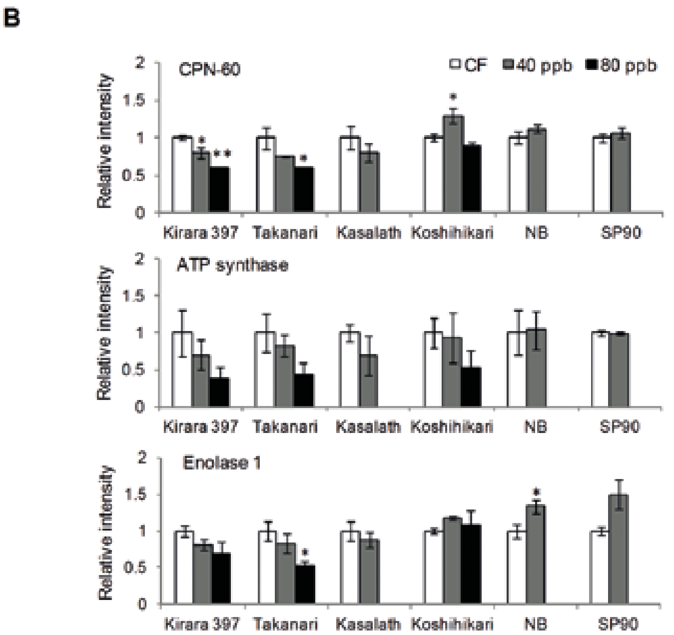


Fig. 2 - (A) Immunoblot analysis of CPN-60, ATP synthase, and enolase 1 in leaves of rice seedlings exposed to charcoal filtered air (CF), or to 40 or 80 ppb of ozone. Immunodetection was performed with antibodies specific to these proteins. (bottom panel) CBB-stained SDS-PAGE showing the quality and loading quantity of the protein samples. (B) Relative intensity for each protein estimated from the immunoblot analysis in panel (A). Values are mean ± SE (n = 3). Asterisk indicates a significant difference compared with CF according to Dunnett's test or *t*-test ($P<0.05$). NB, Nipponbare; SP90, Suphanburi 90.

40 ppb of ozone. The levels of all three proteins increased significantly or were maintained under 40 ppb of ozone in 'Nipponbare' and 'Suphanburi 90', but tended to decrease in 'Kasalath'. Therefore, the levels of CPN-60 and enolase 1 differed between the ozone-sensitive and ozone-tolerant cultivars: they decreased and increased, respectively at least at 40 ppb.

CPN-60 is a molecular chaperone. Many molecular chaperones were originally identified as heat-shock proteins (HSPs), which function in protein folding, assembly, translocation, and degradation during many normal cellular processes, and can assist in protein refolding under stress (Wang *et al*., 2004). CPN-60 (HSP60) appears to be involved in the defense response that mitigates oxidative stresses (Wang *et al*., 2011). Enolase 1 is an enzyme involved in glycolysis in the cytosol. Bohler *et al*. (2007) suggested that the enzymes involved in glycolysis increase to produce more energy and to increase the reduction capacity for detoxification of ROS and repair oxidative damage in response to ozone stress in the leaves of poplar (*Populus*). In *Arabidopsis thaliana*, *CPN60B* (At1g55490), encoding homologous protein to CPN-60 in rice, was upregulated in response to drought, UV-B, heat, wounding and oxidative (Methyl viologen) stress within 30 min (Winter *et al*., 2007). Similarly, *ENO2* (At2G36530) in *A. thaliana*, encoding homologous protein to enolase 1 in rice, was upregulated in response to cold, drought, UV-B, wounding and heat stress (Winter *et al*., 2007). These studies suggest that CPN-60 and enolase 1 are induced by stresses involved in the production of ROS. Therefore, the alterations of these protein levels with ozone exposure might result in ozone-derived ROS rather than ozone itself. However, there has been no report describing whether CPN-60 and enolase 1 influence grain production in crops under environmental stress, although these proteins might not be specific markers to ozone. Further studies will be needed to clarify the relationship between the reduction in grain yield and decreased production of these proteins by ozone-sensitive cultivars.

In order to compare the relative levels of each protein upon short-term ozone exposure with the relative grain yields under chronic ozone exposure we performed a linear regression analysis (Fig. 3). We found significant positive correlations between the levels of CPN-60, ATP synthase, and enolase 1 and the relative grain yield (i.e. yield decreased as the protein concentrations decrease). Therefore, the three proteins may serve as potential markers for chronic ozone stress in rice, although further experiments will be required for ATP synthase that also tended to decrease in 'Koshihikari' at 40 ppb ozone exposure (Fig. 2B). The level of CPN-60 had the highest goodness of fit ($R^2 = 0.786$) with the grain yield. This suggests that the potential ozone-induced yield reduction can be evalu-

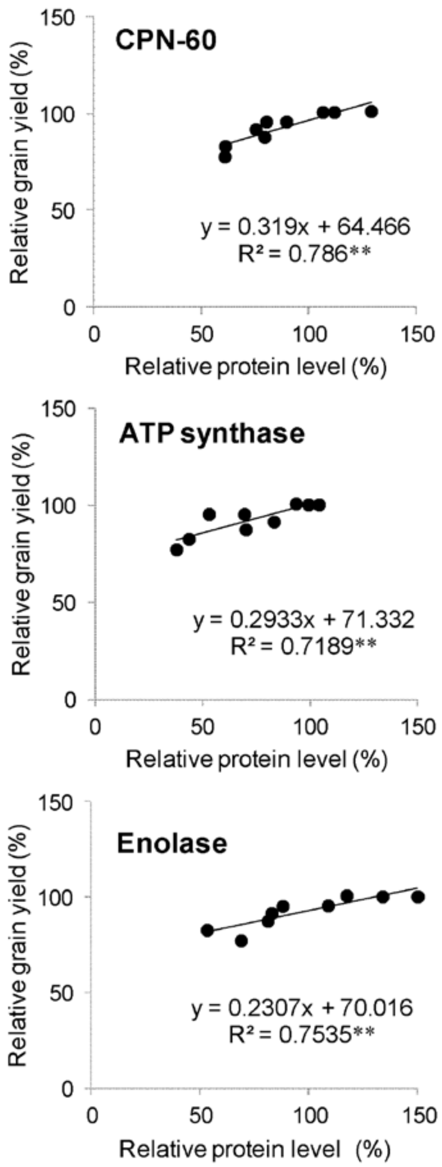


Fig. 3 - Regression analysis for the relative levels of CPN-60, ATP synthase, and enolase 1 in rice seedlings exposed to 40 and 80 ppb ozone, plotted as a function of relative grain yield. The grain yields are relative to the values for six cultivars grown in open-top chambers. Significance levels: **, $P < 0.01$; *, $p < 0.05$.

ated using the level of CPN-60 at the seedling stage in laboratory-scale tests. Moreover, the protein markers that we identified in this study may be useful in crop breeding to quickly select ozone-tolerant rice varieties. Vincent *et al*. (2007) indicated that the inhibition of shoot growth was best correlated with the level of CPN-60 in two wine grape cultivars exposed to salinity and water deficit stress, suggesting that the protein marker is also applicable to other plant or crops.

