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Horticultural Research in Japan. Production of vegetables and ornamentals in hydroponics, constraints and control measures

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Key words: activated charcoal, allelochemicals, autotoxicity, control of minerals, human health, hydroponics, plant factory, vegetables and ornamentals.

Abstract: Japan has a very mountainous topography, steep landforms, and varied climate making it less suitable than other countries for growing horticultural crops. However, good crop production technologies make it possible to grow high quality produce in Japan. The major horticultural crops are mainly produced in greenhouses and high tunnels however, a vast majority of greenhouse systems are outdated. Horticultural production practices could be more sustainable through the use of hydroponics in the greenhouse. Commercial hydroponic systems have proved more productive (20-25% higher) than conventional systems of agriculture. A number of research projects have been conducted to better understand production constraints in hydroponics, such as autotoxicity, and to identify suitable cultivars and supplementation techniques. This review discusses greenhouse production techniques for vegetables and ornamentals in hydroponics, as well as constraints and means for sustainable production.

1. Introduction

Japan is located between 24° and 46° north latitude and has four distinct seasons, spring, summer, autumn, and winter. It has a very mountainous topography and steep landforms that are poorly suitable for cultivation of horticultural crops. However, despite the rough mountainous terrain and poor soils, the Japanese have still managed to be successful in growing many crops. Japan enjoys mostly a temperate zone climate which varies considerably in different parts because the country stretches from north to south and is surrounded by the sea. The main horticultural crops include apples, grape, cherries, melon, onions, sweet corn, carrots, tomatoes, Japanese radish, Chinese yams and beans. Several types or floricultural crops are also produced and exported to other countries (Araki, 2002). Tomato, cucumber, welsh onion, strawberry, watermelon and spinach are mostly produced in greenhouses and high tunnels. Strawberries are usually grown on high beds for comfortable work and lower labor costs whereas, tomatoes are produced in glasshouses and high tunnels in order to prevent crop damage by rain (Araki, 2002).

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Japan uses 69% of its total greenhouse area for vegetable production, only 17% for flowers, and 13% for fruit tree production. Only 3% of greenhouses use the modern facilities suitable for crop production through hydroponics (Nichols, 2008) although a gradual change has been taking place in greenhouse production. More sustainable horticulture practices in greenhouses are desirable and possible through the use of hydroponic methods that allow only small amounts of water to drain off and usually offer a higher yield. Commercial hydroponic systems have proved to be more productive than conventional systems of agriculture. Hydroponics has averaged around 20 to 25% higher yields than conventional soil cultivation. This could truly benefit Japan's current GDP in agriculture while feeding more people with sustainable and fresh horticultural crops. Conventional hydroponic techniques use culture solution with bare root systems and do not use growing media. On the other hand, soilless cultivation is a very sustainable crop production practice because many of the components in modern media such as perlite and vermiculite take many years to decay and go back into the environment. The improved and consistent vegetable quality is second to none because of the elimination of pesticides and herbicides. This keeps toxic chemicals away from the culture system and it will in turn help keep Japan's groundwater and oceans free from contamination. Hydroponics is currently a managed culture technique

that is largely used for the production of vegetables as it is capable of being sustainable with low amounts of water loss and use of soilless media. Therefore, advanced management of this technique has been practiced throughout Japan with an aim toward yield maximization and higher quality assurance. However, constraints have been found and several researchers have tested strategies to overcome them. This review discusses greenhouse production techniques of vegetables and ornamentals in hydroponics, and the constraints and means for sustainable production.

2. Hydroponics for higher yield and production of specialty horticultural crops

Hydroponics is a method for growing plants using mineral nutrient solutions, in water, without soil. In this system plants can be grown with their roots in the mineral nutrient solution only orin an inert medium. It is possibly the most intensive method of crop production providing efficient use of water and mineral nutrients with a minimal use of space. It has been used successfully by commercial growers for fast-growing horticultural crops such as lettuce, strawberries, tomatoes, cucumbers and ornamentals in Japan. This technology enables a more precise control of growth conditions, making it easier to study the variable factors or parameters. One specialty of this technique is the vigorous development of root systems and the efficient uptake of the essential nutrients from culture solution resulting in a better crop yield. Therefore, this technology has gained popularity for producing high value crops with high quality and specialty production providing human health benefits. However, there are limits due to self-toxicity from root exudates in recycled nutrient solutions. Finding suitable control measures to overcome these constraints could result in sustainable horticultural crop production in greenhouses. In the following sections, hydroponic production techniques for root crops (carrot), production of functional foods for kidney dialysis patients (melon), and enhanced crop quality (turnip) will be discussed.

Growing carrots hydroponically using perlite substrate

Root vegetables are often discouraged to grow by hydroponics possibly because of poor root development. Carrot [*Daucus carota* L.] is a root vegetable which forms numerous hairy roots with a reduced tap root in the nutrient solution. Moreover, inside nutrient solution this storage organ form hypertrophy due to the ample supply of water and nutrient resulting decrease in length and weight compared to non-submerged condition (Terabayashi *et al.*, 2008). Therefore, appropriate moisture content of the growth medium is crucial for optimum growth and development of the storage root (Eguchi *et al*., 2008). It has been found that the use of suitable soilless media can increase both marketable yield and quality of root crops by many folds (Hanna, 2009). Perlite is widely preferred as soilless media, as it encourages faster root development, reduces risk of damping off, avoids water logging and provides an optimum balance of air and water. Its strong attraction for water automatically draws up solution from the reservoir at the same rate that the plants remove water, leaving excess solution in the reservoir. Therefore, an optimum moisture level can be maintained around the root, and this is a significant advantage over rockwool with less capillary action. Recently perlite has emerged as an excellent medium with versatile use. It has been widely used to grow many horticultural crops including tomatoes, cucumber, melon, peppers, lettuce and rose (Szmidt *et al*., 1988; Cantliffe *et al*., 2003; Hochmuth and Hochmuth, 2003; Fascella and Zizzo, 2005; Frezza *et al*., 2005; Rodriguez *et al*., 2006).

Suitable perlite size, along with optimal concentration of culture solution, has been suggested for maximizing carrot yield in hydroponics (Asaduzzaman *et al*., 2013 a). It was found that carrot plants grown in 0.6 mm perlite supplied with 100% nutrient solution produced significantly higher root yield compared to larger perlite particles and higher concentrations of nutrient solution. Interestingly it was observed that when carrots are grown in 0.3 mm perlites, shorter roots are produced which are wider near the proximal end and whitish in the distal end due to excessive water content causing oxygen deficiency. This ultra fine perlite can hold excessive water causing oxygen deficiency in the substrate air zone and as a result roots become whitish with a reduced amount of carotenoids. In the study, the feasibility of growing carrot in used perlites from previous culture was also evaluated. The residual nutrients available in the reused perlite lowers in turn the demand of nutrients in the second culture where carrot plants can uptake about 50% residual nitrogen. Therefore, it was recommended to use 0.6 mm perlite and 100% (for first culture) or 75% (for second culture) 'Enshi' nutrient solution for growing carrots hydroponically for maximum yield and higher quality.

Production of low potassium content melon fruits in hydroponics through managing nutrient solution

Potassium is crucial for the normal functioning of muscles, heart, and nerves in the human body. It is one of the main electrolytes and is concentrated within the body cell. About 90% of body K is normally excreted by the kidneys but patients with kidney dysfunction suffering from chronic kidney disease (CKD) cannot completely excrete it and thus it accumulates. Abnormally elevated levels of K in the blood (hyperkalemia) can cause adverse effects (Kes, 2001). This author also reported that a normal kidney has the capacity to excrete in excess of 400 m mol K day^{-1}, and it is unlikely that an individual will become chronically hyperkalemic without some degree of chronic renal impairment. Sometimes hyperkalemia causes arrhythmias, muscle weakness, disturbed consciousness, heart failure, and can even lead to sudden death (Spital and Stems, 1988; Putcha and Allon, 2007). The CKD patient population is increasing year by year and it is expected that the total number of patients will continue to increase progressively. Therefore, preventive measures have been stressed to divert the prevalence of this disease in a de-

creasing trend. Restricted diets are mainly used as a means of treatment for CKD patients. As a primary control measure, foods with high K content are restricted but a normal daily diet including fruits such as melon, fresh vegetables, seaweed, beans and potatoes are rich in K (Weiner and Wingo, 1998). K is a major nutrient, essential for normal growth and development of plants (Schachtman and Liu, 1999). Plants absorb more K than any other mineral element with the exception of N (Tisdale and Nelson, 1975; Mäser *et al*., 2002; Britto and Kronzucker, 2008; Szczerba *et al*., 2009). It is the only monovalent cation that is essential for all higher plants and it is involved in three major functions: enzyme activation, charge balance, and osmoregulation (Mengel, 2007; Szczerba et *al.*, 2009). It is inevitable that reduced K supply will inhibit plant growth and yield. Therefore, investigations on minimal requirements of K in plants to maintain their normal growth and development are important. In the above context, investigations have been conducted for the production of melon fruits with low K content to provide supplementary diet to dialysis patients (Asao *et al*., 2013).

In Japan greenhouse cultured raw melon generally has higher K content: 340 mg 100 g^{-1} fresh weight (MEXT, 2011). This amount of K was decreased considerably, improving the diet of dialysis patients (Asao *et al*., 2013). The researchers applied a hydroponic method which enabled a more precise control of growth conditions and made it easier to study the variable factors or parameters. Regular nutritional testing can be conducted in hydroponics, making it possible to determine if the desired amount of nutritional content is present in the plants or not. Precise control over the concentration and composition of culture solution allows the production of either mineral enriched or deficient fruits or vegetables.

Results indicated that a general trend of decreasing K content in fruit with a decrease of KNO_3 concentration in the nutrient solution. In spring 2009, the K content of fruits harvested from plants grown in the nutrient solution with 1/4th KNO_3 was about 39% lower compared to those harvested from plants grown in standard nutrient solution, while it was about 35% and 43% lower in fruits from plants grown with 1/16th and without KNO_3 in the spring 2010 and 2011, respectively. The content of Na in fruits however increased progressively with the decrease of K in the nutrient solution. A consistent antagonistic relation with fruit K concentration was found due to the reduced levels of KNO_3. Compared to control plants, about 83% (spring 2010) and 51% (spring 2011) increased Na were found in fruit harvested from plants grown in nutrient solution with 1/6th or without K, respectively. Low K content melon fruit can provide human health benefits to dialysis patients but the higher Na content would likely cause hyperpiesia and edema. Therefore, it is necessary to evaluate the benefits of the reduction of K against the risks of the increased Na intake by the dialysis patients, whose K intake should be restricted to 1500-2000 mg day^{-1} (Agondi *et al*., 2011) and NaCl intake (equivalent to 2000-3200 mg Na) should be restricted to 5000-8000 mg day^{-1}. Na intake must be limited to 1.3-1.6 times of K intake. However, the benefits of reducing the intake of K are greater than the risks of increasing the intake of Na.

Nutrient level changes in anthocyanin and nitrate-N contents in hydroponically grown turnip

"Tsuda-kabu" (*Brassica rapa* L. rapifera group) is a local turnip cultivar popularly grown in Shimane prefecture in Japan. The cultivar is red with a comma-shaped root. The crop is normally sown in September and harvested in December. Harvesting turnip in early December is profitable mainly since many people in Japan send it as a year-end gift. However, sowing turnip in September in field conditions is often delayed due to incessant rainfall. Moreover, turnip in soil culture requires extra labor for harvesting and washing. In hydroponics, turnip cultivation seems to be less influenced by environmental changes and harvesting, and processing is also convenient. Another problem with soil cultivation is that most of this turnip root remains above the ground and only a few centimeters of the root remains in the soil. This red turnip has large amounts of anthocyanin in the root, adding quality for processing and delicious foods. Previous research reported that nitrate inhibited the sucrose-induced accumulation of anthocyarine in grapevine (Faust, 1965; Pirie and Mullins, 1976). Thus, an optimum level of nutrients in the culture solution is a precondition for harvesting quality products with maximum yield through hydroponic culture.

In this context, Asao *et al*. (2005) investigated the influence of different nutrient solution concentrations on the growth of turnip in hydroponics. It was found that NO_3^--N contents in the shoots and roots of turnip were decreased with decreasing NO_3^--N levels in the nutrient solution. On the other hand, anthocyanin concentration in the root was inversely proportional to the content of NO_3^--N in the same. However, when NO_3^--N content at 50% nutrient solution concentration was changed from full (8 m mol^{-1}) to half (4 m mol^{-1}), NO_3^--N content and anthocyanin contents in the turnip root were not affected. These results indicate that the effects of other nutrients except NO_3^--N might be associated with the formation of anthocyanin in turnip. Therefore, they suggested that the medium range (50%) nutrient solution with 4 m mol^{-1} NO_3^--N may be treated as optimum concentration for production of quality turnip by hydroponic culture.