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Evaluation of local eggplant cultivars in terms of the suitability as materials for "Yakuzen" dishes

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Key words: functionality, local cultivars, property and taste, *Solanum melongena.*

Abstract: "Yakuzen" is a form of medicinal cooking based on the theories of Oriental medicine. To prepare Yakuzen dishes, in-season materials with appropriate properties and tastes, "Sei-Mi," are selected according to the health status and constitution of each person. In this study, the suitability of eggplant (*Solanum melongena* L.) cultivars for Yakuzen was evaluated by sensory tests and by analysis of the functional constituents considered to be closely related to the taste and functionality of Yakuzen dishes. Twenty-two eggplant cultivars including 21 Japanese and 1 Italian cultivar, and a Thai species (*Solanum xanthocarpum* Schrad & Wendl.) were evaluated. Principal component analysis (PCA) was used for comprehensive evaluation among the cultivars. From the PCA, many of the cultivars with round or oval fruit were characterized as juicy and sweet and considered easy to eat; most of the long-fruit cultivars were characterized as having higher specific amino acid contents. The small and round fruit cultivar Dewako and the Thai species (Makhuea pro) were considered to contain many functional ingredients, such as ash, polyphenols, and specific amino acids, and to have higher suitability for Yakuzen dishes.

1. Introduction

In recent years, malignant neoplasm (cancer), cerebrovascular disease and heart disease have become major causes of death among Japanese people, with approximately 75% of deaths in Japan caused by these diseases (Japanese Ministry of Health, Labour and Welfare, 2009 a). Such diseases are generally called lifestyle diseases because they are thought to be strongly related to a lack of exercise and high intake of fat and salt. Therefore, emphasis has shifted from early diagnosis (secondary prevention) to lifestyle improvement (primary prevention) (Japanese Ministry of Health, Labour and Welfare, 2009 b). In this context, research on foods that have pharmacological effects or physiological functions, such as disease prevention and health maintenance, has become more important (Namba, 1999; Tokui *et al.*, 2003). Regarding the pharmacological effects of food, there is a form of medicinal cooking called "Yakuzen" which is based on the philosophy of Oriental medicine and is intended to maintain good health and improve physical condition. The preparation of Yakuzen dishes draws from the theory of "Yaku-shoku Dou-gen", which means that the same principle underlies the daily diet and medical treatment, and on the yin-yang theory, the five-phase theory in Oriental medicine. As a result, Yakuzen has attracted considerable attention for the prevention of lifestyle diseases.

To prepare Yakuzen dishes, in-season materials with appropriate properties and tastes are selected according to the health status and constitution of each person (Namba, 1995; Lan *et al.*, 2002; Tokui *et al.*, 2003). These properties and tastes are called "Sei-Mi" in Yakuzen theory. Sei-Mi consists of four properties (making the human body hot, warm, cool, or cold) and five tastes (salty, bitter, sweet, pungent, and sour), and each is considered to have its own function in the human body (Namba, 1999; Tokui *et al.*, 2003). If the concept of Sei-Mi can be applied to vegetables, a cultivar that has a strong flavor and a high content of functional constituents related to the properties and taste is considered to have strong Sei-Mi and is suitable for Yakuzen dishes.

However, the inherent flavor of vegetable cultivars has been weakened by breeding because priority has been given to ease of consumption for consumers or ease of production for growers. In the case of eggplant (*Solanum melongena* L.), popular cultivars in Japan today are F_1 (first filial generation) cultivars derived from a parental line with oval fruit, a deep purple pericarp, and high yield; these cultivars have improved fruit quality with less unpleasant or harsh taste.

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However, eggplant was introduced into Japan more than 1,260 years ago, and it is mentioned in Nara-period documents (The Shõsõin documents) edited in 750 A.D. (Yoshida, 2010). Therefore, many cultivars have been developed over the long history of cultivation, and even today there are many cultivars with local origins. The chemical composition, flavor, and texture of the edible parts of plants vary among cultivars. Local cultivars grown for many years may have retained more flavor, and some of them may be more suitable for Yakuzen dishes than the currently popular cultivars.

Regarding the functionality of foods, many studies have focused on certain ingredients and have discussed the relationship between the amounts of those ingredients and the functionality of the food. However, it is important to consider the functionality of foods comprehensively in Yakuzen theory. In previous studies, we used principal component analysis (PCA) to comprehensively evaluate the functionality of local cultivars, and we demonstrated that two local cultivars of Japanese radish (*Raphanus sativus* L.), two of carrot (*Daucus carota* L.), and one of bitter melon (*Momordica charantia* L.) were more suitable for Yakuzen dishes than the widely used F_1 cultivar (Saito *et al.*, 2010; Tsukagoshi, *et al.*, 2011 a, b).

In this study, two F_1 cultivars and 21 local cultivars of eggplant were evaluated for their suitability as materials for Yakuzen dishes according to their taste and content of functional constituents.

2. Materials and Methods

Plant materials and growing condition

Twenty-one Japanese eggplant cultivars with different fruit shapes were selected for this study (Table 1). Most of them were purebred cultivars, but two F_1 cultivars were included. In addition, an Italian cultivar and a Thai species (*Solanum xanthocarpum* Schrad & Wendl.) were used. All cultivars were grown at the Center for Environment, Health and Field Sciences, Chiba University. Seeds of all cultivars were sown in 9-cm plastic pots filled with upland soil on 17 April 2012, and the seedlings were raised in a glasshouse. On 5 June seedlings were transplanted to the open field at a spacing of 50 cm between plants and 100 cm between rows. Fruits were harvested when they reached the regular size for each cultivar. Harvest began on 31 July and ended on 14 September. All other management was carried out according to the conventional methods in Japan (Chino, 2001).

Sensory test

Fruits were harvested on 7 and 9 August and the sensory test was conducted the following day. The fruits were washed, cut to a size of 1.5×1.5×3.0 cm, and steamed at 100°C for 5 min. The characteristics of steamed samples were evaluated by six panelists. The panelists passed recognition tests for five tastes (sweetness, umami, saltiness,

Table 1 - Eggplant cultivars used for the experiment

Fruit shape (country)	Cultivar	Abbreviation	Fruit length, weight and color	Remark
Small, Round (Japan)	Dewako	De	3-8 cm, 10-15 g, deep purple	
	Minden	Min	3-8 cm, 10-15 g, deep purple	
Round (Japan)	Aizu maru	AM	8-10 cm, 200-300g, deep purple	
	Kamo nasu	KN	12-15 cm, 200-350 g, deep purple	
	Tonosama	To	approx. 15 cm, 300-450 g, deep purple	
	Yamatoyo maru	YM	10-12 cm, 250-350g, deep purple	
Money pouch (Japan)	Nagaoka kinchaku	NK	8-10 cm, 300-350g, deep purple	
(swelling toward the bottom)	Saitama ao daimaru	SA	approx. 15 cm, 300-450 g, green	
Oval (Japan)	Heta murasaki	HM	approx. 5 cm, 30 g, deep purple	
	Senryo 2 gou	Se2	10-12 cm, 80-90 g, deep purple	F_1, Control cultivar in this experiment
	Se2 (Control)	SK	10-11 cm, 150-180 g, deep purple	
	Wase shinkuro	WS	10-12cm, 80-120 g, deep purple	
	Yamashina	Ya	10-12cm, 80-120 g, deep purple	
Long (Japan)	Chikuyou	Chi	20-25 cm, 120-150 g, deep purple	F_1
	Hakata naga	Hak	40-45 cm, 200-300 g, deep purple	
	Hhogo naga	Hho	17-18 cm, 85-90 g, deep purple	
	Himo nasu	HN	25-30 cm, 150-200 g, pale purple	
	Kitta chunaga	KC	10-12cm, 80-120 g, deep purple	
	Kurume oh naga	KO	30-35 cm, 250-300 g, deep purple	
	Shikon sendai naga	SS	8-10 cm, 20-30 g, bluish purple	
	Shin nagasaki naga	SN	35-40 cm, approx. 250 g, deep purple	
Big, Oval (Italy)	Zebra	Ze	20-25 cm, 300-400 g, purple and white stripes	
Small, Round (Thailand)	Makhuea pro	MP	4-5 cm, approx.10 g, green	Solanum xanthocarpum

bitterness, and sourness) and the discrimination tests for four solutes (sucrose, sodium chloride, tartaric acid, and sodium glutamate). In addition, they had more than one year experience in evaluating vegetables and were classified as expert assessors (Japanese Society for Sensory Evaluation, 2009). The characteristics listed in Table 2 were evaluated on a scale of -5 (weaker) to 5 (stronger) compared to Se2, the control cultivar in this study.

Taste sensor analysis and amino acid content

Approximately 50 g of fresh fruit was homogenized in 100 mL of water in an ice bath, then filtered through cotton cloth. Although filtrates were prepared separately from three or four fruit samples, the filtrates were mixed to obtain the quantity necessary for measurements. Bitterness, astringency, acridity, and pungency of the filtrates were measured using a taste sensor system (SA402B, Intelligent Sensor Technology, Kanagawa, Japan). Each value was expressed relative to the control cultivar (Se2), which was set at zero. A portion of each filtrate was filtered again through a 0.45-μm filter (DISMIC-25CS, Advantec, Tokyo, Japan) and the amino acid content was measured using an amino acid analyzer (JLC-500/V, JEOL, Tokyo, Japan).

Soluble solids content

Fresh fruit was cut into small pieces and pressed in gauze to extract the juice. The soluble solids content of the juice was measured using a refractometer (PAL-1, ATAGO, Tokyo, Japan) and expressed as percent Brix.

Ash and polyphenol contents

The harvested fruit was stored at -30°C until use. Fresh-frozen fruit samples were freeze-dried and ground into a fine powder. Ash content was determined using the dry ashing method. Briefly, 0.3 g of the powder was put in a crucible and ashed at 550°C for 24-48 hr. After cooling, the weight of the residue was measured. Polyphenol content was determined using iron tartrate spectrophotometry. First, 0.2 g of the powder was mixed with 10 mL of distilled water, and shaken for 10 min at 80°C. After cooling, the sample was centrifuged at 3,000 rpm for 15 min. Then, 3.2 mL of the supernatant was mixed with 1.6 mL of iron tartrate reagent (0.1% (w/v) ferrous sulfate and 0.5% (w/v) potassium sodium tartrate) and 3.2 mL of phosphate buffer (0.1 M, pH7.5). The absorbance at 540 nm was then measured using a spectrophotometer (U-2000, Hitachi, Tokyo, Japan). The polyphenol content was calculated from a

Table 2 - Evaluation of the taste of eggplant cultivars by sensory test (z)

Fruit shape	Cultivar	Aroma		Softness		Juiciness	Sweetness	Bitterness	Astringency
		Good	Grassy	Pericarp	Flesh				& Acridity
Small, Round	De (y)	0 (x)	0	-1	0	0	0	1	1
	Min	1	1	0	-1	0	-1	2	1
Round	AM	0	1	0	-1	0	0	0	1
	KN	1	1	0	-1	0	0	0	0
	To	0	0	-1	0	0	0	1	0
	YM	0	0	0	1	1	1	-1	0
Money pouch	NK	0	2	-3	-2	-1	-1	0	0
	SA	0	2	-4	0	-1	0	0	1
Oval	HM	0	0	0	1	0	0	0	0
	Se2 (Control)	0	0	0	0	0	0	0	0
	SK	0	2	0	1	2	1	0	0
	WS	0	0	-1	0	-1	-1	0	0
	Ya	1	0	-1	0	0	0	0	0
Long	Chi	0	0	0	1	0	-1	0	0
	Hak	0	0	1	1	-1	0	-1	0
	Hho	0	0	0	0	0	0	0	0
	HN	0	1	-1	1	-1	-1	0	0
	KC	0	0	-2	1	0	-1	0	1
	KO	0	0	-2	1	-1	-1	0	0
	SS	0	1	-1	0	0	-1	0	1
	SN	0	2	0	1	-2	0	-1	0
Big, Oval	Ze	2	2	0	-2	0	-1	0	2
Small, Round	MP	0	3	0	-1	0	-1	0	1

(z) Eggplant fruit was cut to the size of 1.5 x 1.5 x 3.0 cm, then steamed at 100 degree C for 5 min before the test.
(y) Amino acid which is considered to be important for the functionality of eggplant in Yakuzen theory.
(x) Tastes were evaluated on a scale of -5 (weaker) to 5 (stronger) as compared to Senryo 2 gou (Se2).

standard curve of ethyl gallate.

Principal component analysis and characterization of cultivars

Data were analyzed by principal component analysis (SPSS for Windows version 13), and the characteristics of cultivars were comprehensively evaluated to determine the suitability of the cultivars as materials for Yakuzen dishes.