3. Production constraints in recycled hydroponic culture

Autotoxicity in cucumber (Cucumis sativus L.) under closed hydroponics

Autotoxicity in cucumber plants was reported in previous research as due to accumulation of phytotoxic phenolic compounds in the substrates used for long-term cultivation (Politycka *et al*., 1984; Yu and Matsui, 1994). Root exudates from the cucumber cv. Shougoin-Aonaga-Fushinari gave maximum inhibitory effect on seedling growth of its own cultivar, as well as other test cultivars of cucumber

(Asao *et al*., 1998 a). The growth of tomato (Yu and Matsui, 1993 a, b) and cucumber plants (Yu and Matsui, 1994) increased upon addition of activated charcoal (AC) to the nutrient solutions. Yu and Matsui (1993 c, 1994) collected the root exudates of cucumber cv. Tokiwa from hydroponic culture through a continuous trapping technique (Tang, 1986) and identified a number of autotoxic chemicals.

Fruit yield of cucumber was improved by the addition of AC to the nutrient solution (Asao *et al*., 1998 b) because the added charcoal adsorbed the phytotoxic root exudates from the nutrient solution and thus favored cucumber growth. It was also found that the fruit yield of cucumber plants decreased significantly at late reproductive stage (2 weeks ahead of final harvest) while this inhibition was recovered if nutrient solutions were renewed biweekly or supplemented with AC to the nutrient solution (Asao *et al*., 1998 a). Characteristic shrunken cucumber fruits were harvested from the plant grown in non-renewed culture solution. Autotoxicity of cucumber was also found to be different among cultivars (Asao *et al*., 1998 b). Fruit harvesting of a susceptible cucumber cultivar grown in a closed nutrient flow system was prolonged by grafting onto a non-autotoxic cultivar (Asao *et al*., 1999 b). Thus, cucumber root exudates from a closed hydroponic system were analyzed and 2,4-dichlorobenzoic acid (DCBA) was found to be the strongest inhibitor among a number of growth inhibitors detected (Asao *et al*., 1999 c; Pramanik *et al*., 2000).

Autotoxicity in strawberry (Fragaria × ananassa Duch.) under closed hydroponics

In Japan, closed hydroponics have been considered feasible for strawberry cultivation (Takeuchi, 2000; Oka, 2002; Koshikawa and Yasuda, 2003), however it was reported that a yield reduction, caused by unknown factors, occurred in this production system for strawberry (Oka, 2002). Many researchers showed that root exudates can be removed by adding AC to the nutrient solution (Koda *et al*., 1977; Asao *et al*., 1998 a, 1999 c; Sato, 2004). Subsequently, Kitazawa *et al*. (2005) identified potential chemicals in root exudates of strawberry and investigated the effects of non-renewed nutrient solution, and on vegetative and reproductive growth of strawberry with the addition of AC.

Non-renewed nutrient solution resulted in a significant decrease in the growth of strawberry plantlets compared to growth when the nutrient solution was renewed. The number of flower clusters, flowers and fruits harvested all decreased in non-renewed nutrient solution. This phenomenon was also evident in cucumber (Asao *et al.*, 1998 a), mitsuba (Koda *et al*., 1980) and rose (Sato, 2004). GC-MS analysis of strawberry root exudates revealed the presence of lactic, benzoic, succinic, adipic, and p-hydroxybenzoic acids. These acids showed phytotoxicity during bioassay and benzoic acid was found to be the strongest inhibitor of vegetative and reproductive growth in strawberry plantlets. Therefore, it was concluded that reduction in performance of strawberry grown in closed hydroponic systems occurred thorough autotoxic root exudates from the strawberry plant itself.

Autotoxicity in some leaffy vegetables when grown in recycled hydroponics

Yield reduction due to continuous culture of numerous crops has been demonstrated by researchers around the world. Allelopathic effects from crop residues and root exudates have been extensively studied in crops such as alfalfa (Miller, 1983; Nakahisa *et al.*, 1993, 1994; Chon *et al*., 2002, Chung *et al*., 2011), asparagus (Young, 1984; Young and Chou, 1985; Hartung *et al*., 1990), cucumber (Yu and Matsui, 1994, 1997), watermelon (Kushima *et al*., 1998; Hao *et al*., 2007), taro (Asao *et al*., 2003), strawberry (Kitazawa *et al.*, 2005), tomato (Yu and Matsui, 1993 b), lettuce (Lee *et al*., 2006) and so on. Thus, autotoxicity is considered to be one of the causes of growth retardation on the successive culture of vegetables.

It was found that many leaffy vegetables demonstrated autotoxicity when grown in recycled hydroponics (Asao *et al*., 2001 a). In another study autotoxic substances from eight leaffy vegetables were identified as lactic, benzoic, m-hydroxybenzoic, p-hydroxybenzoic, adipic, succinic, and vanillic acids (Asao *et al*., 2004 b). Benzoic acid was the strongest inhibitor overall for leaffy vegetables among the substances tested. These results confirm that some unidentified compounds inhibited growth of leaffy vegetables in hydroponic culture (Asao *et al*., 2001 b).

Autotoxicity in taro (Colocasia esculenta Schott.) under closed hydroponics

Yield of taro plants decreased when cultivated consecutively for several years on the same land (Takahashi, 1984). Rotation with other crops for at least three years (Miyoshi *et al*., 1971), in combination with organic matter and soil disinfectants (Murota *et al.*, 1984), has been suggested to improve the yield of taro. However, even in a fixed crop rotation system, there was a great difference in the growth and yields of taro plants. Miyaji and Shirazawa (1979) found that taro residues in soils after harvest were inhibitory to its growth.

Asao *et al.* (2003) identified the chemicals exuded by taro roots and evaluated their phytotoxicity on growth and yield of taro using a hydroponic system. GC-MS analysis of taro root exudates detected methyl esters of lactic, benzoic, m-hydroxybenzoic, p-hydroxybenzoic, vanillic, succinic, and adipic acids. Among them benzoic and adipic acids significantly inhibited the growth of taro plantlets at concentrations ranging from 0 to 400 $\mu M\ L^{-1}$. Benzoic acid affected growth of taro plants even at 50 $\mu M\ L^{-1}$. Their conclusion was that the decline in yield in successive culture of taro appeared to be related to the allelochemicals exuded from taro plants.

Autotoxicity in some beans grown with non-renewed culture solution

Edible beans are grown as vegetables and intensively cultivated in the same farmland year after year. The production of these common bean plants and other perennial legumes declines in replanting conditions owing to autotoxicity, a form of intraspecific allelopathy that occurs when

a plant species releases chemical substances that inhibit or delay germination and growth of the same plant species (Putnam, 1985; Miller, 1996; Singh *et al.*, 1999). Allelopathy has been investigated in some beans such as *Pisum sativum* (Kato-Noguchi, 2003), *Mucun pruriens* (Fujii *et al*., 1991), *Glycine max* (Huber and Abney, 1986; Xiao *et al*., 2006; Yan and Yang, 2008), and *Cicer arietinum* (Yasmin *et al.*, 1999). In field experiments, it has been reported that residues and extracts of pea plants suppressed the growth and population size of several plant species (Purvis, 1990; Schenk and Werner, 1991; Tsuchiya and Ohno, 1992; Akemo *et al*., 2000). Phytotoxic substances in *Pisum sativum* root exudates have been reported by several researchers (Hatsuda *et al*., 1963; Yu and Matsui, 1999) and, recently, pisatin has been identified as an inhibitory chemical from its shoots (Kato-Noguchi, 2003).

Removal of the inhibitory chemicals from soils or culture solution can permit continued crop cultivation on the same land for several years. Hydroponic culture technique has the ability to trap and isolate the chemicals released through plant roots. Elimination of these growth inhibitors from recycling culture solution is desirable from the viewpoint of conservation-oriented agriculture. Thus, identification of the allelochemicals from bean root exudates, evaluation of their phytotoxicity, and their removal would facilitate the maintenance of profitable crop production. Asaduzzaman and Asao, (2012) studied autotoxicity in *Pisum sativum*, *Phaseolus vulgaris*, and *Vicia faba*, and their responsible allelochemicals using hydroponics. They also evaluated phytotoxicity of the identified allelochemicals using seedling growth bioassay of the test plants. Results indicated that yield of these beans decreased greatly (over 50%) when grown in non-renewed culture solution, however addition of AC in the culture improved the yield significantly. The identified allelochemicals were benzoic, salicylic, and malonic acids in root exudates of *P. vulgaris* and lactic, benzoic, p-hydroxybenzoic, vanillic, adipic, succinic, malic, glycolic, and p-hydroxyphenylacetic acids in *V. faba*. Bioassay of the identified allelochemicals revealed that benzoic, salicylic, and malonic acids significantly reduced the growth of *P. vulgaris* even at low concentrations.

Autotoxicity in some ornamental plants evidenced in recycled hydroponics

Asao *et al*. (2007 a,b) investigated autotoxicity in some selected ornamentals along with a possible remedial measure to overcome growth inhibition. Among the 37 plants under study, growth of lily, prairie gentian, corn poppy, farewell-to-spring, rocket larkspur, and carnation was drastically reduced in the absence of AC, compared with those in the presence of AC in the nutrient solution. In this study the added AC adsorbed phytotoxic root exudated from nutrient solution leading to improved plant growth. Thus use of AC showed potentiality of overcoming autotoxicity under recycling hydroponics. Root exudates of some plants were analyzed and several organic compounds were detected. Strong growth inhibitors such as lactic acid in pot marigold, benzoic and p-hydroxybenzoic acids in lily, o-hydroxyphenylacetic acid in rocket larkspur, benzoic and p-hydroxybenzoic acids in sweet pea, and maleic and benzoic acids in prairie gentian were detected in the root exudates. The reduced growth of prairie gentian after prolonged cultivation in a field suggested that it could be avoided by amending the soil with AC at a rate of 60 kg 10 a^{-1}.

4. Causes of crop yield reduction in non-recycled hydroponics

Decrease of cucumber yield in non-renewed culture solution

Investigations were conducted to clarify the reasons for fruit yield reduction during a late growing period of cucumber cultured in hydroponic nutrient solution which was not renewed completely (Asao *et al*., 1998 a). It was found that vegetative growth was unaffected by biweekly renewed or non-renewed nutrient solution whereas, fruit yield decreased when nutrient solutions were restored during culture as compared to total renewed or supplemented with AC.

Influence of isolated phenolics on fruit yield in cucumber

Phenolics isolated from the nutrient solution growing cucumber plant had significant influence on fruit yield (Asao *et al.*, 1999 a, 1999 c). The researchers isolated and identified growth inhibiting substances of unknown origin in the nutrient solution culturing cucumber plants. The growth inhibitors were adsorbed on the AC and extracted by an organic solvent. The active substances were analyzed by GC-MS as benzoic acid, p-hydroxybenzoic acid, 2, 4-dichlorobenzoic acid (DCLBA) and phthalic aicd. Among these substances, DCLBA exhibited the strongest inhibitory activity toward cucumber seedlings and thus it was considered the most effective allelochemical of cucumber. It was also found to cause growth inhibition of cucumber seedlings in a seedling growth bioassay. Therefore, it was assumed that this phenol was one of the compounds responsible for the inhibition of cucumber growth in non-renewed hydroponic culture solution. Other researchers have also shown that combinations of certain phenolics can have interaction effects on the germination of various crops and weed species (Williams and Hoagland, 1982). Combination of DCLBA (20 µmol $liter^{-1}$) with benzoic acid, p-hydroxybenzoic acid, and phthalic acid resulted in growth suppression of cucumber (Asao *et al*., 1999 a).

The effects of DCLBA on the number of harvested cucumbers were evaluated by split-root system hydroponic culture (Asao *et al*., 2001 c). DCLBA applied in the nutrient solution at 10 µmol $liter^{-1}$ severely damaged the roots by disrupting the integrity of epidermal cells, and remarkably inhibited the uptake of NO_3^-, $H_2PO_4^-$, and K^+ ions leading to a decreased number of harvested cucumber fruits. DCLBA is a herbicide and also a synthetic auxin, and it stunts cucumber plants at low concentration but kills them at higher levels. This allelochemical was applied through nutrient solution to cucumber seedlings grown hydroponically and exposed to two microorganisms (TS-22 and TS-29) and one Rhizoplane ACI, isolated from soil

and cucumber roots (Asao *et al.*, 2001 b). It was found that cucumber seedlings not exposed to DCLBA and different microorganisms grew vigorously, whereas those exposed to this chemical were stunted.