3. Results and Discussion

Cultivars NK and SA (with money-pouch fruit shape) tended to have harder fruits, and long-fruit cultivars tended to be less sweet (Table 2). The aroma of the Italian cultivar, Ze was characterized as both "good" and "grassy". However, most cultivars were very similar to Se2 (the control cultivar in this study) in the sensory test.

Taste sensor analysis showed that the local cultivars tended to have a less unpleasant taste than Se2 (Table 3).

Table 3 - Evaluation of the taste of eggplant cultivars by taste sensor [(z)]

Fruit shape	Cultivar	Bitterness	Astringency	Acridity	Pungency
Small, Round	De [(y)]	-0.27 [(x)]	0.06	-1.74	-0.69
	Min	-0.38	0.04	-2.27	-0.96
Round	AM	-0.23	0.20	-1.14	-0.48
	KN	-0.11	0.21	-2.11	-0.94
	To	-0.11	0.13	-2.18	-1.18
	YM	-0.16	-0.02	-1.33	-1.30
Money pouch	NK	-0.26	0.27	-1.78	-0.64
	SA	-0.07	0.30	-2.30	-1.07
Oval	HM	0.22	0.13	-1.83	-0.99
	Se2 (Control)	0.00	0.00	0.00	0.00
	SK	-0.28	0.05	-1.51	-0.76
	WS	-0.06	0.23	-2.26	-1.17
	Ya	-0.09	0.08	-2.57	-1.30
Long	Chi	-0.33	0.12	-1.39	-0.61
	Hak	-0.28	-0.09	-2.55	-1.40
	Hho	-0.15	0.05	-1.61	-0.86
	HN	-0.01	0.15	-2.87	-1.45
	KC	-0.12	0.11	-2.24	-1.28
	KO	-0.43	0.07	-0.52	-0.15
	SS	-0.26	0.04	-2.03	-1.00
	SN	-0.47	-0.05	-2.27	-1.09
Big. Oval	Ze	-0.32	-0.02	-2.73	-1.23
Small. Round	MP	-0.34	0.19	-2.15	-0.75

[(z)] Taste sensor was prepared to express the value of Se2 was zero.

[(y)] Amino acid which is considered to be important for the functionality of eggplant in Yakuzen theory.

[(x)] Positive and negative value means the taste was stronger and weaker than Se2, respectively.

This result did not correspond to the results of the sensory test, and the difference may be due to the heating of samples before the sensory test but not before the taste sensor analysis. Nevertheless, we can conclude that the local cultivars were not unpalatable compared with the commonly used cultivar.

Min, YM and some other cultivars tended to have higher soluble solids contents, but there was no significant difference between cultivars (Table 4). "Mi" (the taste) of eggplant is "Kan" (sweet). In Yakuzen theory, Mi means not only the taste on the tongue but also specific functions in the human body (Tokui *et al.*, 2003). In this study, we could not discern differences of Kan characteristics from the results of sensory test and soluble solids contents among cultivars; therefore, the suitability of cultivars was evaluated on the basis of other characteristics.

Ash content was higher in cultivars De and Min (both of which have small, round fruit) and lower in cultivar WS, and tended to be lower in cultivars with oval fruit. "Sei" (the property) of eggplant is to cool the human body. Potassium accounts for most of the ash of eggplant fruit (USDA, 2013), and the function of this mineral is to release heat inducing diuresis. This function is closely related to the property of eggplant, and higher ash content may be related to greater suitability of cultivars for Yakuzen dishes.

Polyphenol content was also higher in De and Min. Eggplant contains polyphenols such as chlorogenic acid and nasunin, which are considered to have antioxidant activity, and to suppress lipid peroxidation, aging, various lifestyle diseases, and cancer (Kimura *et al.*, 1999; Noda *et al.*, 2000; Kitsuda *et al.*, 2005; Singh *et al.* 2009). Das *et al.* (2011) reported that grilled eggplant had a higher polyphenol content, though the cardioprotective ability was not different. The high polyphenol content in De and Min may increase the pharmacological value of these cultivars.

As mentioned above, Mi (the taste) also encompasses specific functions in the human body, and Mi of eggplant is Kan (sweet). Kan is considered to have functions such as supplying nutrition and energy, promoting relaxation, etc. Some amino acids are considered to have Kan functions. For example, glutamine is an energy source for digestion and plays an important role in the maintenance and improvement of immunity and the repair of organs (Ajinomoto Co. Inc., 2003 a, b), and this may correspond to a Kan function. Alanine has a sweet taste and supplies sugars to the body, and it is also considered to have a Kan function. The amino acids strongly related to the Kan of eggplant include alanine, citrulline, glycine, glutamine, proline and serine. Therefore, these amino acid contents were summed to give specific amino acid content (Table 4): it was highest in Se2 and SN and tended to be higher in the long-fruit cultivars and lower in the round-fruit cultivars. Total amino acid content also tended to be higher in the long-fruit cultivars, especially in SN. However, no other trends in amino acid content were observed. The higher content of specific and total amino acids in SN would indicate greater suitability of this cultivar for Yakuzen dishes.

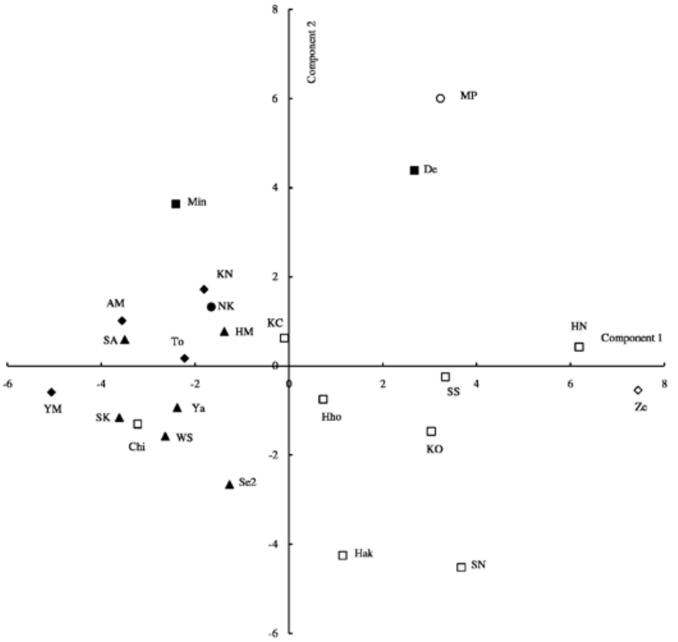

Fig. 1 Two dimensional scatter diagram of the principal component score of eggplant cultivars
■: small, round ◆: round ●: monry pouch ▲: oval □: long ◇: Italy ○: Thailand. Abbreviations as in Table 1.

From the PCA, 23 principal components (PCs) were derived and 14 PCs were considered to be meaningful (contribution rate>1). These 14 meaningful PCs accounted for 96.6% of the total rate (data not shown). Although the first two PCs accounted for only 41.3% of the total rate, a two-dimensional scatter diagram of factor loading was constructed. Specific and total amino acid contents were in the positive direction along the x-axis, and juiciness, sweetness, percent Brix and some unpleasant taste were in the negative direction. Ash, polyphenol content, and bitterness were in the positive direction along the y-axis, and softness was in the negative direction (data not shown). A two-dimensional scatter diagram constructed from the PC1 and PC2 scores of each cultivar enabled classification of the cultivars (Fig. 1). Many of the common F_1 cultivars of eggplant used for commercial production in recent years have been developed to improve ease of consumption and cultivation. Se2 is one of the common cultivars in Japan. It was characterized as juicy and sweet by the PCA and is considered easy to eat. The other cultivars with round or oval fruit were also characterized as juicy and sweet. On the other hand, most of the long-fruit cultivars were distinct from the round-fruit and oval-fruit

Table 4 - Soluble solid, ash, polyphenol and amino acid content of eggplant cultivars

Fruit shape	Cultivar	Soluble solids (% Brix)	Ash (g 100 g^{-1} FW)	Polyphenol [z] (mg 100 g^{-1} FW)	Specific amino acid [y] (mg 100 g^{-1} FW)	Total amino acid (mg 100 g^{-1} FW)
Small. Round	De [x]	5.1 a [w]	0.60 a [w]	673.4 a [w]	120.6	284.9
	Min	5.2 a	0.60 a	434.0 a	71.1	186.0
Round	AM	4.8 a	0.45 ab	169.0 b	37.2	151.2
	KN	5.4 [v]	0.53 [v]	150.0 [v]	48.8	203.1
	To	4.8 a	0.39 b	253.7 ab	52.5	196.6
	YM	5.3 a	0.50 ab	255.0 ab	36.3	144.3
Money pouch	NK	5.0 a	0.45 ab	172.9 b	63.1	197.8
	SA	4.6 a	0.50 ab	247.3 ab	39.5	167.3
Oval	HM	4.7 a	0.50 ab	300.6 ab	64.5	203.1
	Se2 (Control)	4.6 a	0.44 ab	129.1 b	145.6	302.0
	SK	4.3 a	0.39 b	158.4 b	48.0	170.6
	WS	5.1 a	0.37 b	181.9 b	52.8	199.1
	Ya	4.3 a	0.40 b	248.5 ab	58.1	185.4
Long	Chi	4.5 a	0.42 ab	188.1 b	34.1	161.2
	Hak	4.3 a	0.38 b	127.9 b	116.3	286.5
	Hho	4.8 a	0.52 ab	166.2 b	76.8	253.3
	HN	4.5 a	0.52 ab	144.1 b	83.6	288.6
	KC	4.4 a	0.48 ab	236.6 ab	50.8	211.0
	KO	5.1 a	0.45 ab	135.1 b	93.6	314.2
	SS	4.1 a	0.38 b	250.6 ab	106.1	281.8
	SN	4.5 a	0.41 ab	119.2 b	139.2	342.0
Big. Oval	Ze	3.8 a	0.46 ab	124.0 b	95.1	308.9
Small. Round	MP	5.1 a	0.56 ab	245.9 ab	63.9	196.7

[z] Polyphenol content was expressed as ethyl gallate equivalent.
[y] Amino acid strongly related to Kan of eggplant.
[x] Abbreviations as in Table 1.
[w] Different letter within the row indicates significant difference by Tukey's multiple range test at 5% level (n=5).
[v] Number of harvested fruit was not enough for statistical analysis.

cultivars. They were characterized as having higher specific amino acid contents and little unpleasant taste or sweetness. The small and round fruit cultivar De and the Thai species (MP) were considered to contain many functional ingredients, such as ash, polyphenols, and specific amino acids, and to have greater suitability for Yakuzen dishes.

4. Conclusions

Among the local Japanese cultivars used in this study, cv. De is highly suitable for Yakuzen dishes because it contains many ingredients associated with the properties and taste (Sei-Mi) of eggplant in Yakuzen theory. In addition, the Italian cultivar (Ze) and the Thai species were highly distinct. Different results may have been obtained had we grown the cultivars in another area or under different conditions. Nevertheless, we have demonstrated that some local eggplant cultivars have stronger Sei-Mi than current F_1 cultivars. These characteristics could add value to the local cultivars and lead to regional development.

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Physiological and psychological relaxing effects of visual stimulation with foliage plants in high school students

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Key words: heart rate variability, minors, house plant, stress reduction, visual stimuli.

Abstract: With lifestyles in modern society becoming increasingly stressful, there is growing interest in the physiological relaxing effects of the natural environment. Particular interest has been paid to the physiological effects of indoor plants, however no studies have revealed the effects of such visual stimulation on minors. In this study 85 (41 male and 44 female; 16.5±0.9 years; mean±SD) students were exposed, or not as control, to a typical foliage plant, dracaena (*Dracaena deremensis;* Lemon Lime), for 3 min. Physiological indices included heart rate variability (HRV) and pulse rate, using an accelerated plethysmography at the fingertip, were collected continuously during the experiments. The results indicated that the high frequency component (HF), a general index of parasympathetic nervous activity, was significantly higher; the low frequency component [LF/(LF+HF)], a general index of sympathetic nervous activity, was significantly lower; and the pulse rate was significantly lower. After exposure, or not, the subjects completed a questionnaire as psychological evaluation. A 13-point rating scale was used for following parameters: "comfortable-uncomfortable," "relaxed-awakening," and "natural-artificial." Results of the study showed that subjects felt more comfortable, relaxed and natural after visualizing the dracaena plants. Overall, the physiological and psychological relaxing effects of visual stimulation with foliage plants in high school students is confirmed.

1. Introduction

Recent studies have focused on the physiological relaxing effects of the natural environment (Park *et al.*, 2009; 2012). It has been reported that staying in a forest environment enhances parasympathetic nervous activity (Park *et al.*, 2012; Tsunetsugu *et al.*, 2013), suppresses sympathetic nervous activity (Park *et al.*, 2012; Tsunetsugu *et al.*, 2013; Lee *et al.*, 2014), decreases blood pressure and pulse rate (Park and Mattson, 2009; Park *et al.*, 2012), and decreases cortisol concentration (Park *et al.*, 2012). Studies by Li *et al.* (2007, 2008 a, b) demonstrated that staying in a forest environment for three days and two nights improved the immune function of office workers (Li *et al.*, 2007), and this effect was sustained for approximately one month (Li *et al.*, 2008 a, b). Another study reported that walking in an urban park enhances parasympathetic nervous activity and decreases heart rate (Song *et al.*, 2013). In addition, spending time in rooftop gardens enhances parasympathetic nervous activity and suppresses sympathetic nervous activity in elderly people requiring care (Matsunaga *et al.*, 2011).