Effect of temperature and photoperiod on phytotoxins exudation in cucumber

Light and temperature have profound effects on the quality and quantity of exudates because they affect the processess of photosynthesis, translocation, and respiration in plants (Hale *et al*., 1971; Hale and Moore, 1979). An increase in exudation at high temperature has been reported for many crops (Rovira, 1959, Vancura, 1967; Rovira, 1969; Hale *et al*., 1978). The effect of temperature and photoperiod has been assessed on the quality and quantity of growth inhibitors exuded from the root of cucumber (Pramanik *et al*., 2000). The researchers found that exudation rate varied greatly with the kind of acids, temperatures, and photoperiods, ranging from 0.2 to 4.17 $\mu g\ day^{-1}\ plant^{-1}$. Exudation tended to increased with plant growth and maximum exudation rate was recorded with high temperature and long photoperiod, and minimum with low temperature and short photoperiod.

5. Possible measures to control autotoxicity under closed hydroponics

Selection of suitable crop cultivars

Screening for differential autotoxic potential of cucumber cultivars has been conducted in a closed hydroponic system using seedling growth bioassay (Asao *et al*., 1998 b). In this study, some commercial cucumber cultivars were classified into four groups, such as PI 169391, Encore I, Hokushin and Aodai. The cultivars showed intermediate, high, intermediate and low sensitivity to growth retarding substances in culture solutions once used for different cultivars, while growth reduction was not found, except in PI 169391. These findings indicate that there are intraspecific variations in the autotoxic potential of cucumber. Thus, cucumber cultivars with less growth reduction of seedlings in culture solution once used for the same cultivars would be most suitable for culturing in closed hydroponics.

Species differences in autotoxicity susceptibility

In another study species differences in susceptibility to autotoxicity among sixteen leaffy vegetables were studied in hydroponics (Asao *et al*., 2001 a). Among the species, parsley inhibited most severely in the absence of AC followed by celery, edible burdock, garland chrysanthemum, kale, curled lettuce, pak-choi, head lettuce and mitsuba, whereas komatsuna, Chinese cabbage, radish, takana, welsh onion, perilla and spinach were found to be unaffected.

Use of autotoxin-tolerant plant as rootstock

Fruit yield of cucumber in a closed nutrient flow system has been increased by grafting "Shogoin-aonaga-fushinari" on "Hokushin" or "Aodai" seedlings (Asao *et al*., 1999 b). The number of harvested fruit of "Shogoin-aonaga-fushinari" in the summer crop was increased by grafting on the rootstock of "Hokushin" or "Aodai". The decreased weekly number of harvested fruits in the late harvest period on ungrafted plants was not observed on plants grafted on "Hokushin" or "Aodai" seedlings, thereby extending the harvest season. There was no evidence found that rootstocks influence vegetative growth.

Use of bloomless rootstocks to increase number of harvested cucumber fruits

The number of harvested fruits of cucumber cultivar "Shogoin-aonaga-fushinari" grafted on bloomless rootstocks such as "Hikari-power" and "Kitora" was increased slightly when AC was not added, but increased greatly when AC was added in the culture solution (Asao *et al*., 2000). These results indicate that root exudates influence the harvested fruit number, which can be limited in bloomless rootstocks.

Use of AC in addition to dissolved oxygen levels to increase cucumber fruit number

Investigation on the effects of dissolved oxygen levels and addition of AC has revealed a significant influence on fruit yield of cucumber in closed hydroponics (Asao *et al*., 1999 d). Addition of AC had no influence on the dissolved oxygen concentration (1.7-3.1 ppm and 5.0-7.5 ppm after 6 and 24 h aeration, respectively) while fruit yield per plant increased significantly. In this study, aeration hours or dissolved oxygen did not show any effect on yield of cucumber.

Addition of AC to adsorb allelochemicals from recycled culture solution and/or replanting soil

Activated charcoal, with its large surface area, pore volume and polarity, has tremendous adsorption capacity for many organic compounds. In cucumber, tomato, and asparagus, increased productivity has been observed after using AC in non-renewed culture solution or replanting soil (Yu *et al*., 1993; Yu and Matsui, 1994; Asao *et al*., 2003; Motoki *et al*., 2006). Mat-rush does not grow well if it is cultivated consecutively for years in the same land. The continuous cultivation of mat-rush accumulates toxic allelochemicals from roots in soil and inhibits subsequent plant growth. Fujitomi *et al*. (1999) reported an approximate 45% yield reduction in second year cultivation of mat-rush. Asao *et al.* (2007 b) reported that mat-rush seedlings grown in soils collected from fields consecutively cropped with mat-rush for three years had lower shoot dry weight compared to plants grown in soils amended with AC (138 and 165% lower dry weight compared to coarse and fine AC, respectively).

Mitigation of autotoxicity using microbial strain

Microorganisms can degrade chemical substances in soil and water (Sundin and Waechter-Kristensen, 1994) and phytotoxins, both autotoxins and microbial toxins (Caspersen *et al*., 2000; Asao *et al*., 2003, 2004 a; Chen *et al*., 2011). Sim-

ilarly, many isolates from suppressive soils or others can degrade autotoxins in the rhizosphere of continuously cropped plants (Asao *et al*., 2004 a; Chen *et al*., 2011). Growth and yield of cucumber plants significantly decreased with addition of DCLBA (the strongest growth inhibitor released during reproductive stage) to the recycled nutrient solution but growth recovered upon addition of microbial strains. It is suggested that microorganisms, if added to nutrient solution at the reproductive stage of cucumber, can catabolize autotoxic compounds from root exudates into non-toxic compounds and thus increase fruit yield.

Mitigation of autotoxicity by supplementation of 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid

Phenolic compounds disrupt the endogenous hormonal balance in plants (Rice, 1984; Asao *et al*., 2001 c). Benzoic acid or similar structural compound substitution has been considered as anti-auxinous (Keitt and Barker, 1966; Karabaghli-Degron *et al*., 1998). Callis (2005) reported that fruit enlargement and maturation of strawberry depends on auxin. Thus, the effects of foliar applications of 2,4-D and NAA on growth of strawberry were investigated to mitigate the autotoxicity in growing plants in closed hydroponic systems (Kitazawa *et al*., 2007). Supplementation of 5.4 μM NAA was found to be the most effective treatment for alleviating autotoxicity of strawberry and increasing fruit yield.

Mitigation of autotoxicity by supplementation of amino acids

Amino acids are the nitrogenous compounds which form the basic component of all living cells (Furuya and Umemiya, 2002). Therefore, they have great potential for use in culture techniques: recently they were applied as foliar spray to improve the growth, yield and quality of several crops (Mazher *et al*., 2011; Takeuchi *et al*., 2008). Similarly, supplementation of amino acids has been investigated to recover growth and yield of strawberry plants with autotoxicity in closed hydroponics. In greenhouse experiments, 22 water soluble amino acids were sprayed on strawberry plants at 2 ml per plant and among them glutamic acid and hydroxy-proline spray produced 50% greater fruit yield compared to control water spray in plants grown under non-renewed culture (Mondal *et al*., 2013).

Mitigation of autotoxicity through electrodegradation of root exudates

In recycled hydroponic culture, significant growth inhibition of strawberry has been observed due to accumulation of toxic root exudates in the nutrient solution; benzoic acid was found to be the most potent growth inhibitor in root exudates (Kitazawa *et al*., 2005). Electrochemical methods have also been applied for degradation/oxidation of phenols and their derivatives from organic waste or pollutants by several researchers. Phenolic compounds, including phenols, catecol and hydroquinone in aqueous solution and even benzene were found to decompose when treated by electro-degradation (Fleszar and Ploszynka, 1985; Comninellis and Pulgarin, 1991; Feng and Li, 2003). These compounds are oxidized rapidly at the anode and decompose to CO_2. Thus, electrodegradation may cause the decomposition of allelochemicals, including benzoic acid exuded in the nutrient solution from plants, offering a possibly useful tool to mitigate autotoxicity in strawberry. Based on these results, electrodegradation has been attempted for the decomposition of benzoic acid in the culture solution (Asao *et al.*, 2008). It was found that benzoic acid exogenously added to nutrient solution was almost completely decomposed within 24 h. Electrodegradation of nutrient solution could mitigate autotoxicity in strawberry plants grown in closed hydroponic culture and it recovered fruit yield up to 71% of control. The appropriate timing and intensity of electrodegradation of nutrient solution has also been investigated and it was recommended that application of electrodegradation to non-renewed culture solution for 2 h at four-week intervals can recover fruit yield completely (99%) compared to non-renewed culture solution without electrodegradation (Asaduzzaman *et al*., 2012).

Selection of ideal succeeding crops after asparagus, taro and beans

Successive culture of the same crop on the same land for years causes soil sickness or replanting injuries (Rice, 1984; Tsuchiya, 1990) resulting in reductions in both crop yield and quality. Among the possible reasons for this complex natural phenomenon self-allelopathy, or autotoxicity, has often been suggested (Asao *et al*., 2003; Asaduzzaman and Asao, 2012). This phenomenon has been evidenced in asparagus (Hartung and Stephens, 1983; Young and Chou, 1985; Lake *et al*., 1993), taro (Takahashi, 1984) and several beans (Putnam, 1985; Miller, 1996; Singh *et al*., 1999). Therefore, growth performances of 67 vegetable crop cultivars were evaluated through seedling growth bioassay using once used nutrient solution of asparagus and also replanting soil of asparagus, taro and three beans (Asaduzzaman *et al*., 2013 b). Strategies to overcome this problem in replanting soil or reuse of hydroponic culture solution have been suggested by many researchers. Bioassays using asparagus with used nutrient solution, with or without AC, suggest cucumber, garden pea, komatsuna, melon, pak-choi cv. 'Tyoukou', parsley, soybean (except cv. 'Tankurou'), cabbage cv. 'Early Ball' and lettuce cv. 'Shato' as possible succeeding crops. While, bioassays using replanting soil, with or without AC, have suggested that most of the cultivars tested can be planted after asparagus, taro, and three beans (*Vicia faba* L., *Pisum sativum* L. and *Phaseolus vulgaris* L.) with little adverse effects. Among the three methods of bioassay used (i.e. nutrient solution, direct seed sowing and seedling transplanting in replant soil) the nutrient solution bioassay proved more sensitive than replanting soil bioassay. However, results of nutrient solution bioassay may not be reproducible in field conditions. Therefore, the seedling transplanting method can be used as an easy and practical bioassay approach to select succeeding crops for fields with replanting problems.

6. Future challenges and endeavors in horticultural research in Japan

Currently Japan faces problems of an aging population, mainly in rural areas. In fact, many researchers are trying to breed new high quality cultivars to develop labor-saving and environmental-friendly technologies for horticultural cultivation. Tomato production in Japan is an especially up and coming market that needs to be looked at much more closely. In this regard, outdated hydroponic systems should be up-scaled with modern facilities. A number of favorable resources are found in Japan, such as rivers, the ocean, and a relatively temperate climate, and they can make greenhouse productions much more sustainable. Through the use of wind, solar and geothermal power these new greenhouses can be brought into sustainable horticultural production.

Recent advances in solar technology have created an outstanding 17% conversion efficiency in solar panels. These free-standing towers can be set up on the south end of greenhouses on an automatic swivel to rotate with the movement of the sun to ensure optimal light intensity at all times of the day. This solar energy collected during the day can be used to heat water tanks under the greenhouse for use during the night to heat the greenhouses as needed. Through the generation of electricity with wind and solar power, heating and electricity needs beyond these systems will be minimal, greatly reducing greenhouse electricity consumption from less sustainable sources. Therefore, development of Plant Factory supported facilities with this geothermal energy system should be expanded throughout Japan.

According to "Japanese Society of Nephrology" about 13.3 million people suffer from CKD (i.e. one in every eight adults) and it expected that the total number of patients will continue to increase progressively. Therefore, preventive measures should be stressed to divert the prevalence of this disease toward a decreasing trend. CKD patients are restricted from consuming K rich foods but our typical daily diet includes fruits, fresh vegetables, seaweed, beans and potatoes with high K content, making it difficult for sufferers to eat their usual diet with other family members. This restriction impacts on their quality of life greatly.In general, research efforts are directed at controlling the growth and yield of plants, however the complexity of plant responses to culture systems and environmental factors should be stressed in particular cases. Indeed, horticultural techniques can be a good way to improve fruit quality and increase the bioactive compounds in plants. Toward this aim, simple management of nutrient solutions, together with good knowledge about cultural practices of each particular crop and application of suitable crop technologies are key for controlling the nutritional composition of plants.

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Adv. Hort. Sci., 2014 28(4): 179-183 *Review article*

Flowering response to blue light and its molecular mechanisms in *Arabidopsis* and horticultural plants

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Key words: cut flower, light-emitting diode, lighting culture, plant factory, vegetable.

Abstract: Using light-emitting diodes (LEDs) in plant factories and protected horticulture is expected to decrease energy costs and environmental burden. Because LEDs emit monochromatic light, detailed knowledge of plant responses to light quality is essential for efficient and appropriate utilization of LEDs in horticultural production. Timing of flowering is important in cut flower and fruit/vegetable production, and it is often affected by light quality. Red/far-red light is well known to be effective in flowering, and there is abundant knowledge about the effects of red/far-red ratio on flowering of horticultural plants as well as of the model plant *Arabidopsis thaliana*. However, studies have not focused on the effects of blue light on flowering. Therefore, this review describes the progress in the promotion of flowering by blue light and its molecular mechanisms in *Arabidopsis* and horticultural plants.

1. Introduction

The utilization of light-emitting diodes (LEDs) is increasing because of their technological improvements and reduction in prices. Because LEDs emit monochromatic light, studying the effects of light quality on plant growth has become important for efficient utilization of LEDs in horticultural production. Plant factories are a promising system of horticultural production where the completely artificial environment may lead to efficient photosynthesis, year-round production unaffected by climate, farming without insecticides, and effective utilization of resources such as water (Kozai, 2013). However, plant factories consume large amounts of energy for lighting and air conditioning, which may result in global warming and high fuel costs and electricity bills. Using LEDs, which consume less electricity and have a long life, may reduce energy consumption and production costs. LEDs emit little thermal irradiation and can be used in close proximity with plants, making them suitable for multistage production to increase unit production (Goto, 2012).

LEDs are also expected to be useful in lighting culture, where flowering time is controlled by night-time lighting for cut flower production in greenhouses, because incandescent lamps, which have been commonly used for long-day treatments, are characterized by high energy consumption and short life. *Chrysanthemum morifolium* (hereby referred to as chrysanthemum), the most commonly used cut flower in Japan, is a short-day plant; it is produced year-round by lighting culture, repressing flowering by long-day treatments. Long-day cut flowers can also be produced under short-day conditions during autumn and winter by long-day treatments to promote flowering.

The effects of monochromatic light quality on plant growth, morphogenesis, and metabolism need to be investigated for the development of horticultural production using LEDs. Blue, red, and far-red lights are already known to be effective in plant morphogenesis and flowering, and molecular mechanisms underlying the regulation of flowering by each light color have been proposed in studies using the model plant *Arabidopsis thaliana*, which is a long-day plant. Far-red light or a low red/far-red ratio, which promotes flowering in *Arabidopsis*, reportedly promotes flowering in long-day cut flowers such as *Eustoma grandiflorum* and *Gypsophila paniculata* (baby's breath) (Yamada *et al.*, 2008, 2009; Nishidate *et al*., 2012). However, studies have not focused on the effects of blue light on flowering; blue light could also be important in timing of flowering. Therefore, this review describes flowering response to blue light and its molecular mechanisms in *Arabidopsis* and horticultural plants with the aim of advancing research on the utilization of LEDs in horticultural production.

2. Flowering response to light quality and its molecular mechanisms

Flowering is triggered upon the expression of *flowering locus T* (*FT*), which is a key flowering integrator, in

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the phloem of leaves induced by CONSTANS (CO) under long-day conditions in the photoperiodic flowering pathway proposed for *Arabidopsis* (An *et al*., 2004). *CO* expression is regulated downstream of the circadian clock, and *CO* mRNA is expressed during the dark period under short-day conditions and during the light period (late afternoon) under long-day conditions (Suârez-Lôpez *et al*., 2001). Because CO protein translated from CO mRNA is degraded through ubiquitination by an E3 ubiquitin ligase, CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1), during the dark period, the expression of *FT* and consequent induction of flowering are not triggered under short-day conditions (Jang *et al*. 2008; Liu *et al*. 2008 b).

In *Arabidopsis*, flowering is promoted under long-day conditions with blue and far-red lights but not with red light (Eskins, 1992). Blue light may induce *FT* expression by stabilizing CO, as described below. Far-red and blue light signaling through phytochrome A and through CRYPTOCHROME 1 (CRY1) and CRY2, respectively, stabilize CO protein by an antagonistic function to red light signaling through phytochrome B that induces CO degradation (Mockler *et al*., 1999, 2003; Valverde *et al*., 2004). As a result, the accumulation of CO protein increases during the light period (late afternoon), and the expression of *FT* and consequent induction of flowering are triggered under long-day conditions.

FT protein expressed in leaf phloem moves to the apical meristem through sieve tubes (Corbesier *et al*., 2007) and activates floral identity genes such as *APETALA1* and *FRUITFUL* by interacting with FLOWERING LOCUS D (FD), a bZIP transcription factor (Abe *et al*., 2005; Wigge *et al*., 2005). In addition, FT activates *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) to promote flowering (Yoo *et al*., 2005).

3. Molecular mechanism underlying the promotion of flowering mediated by blue light signaling via FKF1

Flavin-Binding Kelch repeat F-box 1 (FKF1), which has flavin mononucleotide as a chromophore, and GIGANTEA (GI) mediate *CO* transcription in the photoperiodic flowering pathway (Fig. 1) (Nelson *et al*., 2000; Imaizumi *et al*., 2003; Martin-Tryon *et al*., 2007). They are important for the promotion of flowering because in the *CO* promoter region, FKF1 forms a complex with GI in a blue light-dependent manner, induces the degradation of CYCLING DOF FACTOR 1 (CDF1) to repress *CO* transcription, and promotes *CO* transcription (Fig. 2) (Imaizumi *et al*., 2005; Sawa *et al*., 2007). In detail, the F-BOX domain of FKF1 interacts with *Arabidopsis* Skp1-like proteins to form the E3 ubiquitin ligase complex, and this complex induces CDF1 degradation by ubiquitination (Yasuhara *et al*., 2004; Imaizumi *et al*., 2005; Sawa *et al*., 2007). *Arabidopsis* has multiple CDF family proteins, which redundantly repress *CO*, and the degradation of another CDF family protein CDF2 is also induced by the interaction of FKF1 with GI in a blue light-dependent manner (Fornara *et al*., 2009).

In addition, FKF1, GI, and CDF1 are proposed to regulate *FT* transcription in the same manner as *CO* transcription regulation (Sawa and Kay, 2011; Song *et al*., 2012). Furthermore, FKF1 stabilizes CO by interaction of the LOV domain of FKF1 with CO protein, which is enhanced by blue light (Song *et al*., 2012). Thus, FKF1 transduces blue light signals to promote flowering in a complex manner.

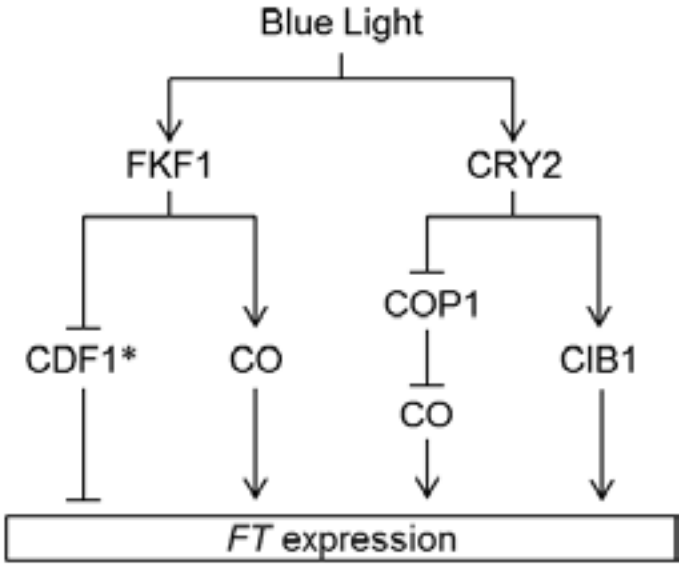

Fig. 1 - Proposed model for the induction of *FT* expression by blue light through two photoreceptors (Jang *et al*., 2008; Liu *et al*., 2008 a, b; Sawa and Kay, 2011; Zuo *et al*., 2011; Song *et al*., 2012). FKF1 stabilizes CO protein to activate *FT* expression in a blue light-dependent manner. CRY2 activates CIB1 as a transcription factor to induce *FT* expression and inhibits COP1, inducing the degradation of CO protein during the light period in a blue light-dependent manner. *See figure 2.

4. Molecular mechanism underlying the promotion of flowering mediated by blue light signaling via CRY2

CRY is a blue/UVA photoreceptor in plants (Cashmore *et al*., 1999). In *Arabidopsis*, the expression and function of CRY2 in vascular bundles regulates flowering (Endo *et al*., 2007). COP1 controls the accumulation of GI and CO proteins, that is, COP1-mediated degradation of GI and CO proteins during the dark period is repressed by CRY2 during the light period to promote flowering (Fig. 1) (Jang *et al*., 2008; Liu *et al*., 2008 b; Yu *et al*., 2008). COP1-mediated degradation of CO is repressed by blue light-dependent interactions between CRY2, COP1, and SUPPRESSOR OF PHYA-105 1 (SPA1) (Zuo *et al*., 2011).

CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP-HELIX 1 (CIB1) has been isolated as an interacting factor of CRY2, and its interaction with CRY2 is promoted by blue light. CIB1 induces *FT* transcription to promote CRY2-dependent floral initiation (Liu *et al*., 2008 a). CIB1 is believed to bind to E-box (CANNTG) elements in the promoter region of *FT* and stimulate *FT* mRNA expression (Liu *et al*., 2008 a). CIB family proteins, containing CIB1, form heterodimers, and notably, in comparison with homodimers, some of these heterodimers have higher binding

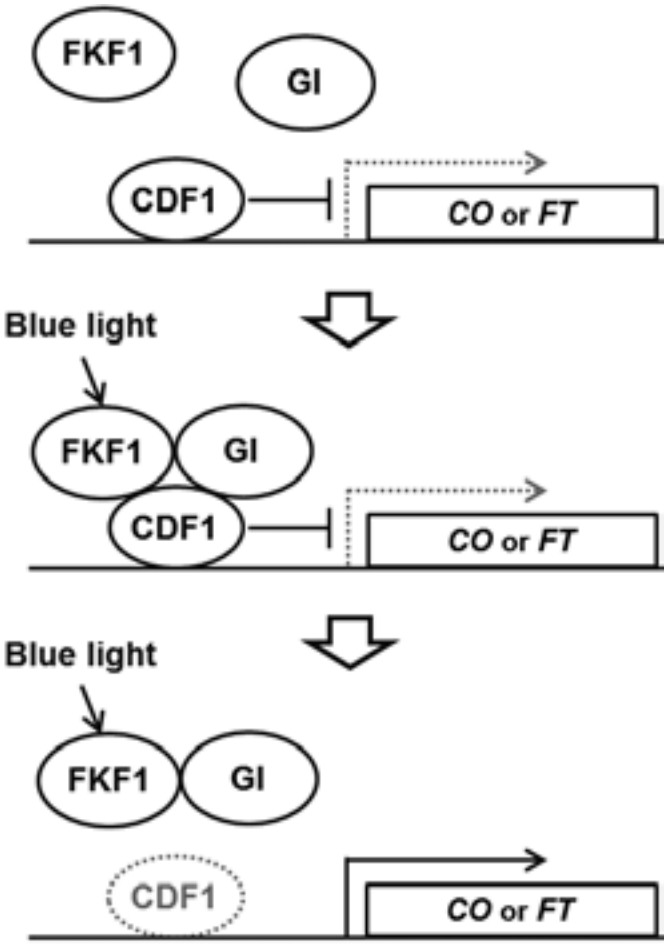

Fig. 2 - Proposed model for the induction of *CO* and *FT* expression by blue light through FKF1 (Yasuhara *et al*., 2004; Imaizumi *et al*., 2005; Sawa *et al*., 2007; Sawa and Kay, 2011; Song *et al*., 2012). CDF1 represses *CO* and *FT* expression under short-day conditions. FKF1 forms a complex with GI and CDF1 on *CO* and *FT* promoters in a blue light-dependent manner in the late afternoon under long-day conditions, and then induces the degradation of CDF1 protein by ubiquitination. As a result, the expression of *CO* and *FT* are induced because of the absence of CDF1.

affinity to E-box elements. This suggests the importance of the interaction between CIB proteins and E-box elements in the induction of *FT* expression (Liu *et al*., 2013 b).

Although the interaction of CRY2 with CIB1 plays a role in *FT* expression in response to blue light, the stabilization of CIB1 protein may be controlled by other blue light photoreceptors such as ZEITLUPE (ZTL) and LOV KELCH PROTEIN 2 (LKP2). ZTL and LKP2, belonging to the LOV domain of blue light receptors containing FKF1, are required for suppressing the degradation of CIB1 protein by blue light (Liu *et al*., 2013 a).