Evidence-based medicine has been attracting attention globally, with physiological data from field tests making a significant contribution. We expect that accumulating physiological data from field experiments will continue to demonstrate the preventive medical effects of nature therapy in the future (Lee *et al.*, 2012).

In modern society, many individuals spend the majority of their time in intensely stressful states, and they have no time to make contact with nature outside of their immediate surroundings. High school students, who spend most of their everyday life at school, are typical examples. Previous studies have evaluated the psychological stress levels in high school students (Anda *et al.*, 2000; Takakura and Sakihara, 2001). Moreover, many high school students have stressful relationships with friends or teachers (Miura and Kawada, 2008). In a document by the Japanese Ministry of Education, the percentage of students who progressed to universities or junior colleges in 2010 was 56.9%, which was 18.5% higher than the rate in 1975 (Statistics Bureau, Ministry of Internal Affairs and Communications, 2012), and the pressure from entrance examinations is extremely high among high school students (Equal Employment, Children and Families Bureau, Ministry of Health, Labour and Welfare, 2009).

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Flowers and foliage plants are common natural surroundings that can be incorporated into the school and home. We previously conducted surveys to evaluate the physiological effects of visual stimulation with fresh rose flowers in high school students (Ikei *et al*., 2013), middle-aged and elderly medical staff (Komatsu *et al*., 2013), and office workers (Ikei *et al*., 2014). The results showed enhanced parasympathetic nervous activity (Ikei *et al*., 2013; Komatsu *et al*., 2013; Ikei *et al*., 2014), suppressed sympathetic nervous activity (Ikei *et al*., 2013), and decreased pulse rates (Komatsu *et al*., 2013) during visual stimulation.

It was reported in a previous study that natural views from hospital windows or the presence of indoor plants hasten the recovery of patients after surgery and decrease systolic blood pressure (Park and Mattson, 2009). These effects have also been studied in a classroom, demonstrating that the ambience of indoor space can be improved by including foliage plants (Doxey *et al*., 2009), as reflected by enhanced feelings of comfort among the students (Han, 2009). However, there have been no reports on the influence of visual stimulation with foliage plants on heart rate variability (HRV) and subjective feelings in minors. Therefore, this study was conducted to examine the effects of exposure to the foliage plant dracaena (Ministry of Agriculture, Forestry and Fisheries, 2008) on physiological and psychological variables (HRV, pulse rate, and subjective responses) in high school students.

2. Materials and Methods

The experiments were conducted in a classroom of the Chiba Prefectural Kashiwanoha Senior High School in October 2012. The room temperature was approximately 25.9°C, relative humidity approximately 52.6%, and illumination approximately 900 lux. Eighty-five high school students (41 male and 44 female; 16.5±0.9 years; mean±SD) participated in the experiment. The study was conducted with the approval of the Ethics Committee of the Center for Environment, Health and Field Sciences, Chiba University. All subjects provided written informed consent.

Three dracaena plants (*Dracaena deremensis*, Lemon Lime), 55-60 cm high, were placed at intervals of 8 cm on a desk in front of each subject (test situation). The distance from the subject's eyes to the plants was approximately 55 cm, and they were adjusted according to the height of the subjects. No exposure to foliage plants was used as the control condition. Before visual stimulation, the plants and the control were covered by a corrugated cardboard box (rest condition). Figure 1 shows the study protocol, figure 2 the rest condition, and figure 3 the visual stimuli (the dracaena

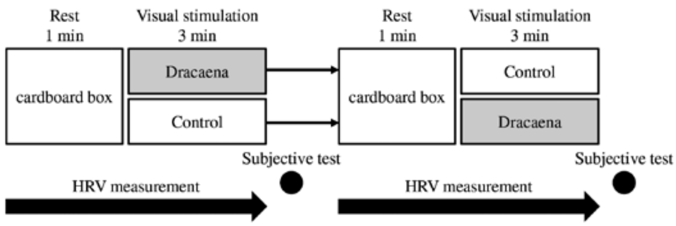

Fig. 1 - Study protocol for testing the physiological and psychological relaxing effects of visual stimulation with foliage plants in high school students.

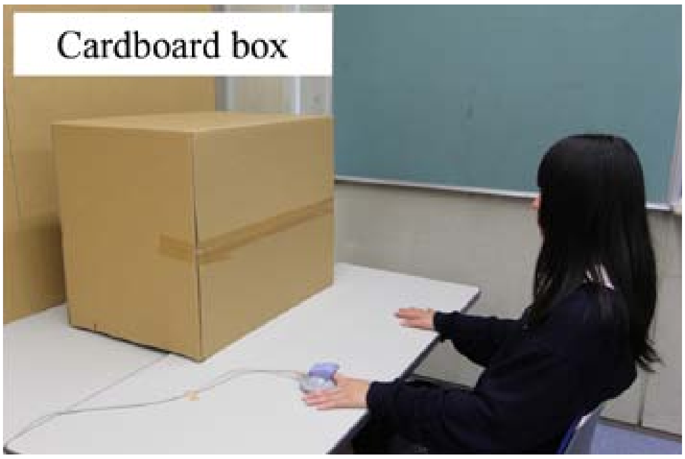

Fig. 2 - The rest condition.