5. Flowering response to blue light in long-day horticultural plants

In some long-day horticultural plants, similar in behavior to *Arabidopsis,* the promotion of flowering by blue light under long-day conditions has been reported. Flowering is promoted by inducing the expression of *FBP28*, a petunia homolog of the flowering accelerator *SOC1* in *Petunia*, an important potted and bedding plant (Fukuda *et al*., 2011). The flowering of a popular cut flower *E. grandiflorum* can be promoted under short-day conditions by a night break with blue light as well as with far-red light or a low red/far-red ratio (Yamada *et al*., 2011). The effect of light quality on flowering has also been investigated in long-day fruit/vegetable production. Ever-bearing strawberry cultivars (*Fragaria x ananassa*) are long-day plants in contrast with June-bearing strawberry cultivars, which are short-day plants. Similar to *Arabidopsis*, the flowering of ever-bearing strawberry cultivars is promoted by long-day treatments with blue as well as far-red light (Nishiyama and Kanahama, 2009; Yoshida *et al*., 2012).

These reports indicate that the flowering of various long-day horticultural plants is promoted under long-day conditions with blue light. However, flowering is not promoted by long-day treatments with blue light, but it is promoted with far-red light in *G. paniculata* 'Bristol Fairy,' which is a popular cut flower frequently used in flower arrangements (Hori *et al*., 2011; Nishidate *et al*., 2012). In a study conducted by Hori *et al*. (2011), flowering in *Arabidopsis* was reportedly induced with *SOC1* expression by long-day treatments with far-red light, whereas it was induced with both *FT* and *SOC1* expression by long-day treatments with blue light. In contrast, although flowering in *G. paniculata* 'Bristol Fairy' was induced with the expression of the *SOC1* homolog of *G. paniculata* (*GpSOC1*) by long-day treatments with far-red light, flowering was not induced because of the low expression of the *FT* homolog of *G. paniculata* (*GpFT*) and *GpSOC1* by long-day treatments with blue light. To the best of our knowledge, there has been no report of blue light receptors FKF1 and CRY in these horticultural plants. Further studies are needed to understand the molecular mechanisms underlying the diversity in flowering response to blue light.

6. Flowering response to blue light in short-day and day-neutral horticultural plants

In short-day horticultural plants, flowering is usually repressed by a night break with red light under short-day conditions; there are only a few studies on flowering response to blue light in short-day horticultural plants. In chrysanthemum, a major short-day cut flower, flowering is repressed by a 4-h night break with blue light or far-red light under short-day (12 h) conditions with blue light (Higuchi *et al*., 2012). However, flowering of chrysanthemum is not repressed by 4-h end-of-day irradiation with blue light under 11-h photoperiods with mixed red and blue lights (Jeong *et al*., 2014). These studies suggest that in chrysanthemum, blue light affects flowering under some limited conditions, but red light plays a major role in the repression of flowering.

Solanum lycopersicum (tomato), the most important fruit/vegetable, is well known as a day-neutral plant. Tomato plants flower after vegetative growth regardless of photoperiods, and their flowering is believed to be unaffected by photoperiods. However, the node position of the first flower truss under a lower blue/red ratio has been reported to be lower than that under a higher blue/red ratio (Nanya *et al*., 2012); the constitutive expression of the *CRY2* homolog of tomato *LeCRY2* delays flowering (Giliberto *et al*., 2005), suggesting that blue light represses flowering in tomato plants. It is important to investigate the tomato homologs of genes that play roles in light signaling in the photoperiodic flowering pathway because the *FT* homolog of tomato *SFT* is a flowering activator, and heterozygosity for loss-of-function alleles of SFT increases the yield (Krieger *et al*., 2010).

7. Concluding remarks

Similar to *Arabidopsis*, blue as well as far-red light-dependent promotion of flowering in long-day horticultural plants has been reported. On the other hand, flowering was not promoted by blue light, but by far-red light in a cultivar of another long-day plant, *G. paniculata*. This diversity in flowering response to blue light is notable, and gaining knowledge about it is important for promoting the utilization of LEDs in plant factories and lighting culture.

The flowering of *Arabidopsis* is promoted by blue light in a complex manner through FKF1 and CRY. There is a paucity of information on the molecular mechanisms underlying blue light signaling in the promotion of flowering in horticultural plants, although there are few studies on the relationship between flowering and expression of flowering-related genes under long-day conditions with blue light. Gaining knowledge at the molecular level about blue light signaling in long-day and day-neutral horticultural plants would contribute to the introduction of novel traits in molecular breeding.

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A novel aeroponic technique using dry-fog spray fertigation to grow leaf lettuce (*Lactuca sativa* L. var. *crispa*) with water-saving hydroponics

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Key words: ascorbic acid, hydroponics, root respiration, soilless culture, sprayponics.

Abstract: Growth characteristics of lettuce cultivated using a novel "dry-fog" hydroponic technique were investigated and compared to lettuce cultivated using deep flow technique (DFT) as the prevailing hydroponic technique. Dry-fog hydroponics is an aeroponic technique that sprays a very fine foggy nutrient solution with an average droplet diameter of less than 10 μm into the root zone. The roots extend into the chamber filled with dry-fog of the liquid fertilizer and absorb water and nutrients from the dry-fog that fills the rhizosphere. This soilless culture system needs less water than any other hydroponic technique, and no differences were found in growth and harvest quality of plants between the two tested sytems. For dry-fog culture, root growth was encouraged and root hair significantly developed to catch the foggy nutrient solution efficiently. The contents of ascorbic acid, nitrate nitrogen, Ca^{+} and chlorophyll of leaves were not significantly different between the two hydroponic cultures. However, respiration rate of roots and photosynthetic rate of leaves significantly increased with dry-fog culture. Because the amount of water around the roots is less with dry-fog, horticultural crops are expected to grow well with this novel hydroponic technique which optimizes the growth and quality of plants with water-saving hydroponics.

1. Introduction

To address the food shortages in the near future caused by a decrease in arable land or increase in the world population, greenhouse horticultures, especially hydroponic cultures without soil, are becoming more important. While one advantage of a hydroponic culture is that plants can be cultivated under optimally controlled conditions for nutrients regardless of the soil conditions, a large amount of water is required to grow crops with nutrient solution. New hydroponic systems that enable stable crop production while saving water have been anticipated in recent years. Aeroponics is a water-saving hydroponic technique without rooting media (Weathers and Zobel, 1992; Ritter *et al*., 2001; Farren and Mingo-Castel, 2006). In this culture, liquid fertilizer is periodically sprayed on the roots from nozzles with pipes in the rooting zone. The sprayed nutrient solution that is not absorbed by the roots is usually re-sprayed using a re-circulation system or it is discarded directly. Tomato, one of the most important crops world-wide, has recently been cultivated using aeroponics (Biddinger *et al*., 1998; Zhao *et al*., 2010). One of the features of this culture system is nutrient accessibility in which nutrients are absorbed efficiently by the roots. Furthermore, root growth in most vegetable crops is expected to increase under the aerobic conditions of the rhizosphere environment because there is no solid phase and less liquid phase compared to the physical composition of soil or any other rooting media (Hillel and David, 1988; Cherif *et al*., 1997).

Dry-fog aeroponics is a novel hydroponic technique that sprays a very fine fog of atomized liquid fertilizer using a specialized nozzle as a double-fluid atomizer with nutrient solution and compressed air (developed and patented by Ikeuchi Co., Ltd., Osaka, Japan). Dry-fog is the finest fog and is less than 10 μm in diameter on average per droplet. The droplets are too small to wet objects, such as roots. Currently, dry-fog is mainly used to humidify factories producing electronic components or printing to keep the environment static-free and has not yet been applied to crop cultivation in commercial horticulture. In dry-fog aeroponics, dry-fog fertilizer is atomized using a specialized nozzle in the rhizosphere where the plant roots directly absorb water and nutrients from the dry-fog (Fig. 1), like an aerial root of epiphytic orchids. Because the fine, foggy, nutrient solution is atomized, only one specialized nozzle can occupy the large space for the rooting zone in a chamber (1000×660×300 mm), with a smaller amount of water compared to the other types of predominant spray hydroponic techniques; therefore, dry-fog aeroponics can reduce water usage and the number of nozzles that are needed, resulting in a lower running cost and greater environmental conservation. The dry-fog nutrients that are not being absorbed by the roots accumulate as condensation on the inner wall of the cultivation chamber, and the collected condensation in the nutrient reservoir is atomized again. Thus, there

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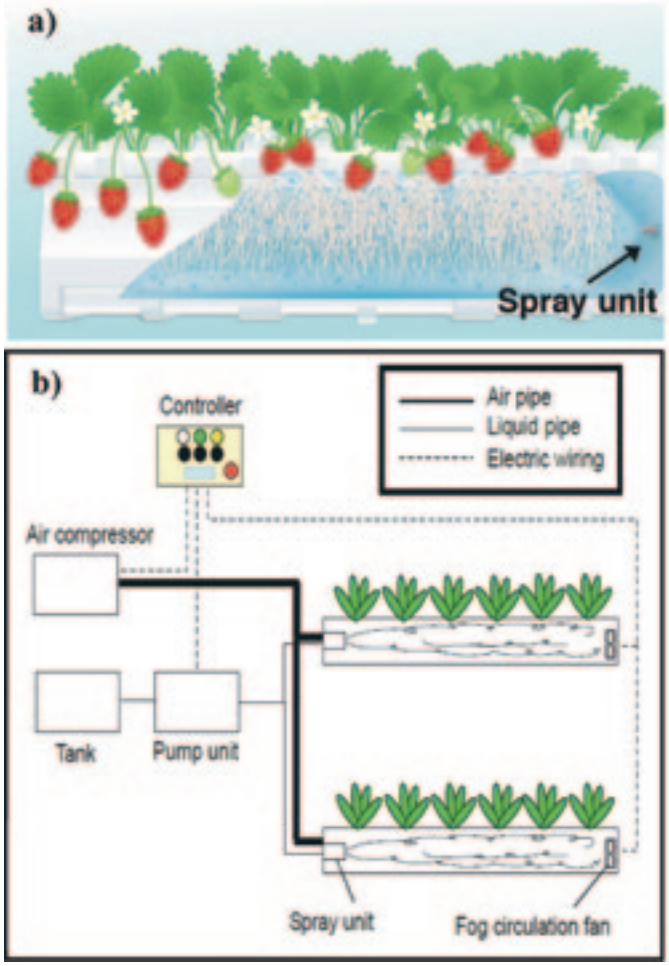

Fig. 1 - a) Cutaway drawing of the dry-fog hydroponic chamber. b) System chart of the dry-fog hydroponic technique.

is little puddling of the fertilizer solution in the chamber, and the roots hang in the dry fog. An ultrasonic humidifier also can atomize the very fine fog, and has been used for many bioreactors to generate the nutrient fog (Mohammad *et al*., 2000; Lin *et al*., 2003; Jing and Yunwei, 2013). In contrast, it is believed that the nozzle used for dry-fog aeroponics atomizes a larger amount of fine fog at a low cost and without problems. Dry-fog aeroponics has not yet been investigated or established as a commercial cultivation system for horticultural crop production.

In the present study, leaf lettuces were cultivated using dry-fog aeroponics in a controlled environment. The growth and leaf constituents were measured and compared to plants cultivated with traditional hydroponic techniques (Deep Flow Technique) to evaluate the efficacy of dry-fog aeroponics. Root respiration and leaf photosynthetic rate were also investigated and compared in order to evaluate affects on water and nutrient absorption resulting from differences in the rhizosphere environment in the two hydroponic systems.

2. Materials and Methods

A plastic chamber (1000×660×300 mm) was fitted with a dry-fog atomizing nozzle to establish a dry-fog cultivation system (Fig. 1). A double-fluid atomizer nozzle was connected to the nutrient solution reservoir with a plastic tube (6 mm across) and a compressor with Teflon tube (8 mm across), with an air pressure of 0.3 MPa. Size fractionation of atomized droplets was measured using LDSA-SPR (Tohnichi computer applications Co. Ltd., Tokyo, Japan), which analyzes the droplets by Fraunhofer diffraction and calculates the size distribution of the droplets by Rosin-Rammler distribution. A commercial liquid fertilizer (Otsuka Chemical Co. Ltd., Tokyo, Japan, EC 1.2 mS cm^{-1} , pH 6.0, N: 130 ppm, P: 60 ppm, K: 200 ppm, Ca: 115 ppm, Mg: 30 ppm, Fe: 1.4 ppm, Mn: 0.8 ppm, Zn: 0.05 ppm, Cu: 0.02 ppm, B: 0.75 ppm, Mo: 0.02 ppm) was added to the nozzle from a nutrient solution reservoir equipped at the end of an aeroponic chamber. The nozzle atomized fine foggy nutrients that continuously filled the chamber. Each chamber had 48 holes (20 mm across) across the top to hold plants and only the roots remained in the chamber. As a control experiment, a Deep Flow Technique (DFT) hydroponic chamber (800×665×310 mm) was filled with 30 L of the same liquid fertilizer. Leaf lettuce (*Lactuca sativa* L. cv. Okayama Saradana, Takii Co. Ltd., Kyoto, Japan) was sown on sponge blocks (10×10×20 mm) supplied with enough pure water. The air temperature and relative humidity throughout the cultivation were maintained at 26°C and 60% respectively. The photosynthetic photon flux density was 250 μmol m^{-2} s^{-1} supplied by cool white tubular fluorescent lamps with a 16-h day length. After germination, the liquid fertilizer (EC 0.6 mS cm^{-1}, pH 6.0) was supplied by bottom irrigation for two weeks, and 48 seedlings that had grown uniformly were transplanted into each hydroponic system and grown for four weeks. Every week, plants were sampled from both hydroponic systems and the fresh weight, leaf length, and fresh weight of roots were recorded. After sampling, the leaves and roots were dried in an oven at 80°C for two days and the dry weights were recorded. At three and four weeks after transplantation, intact root samples (1 g FW) were taken from a growing plant that developed new roots under hydroponic conditions, and the rate of root respiration was measured polarographically at 26°C using a Clark-type gas-phase oxygen electrode (CB1D, Hansatech, Norfolk, UK) with incoming humidified air and 21% O_2 under dark conditions.

The contents of nitrate nitrogen, calcium ion, ascorbic acid and chlorophyll in the leaves were measured at three and four weeks. Leaves (10 g FW) were sampled to determine nitrate nitrogen and calcium contents. The leaves were homogenized in de-ionized water and the nitrate nitrogen and calcium contents in the filtrated fraction were measured using RQ-FLEX (Merck Millipore, Darmstadt, Germany). The other leaves (10 g FW) were also sampled and homogenized in a 5% metaphosphoric acid solution, and the content of ascorbic acid in the filtrated fraction was measured using RQ-FLEX. The content of leaf chlorophyll was measured using SPAD meter (SPAD-502, KONICA MINOLTA, INC., Tokyo, Japan). At four weeks after planting, CO_2 assimilation rates (A) of fully expanded mature leaves were measured under different CO_2 concentrations (50-1500 μmol mol^{-1}) using an LI-6400 portable photosynthesis system

(Li-Cor, Lincoln, NE, USA). The measurement conditions were leaf temperature, 20°C, and photosynthetic photon flux densities, 1200 μmol m^{-2} s^{-1}. The maximum rate (Max) and apparent CO_2 fixation efficiency (ϕ) were estimated as parameters of the best-fitted non-rectangular hyperbola for the photosynthetic responses to intercellular CO_2 concentrations (Ci). All data were subjected to one-way analysis of variance (ANOVA), and the mean differences were compared using the Tukey HSD test when the *F*-test indicated a significant difference at $P \leq 0.01$. Each data point was the mean of six replicates and a comparison with $P \leq 0.05$ was considered significantly different.

3. Results and Discussion

In order to characterize dry-fog spray fertigation conditions, the fog was measured using LDSA-SPR of droplets of various sizes (Fig. 2). The minimum and maximum diameter of droplets were 1.64 μm and 149 μm, respectively. LDSA-SPR analyzed the size distribution of droplets, and the Sauter mean diameter, which commonly means the average particle size of powders and liquid droplets, was found to be 8.85 μm by calculation. Therefore, the fog in the chamber was certainly dry-fog.

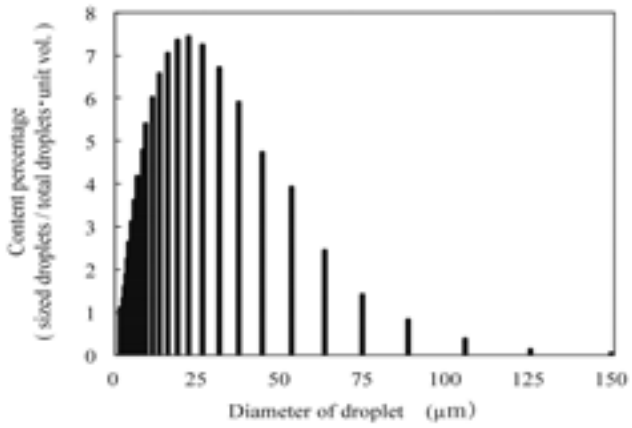

Fig. 2 - The particle size distribution of droplets using a double-fluid atomizer nozzle to establish a dry-fog hydroponic technique measured by LDSA-SPR. The mean diameter of droplets was analyzed and calculated based on this distribution.

There was no significant difference in the fresh and dry weight of leaves between the dry-fog and DFT hydroponic cultures (Fig. 3). Root growth was not significantly different at three weeks after planting, but during the fourth week of dry-fog culture the root fresh weight increased by 29% compared to roots grown using DFT. The roots from the dry-fog culture had many lateral roots, and many root hairs developed on the surface. This development was not observed in the roots grown using DFT (Fig. 4). The root respiration rate at four weeks after planting was significantly high in the dry-fog culture, but there was no significant difference at three weeks between the two hydroponic

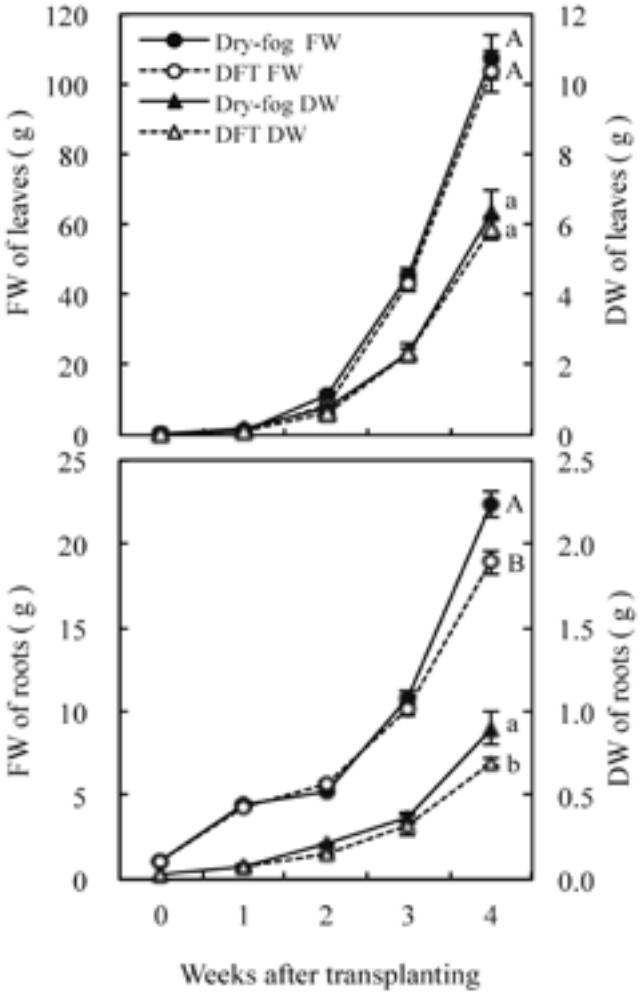

Fig. 3 - Changes in fresh and dry weights of lettuce leaves and roots grown using dry-fog or DFT hydroponics during the four weeks after transplanting. Different uppercase and lowercase letters show a significant difference ($P \leq 0.05$), bars represent ±SE (n=6).

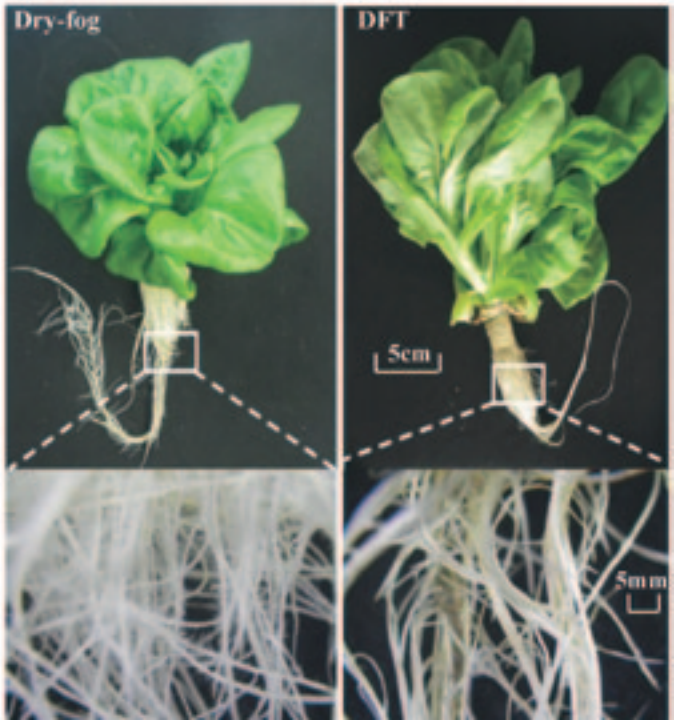

Fig. 4 - Leaves and roots of the lettuce grown using dry-fog or DFT hydroponics at four weeks after transplanting.

systems (Fig. 5). The photosynthetic rates of the mature leaves grown for four weeks dry-fog culture significantly increased in both limited and saturated Ci compared to those in DFT (Fig. 6). In both hydroponic cultures, the photosynthetic rate increased linearly up to approximately 300 µmol mol^{-1} Ci and saturated at approximately 600 µmol mol^{-1} Ci. Leaf nutrient contents were compared between the two hydroponic cultures at three and four weeks after transplanting (Fig. 7). The nitrate nitrogen content in the leaves grown using dry-fog culture significantly

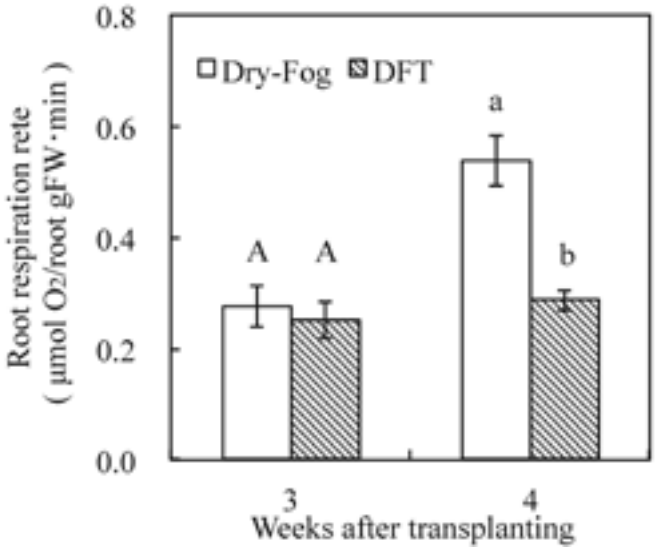

Fig. 5 - Respiration rates of lettuce roots grown using dry-fog or DFT hydroponics for three and four weeks after transplanting. Different letters show significant difference ($P \leq 0.05$), bars represent ±SE (n=6).

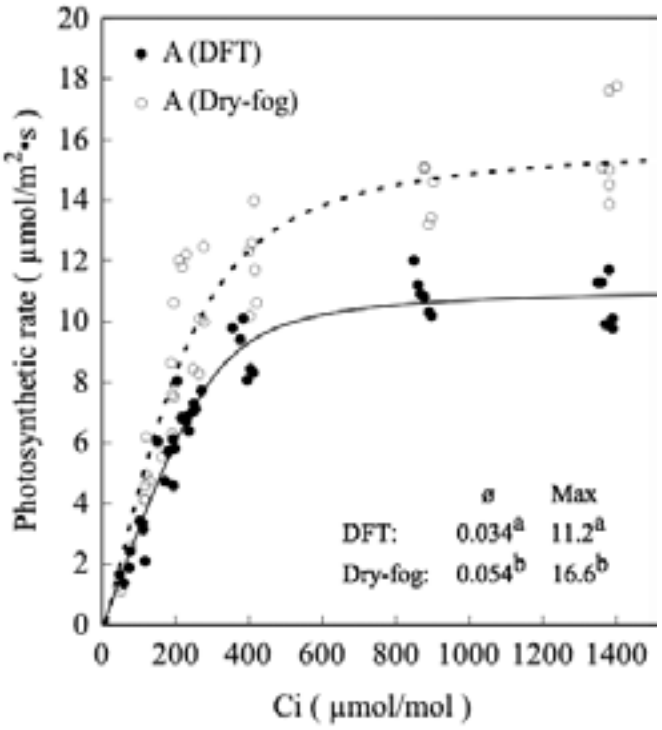

Fig. 6 - Relationships between CO_2 assimilation rates (A) and intercellular CO_2 concentration (Ci) measured for the attached mature leaves of lettuce grown using dry-fog or DFT hydroponics for four weeks after transplanting. The saturated rate (Max) and the apparent CO_2 fixation efficiency (ϕ) were estimated as parameters of the best-fitted non-rectangular hyperbola response curve to Ci. Different letters show significant difference ($P \leq 0.05$).