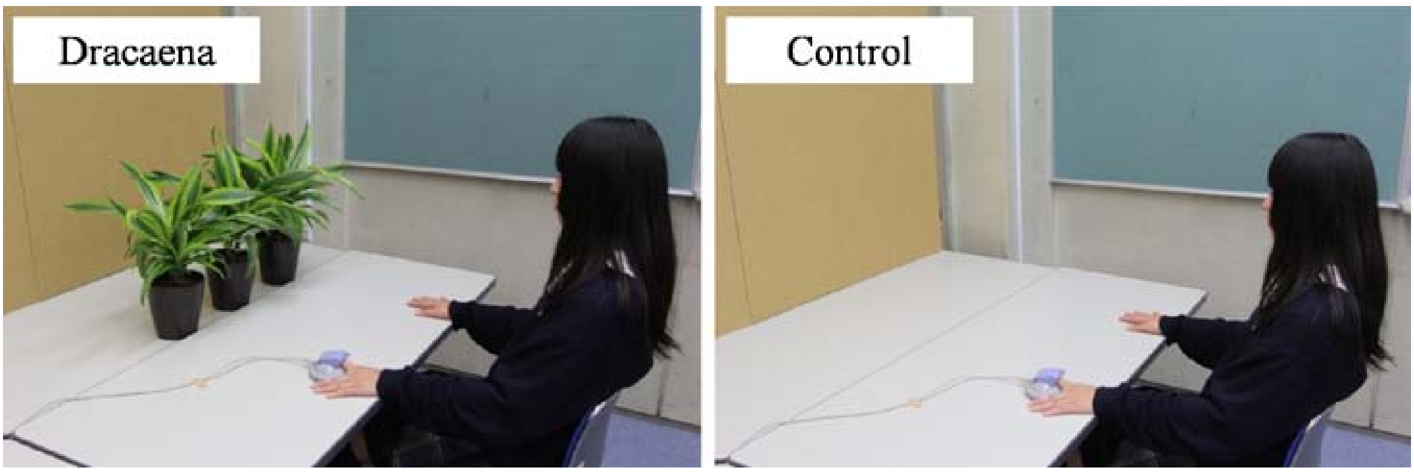

Fig. 3 -The visual stimulation condition.

plants or control). After viewing the cardboard box at rest in a sitting posture for 1 min (Fig. 2), the subject was exposed to the plants or control for 3 min (Fig. 3). After the experiments, each subject completed a questionnaire. The order of stimuli was counterbalanced among subjects.

HRV and pulse rate were measured as physiological indices. HRV was calculated by spectral analysis of the coefficient of variation of the a-a interval on an accelerated plethysmograph (APG; ARTETT, U-Medica Inc., Osaka, Japan). Previous studies have reported that the a-a interval on an APG and R-R interval on an electrocardiogram are strongly correlated (Takada and Okino, 2004; Takada *et al.*, 2008). The sampling frequency was set at 1000 Hz. The maximum entropy method was used for frequency analysis, and variance of the low frequency (LF; 0.04-0.15 Hz) and high frequency (HF; 0.15-0.40 Hz) components were calculated. The LF/(LF+HF) ratio for R-R interval variability was also assessed. The HF component was used as an index of parasympathetic nervous activity and the LF/(LF+HF) ratio was used as an index of sympathetic nervous activity (Weise and Heydenreich, 1989; Cacioppo *et al.*, 1994; Sawada *et al.*, 1997). Generally, parasympathetic nervous activity is enhanced during relaxation and sympathetic nervous activity is enhanced at the time of awakening and stress (Ackerknecht, 1974). Therefore, the pulse rate was converted by dividing 60 by the a-a interval on APG. The HRV and pulse rate data were collected continuously during the 3-min experiments and averaged.

In addition, the subjects subjectively evaluated the emotional effects of the dracaena plants and control using the modified semantic differential (SD) method (Osgood *et al.*, 1957), which uses three pairs of adjectives on 13 scales, including "comfortable-uncomfortable," "relaxed-awakening," and "natural-artificial."

The Statistical Package for Social Sciences software (v20.0, IBM Corp., Armonk, NY, USA) was used for all statistical analyses. A paired t-test was used to compare the physiological responses to visual stimulation with dracaena plants or control, followed by Holm correction of the changes in each 1-min average, while the Wilcoxon signed rank test was used to compare the psychological responses to visual stimulation with dracaena plants or control. For both conditions, one-sided tests were used because of the hypothesis that humans are relaxed by visual stimulation with foliage plants. Statistical differences were considered significant at $P<0.05$.

3. Results

Significant differences were found in the values of the HF component and the LF/(LF+HF) ratio of HRV between dracaena and the control.

Figure 4 shows the HF component of HRV, an estimate of parasympathetic nervous activity. HF between 0 and 1 min was 1210.7±120.3 (mean±SE) ms^2 during the test condition and 1032.9±95.2 ms^2 during the control condition, showing a significant increase of 17.2% ($P<0.05$) (Fig. 4A) during

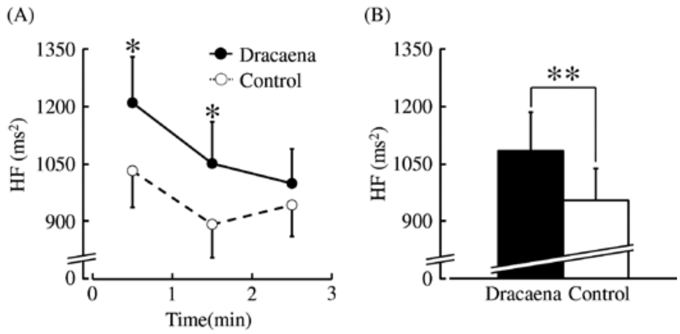

Fig. 4 - The 1-min averages and overall mean high frequency component (HF) of heart rate variability (HRV) during visual stimulation with dracaena plants and control.
(A) Changes in each 1-min average HF value over 3 min.
(B) Overall mean HF values.
n=85.
mean±SE.
$*P < 0.05$, $**P < 0.01$ as determined by the paired *t*-test, Holm correction.

the test condition. Similarly, HF between 1 and 2 min was 1052.0±109.3 ms^2 during the test condition and 893.1±87.1 ms^2 during the control condition, showing a significant increase of 17.8% ($P<0.05$) (Fig. 4A) during the test condition. There was no significant difference in HF between 2 and 3 min. The overall HF during the 3-min experiment was 1083.9 ± 101.5 ms^2 in the test condition and 954.8±83.5 ms^2 in the control condition, showing a significant increase of 13.5% ($P<0.01$) (Fig. 4B) with the test condition, indicating that parasympathetic nervous activity was significantly higher during dracaena plant exposure.

The results of the LF/(LF+HF) ratio, a marker of sympathetic nervous activity, are shown in figure 5. For 1-min segment analysis, the LF/(LF+HF) ratio between 0 and 1 min was 0.47±0.02 during the test condition and 0.52±0.02 during the control condition, showing a significant decrease of 9.6% ($P<0.05$) (Fig. 5A) with the former.

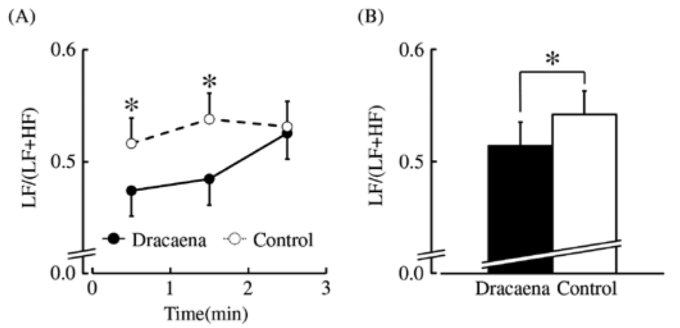

Fig. 5 - The 1-min averages and the overall mean LF/(LF+HF) ratio of heart rate variability. (HRV) during visual stimulation with dracaena plants and control.
(A) Changes in each 1-min average LF/(LF+HF) value over the 3 min.
(B) Overall mean LF/(LF+HF) values.
n=85.
mean±SE.
$*P<0.05$ as determined by the paired *t*-test, Holm correction.