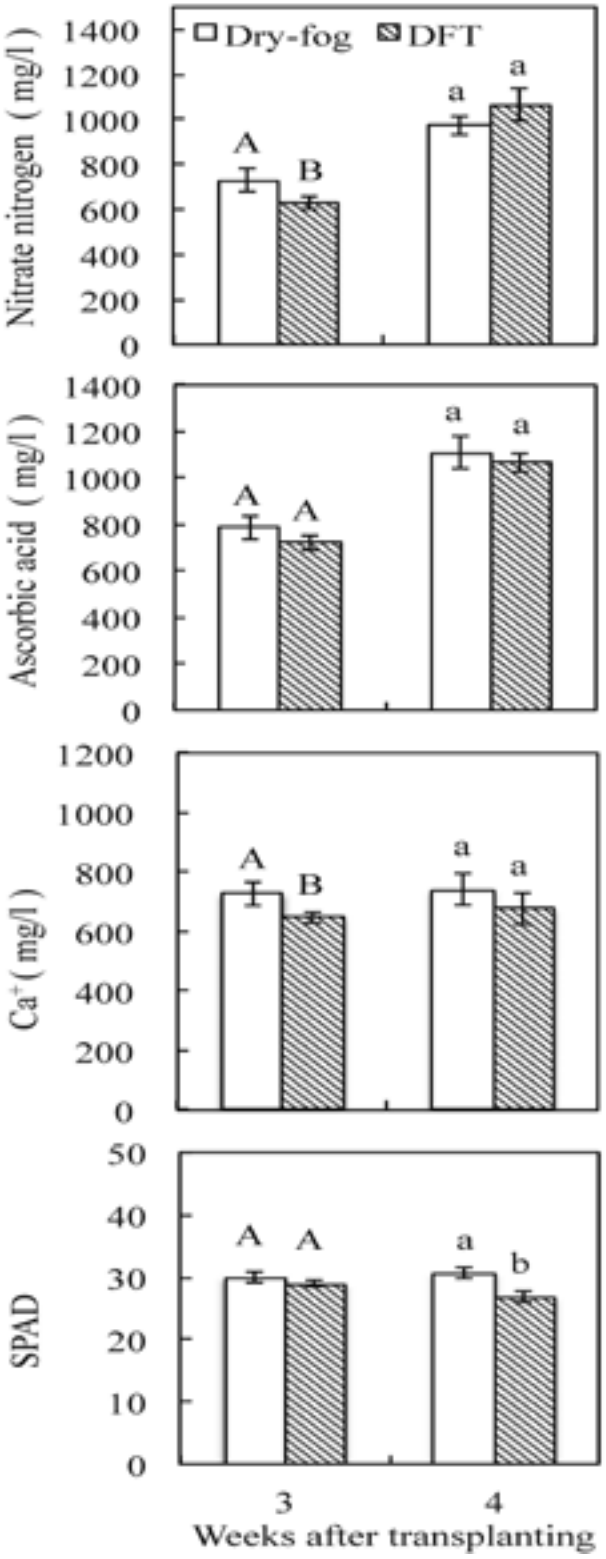

Fig. 7 - Nitrate nitrogen, ascorbic acid, calcium ion and chlorophyll content of mature lettuce leaves grown using dry-fog or DFT hydroponics for three and four weeks after transplanting. Different letters show significant difference ($P \leq 0.05$), bars represent ±SE (n=6).

increased by 16% compared to those grown using DFT at three weeks after planting, but there was no significant difference at four weeks. The content of ascorbic acid in the leaves grown using dry-fog culture increased by 9% at three weeks and 4% at four weeks, compared to the leaves grown using DFT, however there were no significant differences. The calcium content in the leaves grown using dry-fog culture significantly increased at three weeks after planting, but there was no significant difference at four weeks. The chlorophyll content of mature leaves significantly increased with dry-fog culture only at four weeks.

To evaluate the efficacy of dry-fog culture as a novel aeroponic system, the growth of lettuce was investigated and compared to DFT hydroponic culture. One of the features of dry-fog culture is water conservation. For the systems used in this study, the DFT always needed at least 30 L of liquid fertilizer, while dry-fog culture could fill a chamber with only 1 L of solution because the droplets of dry fog are very fine. In addition, the total amount of liquid fertilizer in a whole dry-fog culture system was less than 10 L. This aspect is an advantage not only in terms of water resource depletion but also because the Styrofoam chamber culturing unit remains light, making it possible to install the system almost anywhere. Chamber units can also be multistaged easily with low costs, meaning this system is suitable for a plant factory or urban farming. Furthermore, because the effort to maintain the chamber units and fertigate plants are also greatly reduced, dry-fog aeroponics is expected to be usable not only for commercial crop production but also for welfare and food education.

Despite using less liquid fertilizer in the rhizosphere for the dry-fog culture, there was no difference in leaf growth in the two different hydroponic techniques, probably due to changes in root morphology and physiology. Root hairs that developed only in the dry-fog culture had a normal increase in the efficiency of nutrient absorption from heterogeneous soil due to a significant increase in the root surface area (Bates and Lynch, 1996; Ma *et al*., 2001). Root hairs are not needed in DFT hydroponic technique because there is adequate water and liquid nutrients in the root zone. They are not formed in traditional aeroponics because nutrient solutions are sprayed on roots which are always kept wet. In dry-fog culture, because the rhizosphere is almost an air phase, roots have to catch the very fine droplets of nutrient solutions floating in the air in a chamber. Importantly, as an adaptation mechanism, root hairs develop to catch more droplets of fertilizer. In the present study, the significant increase in root growth was observed only at four weeks after planting. Also the respiration rate of the roots was significantly higher in the dry-fog cultures than in the DFT hydroponic system at four weeks (Fig. 5). The respiration rate of roots has a close relationship with water and nutrient absorption in the roots (Hansen, 1980). Indeed, concomitant increases in both root growth and respiration rate in the dry-fog culture were observed, which might result in promotion of water and nutrient absorption and increases of growth and yield. However, no significant increase in leaf growth was observed in dry-fog culture as compared with DFT. Because the growth and respiration activity of roots significantly increased at four weeks and the morphology of new roots developed in the dry-fog culture changed, it may take longer for plant roots to adapt to the dry-fog culture environment as compared with other hydroponic systems. The ascorbic acid content in the leaves also tended to be slightly higher in the dry-fog culture, but there were no significant differences. Leaf ascorbic acid is a secondary metabolite, and its content in leaves increases under environmental stress conditions (Robinson and Bunce, 2000; Reddy *et al*., 2004, Koyama *et al*., 2012). Because there is nearly no extra water in the rhizosphere in dry-fog culture, roots might suffer from slightly drought conditions. The increase in secondary metabolites caused by environmental stresses generally comes with a decrease in growth and harvest (Champolivier and Merrien, 1996; Kirda *et al*., 2004, Koyama *et al*., 2012). However in the present study, such decreases were not observed. Under drought conditions, the development of root hairs and increase in root surface area are countermeasures to growth depression due to a water shortage. In dry-fog cultures, plants develop root hairs to catch the very fine droplets of nutrient solution, and this development is thought to effect secondary metabolism. Dry-fog culture might be useful to adjust mild drought conditions in rhizosphere by changing the airflow speed at the frequency of atomization, which affect harvest quality (Brix of secondary metabolite content). Furthermore, the atomized dry-fog from the nutrient solution in a chamber fills the rhizosphere space immediately, meaning the rhizosphere environment is changed quickly and drastically. This aspect is expected to lead to new cultivation methods that can increase the content of secondary metabolites by controlling environmental stresses without decreasing growth or harvest.

With regard to the photosynthetic rates of mature leaves grown in the dry-fog culture, significant increases were shown in both ϕ and Max compared with DFT culture. This indicates that the rhizosphere environment of dry-fog culture may affect the photosynthetic ability of lettuce leaves by improving CO_2 assimilation efficiency of mesophyll cells. The content of leaf chlorophyll was significantly increased in the dry-fog culture at four weeks, and this may be considered to be due to improvement in the activity or quantity of photosynthetic light or dark reaction components. The relationship between enhancement of photosynthetic activity and rhizosphere environment in the dry-fog culture remains unclear.

In summary, dry-fog aeroponics enables lettuce plants to grow with less water without sacrificing yield and leaf quality as compared to traditional hydroponics. The lightweight cultivation chamber can decrease the workload for growers and is easy for new growers to maintain. Dry-fog aeroponics posses potential as a novel hydroponic system to promote high-value crop production and yield more than other hydroponic systems by controlling the rhizosphere environment while saving water and nutrients. It is worth noting that the nozzle used in this study

can atomize enough dry-fog to fill a much larger chamber (6000×700×450 mm). More study is needed to determine the utility of a dry-fog culture in horticulture. Further research would be helpful to determine rhizosphere environmental conditions (density of dry-fog, frequency of atomizing, flow speed of dry-fog) to maximize plant growth and the amount of secondary metabolites produced. In addition, optimization of the composition and strength of the nutrient solution, especially for dry-fog culture, is needed and could be determined through investigation of the absorption nutrients in the plants.

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Effects of different wavelengths of LED light on pollen germination and direction of pollen tube elongation in *Cyrtanthus mackenii*

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Key words: Cyrtanthus mackenii, direction of pollen tube elongation, germination rate, light emitting diode, light wavelength.

Abstract: The effects of different light wavelengths on pollen germination and determination of the direction of pollen tube elongation of *Cyrtanthus mackenii* was examined *in vitro* using light-emitting diodes (LED) with five peak wavelengths: 405 nm (violet), 465 nm (blue), 630 nm (orange), 660 nm (red), and 735 nm (far-red). Pollen grains were cultured on a medium solidified with agar, and maintained at 21°C in an incubator under 3-4 h of continuous lighting or darkness. Neither the pollen germinated under LED lighting and dark conditions nor the rate of pollen germination differed among light conditions, including darkness. However, pollen tubes elongated less in the direction toward light under LED, and elongated with no directional trend in darkness. Moreover, pollen tubes did not elongate toward far-red LEDs. These results suggest that light exerts effects on the factors determining the direction of pollen tube elongation, but not on those controlling pollen germination.

1. Introduction

Light is important for plants, not only as an energy source but also as an environmental signal. Plants respond appropriately to light in their environment, as in seed germination and seedling growth (Godo *et al*., 2011), floral transition (Cerny *et al*., 2003), phototropism (Palmer *et al*., 1993), and so on. Light wavelength is also involved in cell elongation (Braidwood *et al*., 2014).

Pollen tube elongation is of one of the fastest growing plant cell types. The mechanism of pollen tube growth is a multi-stepped one, and it is co-regulated by a variety of essential cellular processes, including exocytosis, actin cytoskeleton organization and activity, calcium and proton physiology, and cellular energetics (Hepler *et al*., 2013). The main factor that attracts the growth tip of the pollen tube is considered to be chemo-attractants from the female gametophyte (Higashiyama and Hamamura, 2008).

In vitro, the effects of light wavelength on pollen germination or pollen tube elongation have been studied in *Pinus roxburghii* (Dhawan and Malik, 1981): pollen tube growth decreased in white light compared to the dark, and increased in red light, although this was counteracted by far red light. In a study of two cultivars of wheat × maize crosses, pollen tube growth was significantly affected by light intensity in one cultivar but not in the other (Campbell *et al*., 2001). Furthermore, the directional growth of pollen tubes of *Nicotiana alata* occurred in cultures incubated in the dark as well as in the light (Lush *et al*., 1998). These results highlight the effects of different wavelengths of light on pollen germination and pollen tube elongation, which vary between species, while few studies have described these effects in detail.

The aim of this study was to verify the effect of different wavelengths of light-emitting diode (LED) light on pollen germination and direction of pollen tube elongation in *Cyrtanthus mackenii* using an *in vitro* experimental system for pollen germination developed by Hirano and Hoshino (2010).

2. Materials and Methods

Plant materials and pollen culture

Mature pollen with anthers that showed dehiscence was collected from *C. mackenii* Hook.f. (Amaryllidaceae) and stored at -20°C. Pollen culture was performed using 1% (w/v) agar, medium that contained 0.01% (w/v) $CaCl_2$, 0.01% (w/v) H_3BO_3, 0.0007% (w/v) KH_2PO_4, 10% (w/v) sucrose, and 0.01% (w/v) yeast extract at pH 5.8 (Hirano and Hoshino, 2009, 2010). The culture medium was sterilized by autoclaving at 121°C for 15 min.