Similarly, between 1 and 2 min, the ratio was 0.48±0.02 during the test condition and 0.54±0.02 during the control condition, showing a significant decrease of 11.1% ($P<0.05$) (Fig. 5A) during the test. No significant difference was observed between 2 and 3 min. For the entire 3-min duration, LF/(LF+HF) was 5.6% lower during the test condition than during the control condition (dracaena: 0.51±0.02, control: 0.54±0.02; $P<0.05$) (Fig. 5B), indicating that sympathetic nervous activity was significantly lower during dracaena plant exposure.

Clear differences in pulse rate were observed between dracaena and control exposure. The pulse rate between 0 and 1 min was 71.9±1.2 beats/min during the test condition and 72.7±1.2 beats/min during the control condition, showing a significant decrease of 1.1% ($P<0.05$) (Fig. 6A) during the test condition. Similarly, between 1 and 2 min, the pulse rate was 72.6±1.2 beats/min during the test condition and 73.3±1.2 beats/min during the control condition, showing a significant decrease of 1.0% ($P<0.05$) (Fig. 6A) during dracaena plant exposure. No significant difference was observed between 2 and 3 min. The mean pulse rate was 0.1% lower during the test condition than during the control condition (dracaena: 72.4±1.2 beats/min, control: 73.0±1.2 beats/min; $P<0.05$) (Fig. 6B).

The subjective evaluation data clearly showed the effect of the two different visual stimuli on the psychological states of participants. Participants felt significantly more comfortable (dracaena: "slightly comfortable"; control: "indifferent"; $P<0.01$) (Fig. 7, left), relaxed (dracaena: "slightly relaxed"; control: "indifferent"; $P<0.01$) (Fig. 7, center), and natural (dracaena: "slightly natural"; control: "indifferent"; $P<0.01$) (Fig. 7, right) in the dracaena condition than in the control condition after stimuli.

4. Discussion and Conclusions

An improved ambience resulting from the placement of foliage plants in a classroom may significantly enhance physiological relaxation and mental health in high school students, as shown in the present investigation.

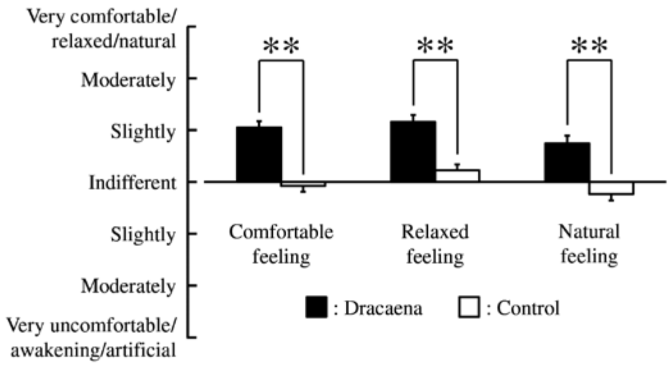

Fig. 7 - Changes in subjective evaluation for "comfortable–uncomfortable," "relaxed–awakening," and "natural–artificial" with dracaena plant exposure and control exposure.
n=85.
mean±SE.
**$P < 0.01$ as determined by the Wilcoxon signed-rank test.

Relatively brief (3 min) visualization of foliage plants resulted in significantly enhanced parasympathetic nervous activity (13.5%), suppressed sympathetic nervous activity (-5.6%), and decreased pulse rate (-0.8%), results which are consistent with previous studies involving the visualization of a forest scene (Park *et al.*, 2010; Tsunetsugu *et al.*, 2010; Park *et al.*, 2011). Furthermore, our findings are consistent with those of our previous report on the calming effects of roses in high school students (Ikei *et al.*, 2013), where similar physiological responses were found. Also in line with our findings, visual stimulation with fresh roses enhanced parasympathetic nervous activity and significantly decreased the heart rate in middle-aged and elderly medical staff (Komatsu *et al.*, 2013), while it enhanced parasympathetic nervous activity in office workers (Ikei *et al.*, 2014).

According to our analysis of the three questionnaires, the subjects in the present study felt more comfortable, relaxed, and natural after visualizing the dracaena plants. This result is consistent with that of our previous report on the calming effects of roses (Ikei *et al.*, 2013; Komatsu *et al.*, 2013; Ikei *et al.*, 2014).

The results of this study support the hypothesis that placement of foliage plants in classrooms can induce a relaxing effect, improve physiological activity, and improve the psychological state in high school students. Because of the growing interest in mental health in modern times (Murray and Lopez, 1996), the psychological benefits of indoor plants are expected to play an important role in the promotion of mental health in the future.

However this study had limitations. First, we only evaluated HRV. Thus, the results cannot be interpreted in terms of a complete physiological evaluation. Other experimental indices such as brain activity and stress hormone levels should be assessed to determine the effects of visual stimulation with natural objects, such as foliage plants on human response. Second, only dracaena plants were used.

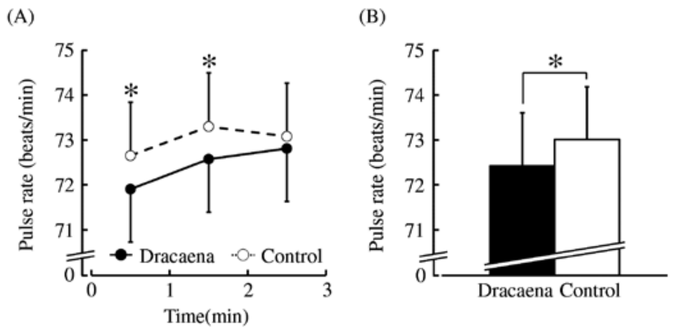

Fig. 6 - The 1-min averages and overall mean pulse rate during visual stimulation with dracaena plants and control.
(A) Changes in each 1-min average pulse rate over the 3 min.
(B) Overall mean pulse rates.
n=85.
mean±SE.
*$P < 0.05$ as determined by the paired *t*-test, Holm correction.

In future experiments, we will examine human responses to exposure to multiple types of foliage plants. We predict that the physiological data will support the physiological and psychological relaxing effects of foliage plants, which may subsequently lead to their increased use in educational establishments in attempts to decrease stress among students.

A brief visual stimulation of foliage plants shifted the sympathetic/parasympathetic balance and improved mood, suggesting a simple method to decrease stress and improve the health of high school students.

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