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The effect of light condition on pollen germination rate

Five spots of culture medium with a diameter of 1 cm were put on a glass slide at *ca*. 5 mm intervals. A tiny amount of frozen pollen grains were sown on each coagulated spot of medium using a paintbrush. Two or three prepared slides were horizontally positioned in a petri dish, which was kept at high humidity (Fig. 1A), with toothpicks between the slides to avoid overlapping. Samples were then cultured at 21°C in an incubator (BIOTRON LPH200, NK system, Japan) for 3-4 h under different light conditions. The incubator was divided into five rooms fitted with overhead LED panels (40×10 cm) fabricated using 2,800 indicator-type LEDs of 3 mm diameter (Fujiwara *et al*., 2011; Yano and Fujiwara, 2012). The LEDs were of five types: violet (L405R-36; Epitex Inc., Kyoto, Japan), blue (L460-36; Epitex Inc., Kyoto, Japan), orange-red (L630-36; Epitex Inc., Kyoto, Japan), red (SRK3-3A80-LE; Toricon Co., Shimane, Japan), and far-red (L735-36AU; Epitex Inc., Kyoto, Japan). Each LED emitted a specific peak wavelength (λ_p) of light: 405 nm, 460 nm, 630 nm, 660 nm, and 735 nm, respectively. The petri dishes were set 2.5 cm below the LED panels. In addition to these treatments, lightproof petri dishes wrapped in aluminum foil were set in a room to confirm whether pollen germination was stimulated or suppressed under dark conditions. Five replicated culture slides were prepared per treatment (Fig. 1B).

After culturing, all slides were observed using a microscope (Axiovert 40 CFL, Carl Zeiss, Germany), and germinated pollen grains were counted. Germinated pollen grains with pollen tubes that were shorter than the pollen grain diameter were not counted. We repeated the experiment three times under the same conditions, and are referred to as G1, G2, and G3.

The effects of light condition on the direction of pollen tube elongation

Cover glasses placed over spots of medium with a diameter of 1 cm were prepared. Frozen pollen grains were sown on the spots of medium in the same manner as for the pollen germination experiment. Five cover glasses were vertically set in a humid petri dish using a paper stand to prevent the glasses from sticking. The petri dishes were wrapped in aluminum foil with a slit (8 cm × 0.5 cm) on the upper side for LED light irradiation, and were then cultured at 21°C in the previously described incubator for 3-4 h. To verify the effects of light intensity on pollen tube elongation, two petri dishes were prepared for each light condition: one was placed 3 cm below the LED panel (strong light) and the other was placed 22 cm below (weak light).

After culturing, the direction of pollen elongation was observed using the previously described microscope. The direction of pollen tube elongation was determined as follows:

1. Elongation of the pollen tube tip within a 60° arc centered on the line connecting the LED panel, pollen grain, and floor, and widening in a direction toward the LED light, was defined as "Light" (under LED lighting conditions) or "Upward" (under dark conditions) (Fig. 1C);
2. "Dark" or "Downward" defined the occurrence of pollen tube elongation in the opposite direction of the Light or Upward response;
3. Pollen tube elongation toward a direction that was neither Light/Upward nor Dark/Downward was defined as "Vertical."
4. This experiment was also repeated three times under the same experimental settings and is referred to as D1, D2, and D3.

Statistical analysis

All statistical analyses were conducted with the statistical package R (v.3.0.2) (R Foundation for Statistical Computing, 2014). A generalized linear mixed-effects model (GLMM) with an offset term was used to investigate determinants on the number of germinated pollen grains, assuming Poisson distribution. The light condition, triple experiment design, and subsequent interactions were explanatory variables in the model, and an individual spot of medium was a random factor that considered effects seen on an individual spot compared to other spots on the same slide. The total number of pollen grains per medium was

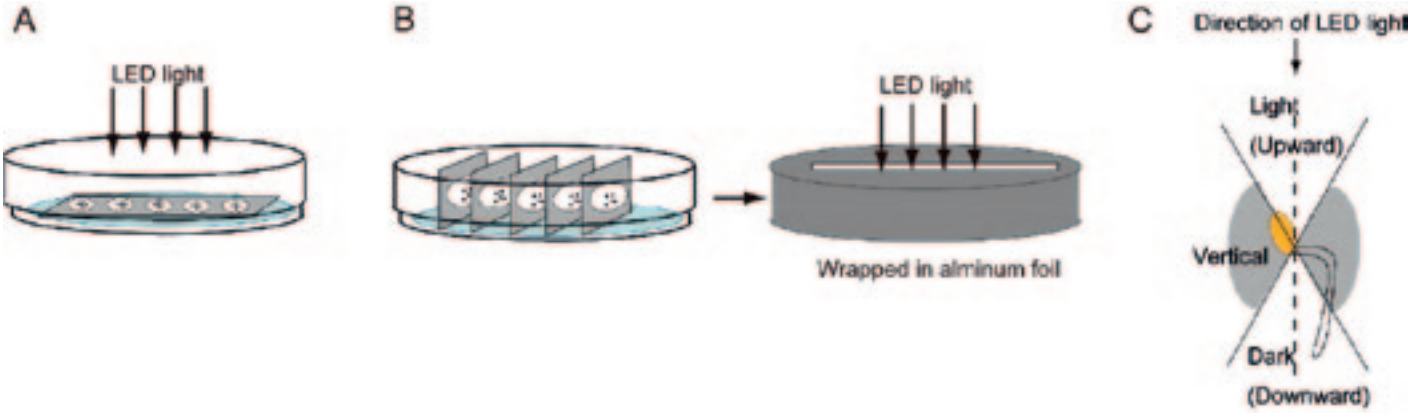

Fig. 1 - Diagrammatic representation of the experimental systems and germinated pollen. A, Petri dish set in prepared slides for pollen germination experiment. B, Petri dishes set in cover glasses and then wrapped in aluminum foil with a slit on the upper side for pollen tube elongation experiment. C, Criterion for determining of the direction of pollen tube elongation.

an offset term. The Akaike information criterion (AIC) was used to select the best models of the GLMMs.

To estimate the effects of light intensity and light wavelengths on the direction of pollen tube elongation, multinomial logistic regression (Venables and Ripley, 2002) was performed using the R package's vector generalized linear and additive model (VGAM). The direction of pollen tube elongation was considered a categorical response variable (Light/Upward, Vertical, Dark/Downward). The explanatory variables were distance from LED panel (long or short); wavelength (405, 460, 630, 660, or 735 nm); and triple experiment design in the case of LED lighting conditions. In the case of analysis for dark conditions, the experiment was the explanatory variable.

3. Results

The effect of light condition on pollen germination rate

The total number of *C. mackenii* pollen grains sown on spots of medium were 9 017 (G1), 6 769 (G2), and 4 257 (G3). The pollen germinated under both LED lightning and dark conditions (Fig. 2). The mean and standard deviation of germination rate under each light condition were 0.30±0.015 at 405 nm, 0.38±0.019 at 465 nm, 0.28±0.018 at 630 nm, 0.34±0.020 at 660 nm, and 0.31±0.023 at 735 nm peak wavelength LEDs, and 0.37±0.018 in darkness. The germination rate varied among the three experiments, but did not differ among the five types of LED and darkness (Table 1).

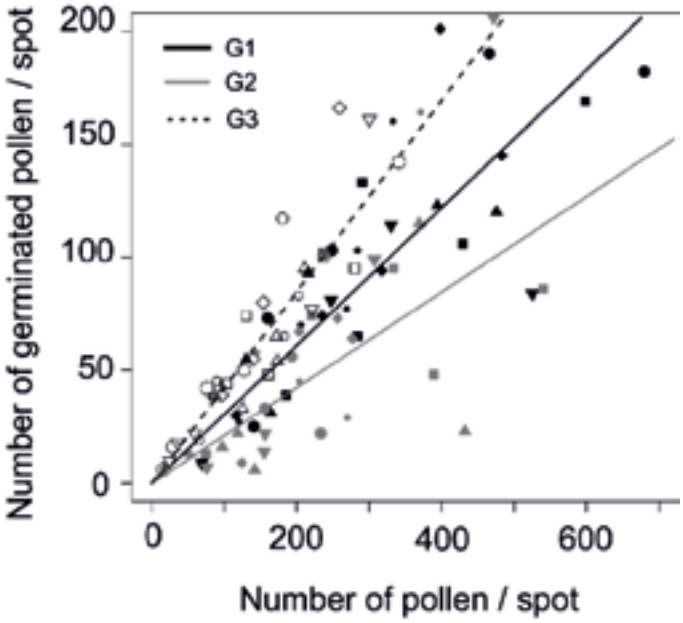

Fig. 2 - Relationships between light condition and each germination experiment and germination rate of pollen. Germinated pollen per total sown pollen on each medium spot under each irradiated LEDs and darkness: 405 nm (squares), 465 nm (circles), 630 nm (triangles), 660 nm (diamonds), 735 nm (upside-down triangles) peak wavelength of the LED, and darkness (small circles). Three lines show the estimated mean number of germinated pollen for three experiments by GLMM. Results of GLMM are shown in Table 1.

Table 1 - Parameters estimated by GLMM to predict number of geminated pollen in different light conditions for three times of germination experiment. The light condition was discarded explanatory variable by AIC

	Coefficient	Z score
Intercept	-1.19	-15.98 ***
Experiment G2	-0.37	-3.40 ***
Experiment G3	0.33	3.06 **

Significance is determined by *Z* scores.
***: significantly different from G1 at $P < 0.001$ and ** $P < 0.01$.

The effect of light condition on the direction of pollen tube elongation

The total number of pollen tubes elongated in the three defined directions as detailed in Fig. 1C for the three experiments was 1 410 for Light, 2 994 for Vertical, and 1 223 for Dark under LED lightning, and 187 for Upward, 421 for Vertical, and 161 for Downward in darkness (Fig. 3). Under LED lightning, the probability of pollen tube elongation classified as Light or Vertical was higher than that classified as Dark (Table 2). However, under darkness, the probability of pollen tube elongation classified as Upward was equivalent to that of Downward, although the Vertical classification was higher. Variability among the three experiments was not found in Light and Upward pollen tube elongation, but was found in Vertical elongation. There was no effect of the distance from LED panel on pollen tube elongation. Under lighting from 735 nm peak wavelength LEDs, the number of Light pollen tube elongations was lower than for Dark pollen tube elongations.

4. Discussion and Conclusions

In this study, light neither inhibited nor promoted *Cyrtanthus* pollen germination, and the different wavelengths

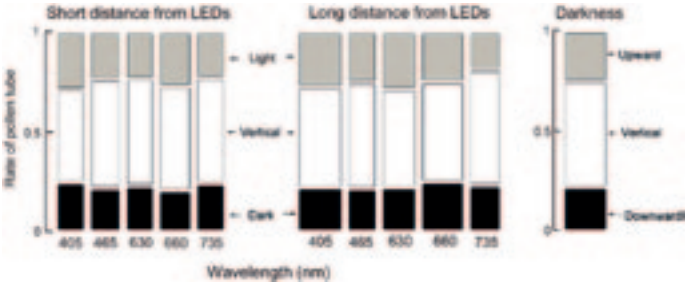

Fig. 3 - Rates of pollen tube elongation in three directions for each irradiated LEDs for the two distances (3 and 22 cm) from LED panels and for shading. The three directions are expressed as follows: toward light or upward (Light or Upward, grey culms), away from light or downward (Dark or Downward, black culms), and in a direction that is neither Light/Upward nor Dark/Downward (Vertical, white culms). The rates are calculated using sum of the number of pollen tubes in five replications of all experiments. The width of culm shows the number of pollen tubes.

Table 2 - Coefficients estimated by multinomial logistic regression models that predict probability of pollen tube elongation toward to the Light/Upward and Vertical directions based on Dark/Downward direction in short distance from 405 nm peak wavelength LED panel in D1

		LEDs irradiation		Darkness	
Direction		Light	Vertical	Upward	Vertical
Intercept		0.23*	0.72***	-0.02 N.S	0.59 **
Experiment	D2	0.05 N.S	0.30***	0.19 N.S	0.42 N.S
	D3	0.03 N.S	0.11 N.S	0.27 N.S	0.54 *
Distance	long	-0.007 N.S	-0.02 N.S		
Wavelength	465 nm	-0.09 N.S	0.14 N.S		
	630 nm	-0.05 N.S	0.08 N.S		
	660 nm	-0.10 N.S	0.03 N.S		
	735 nm	-0.37**	0.10 N.S		

Significant effects of distance and type of LED lights on the direction of pollen tube elongation are determined by *Z* scores.
***: significantly different at $P < 0.001$, **$P < 0.01$, *$P < 0.05$ and NS: non-significant.

of LED light also did not affect the pollen germination rate. However, pollen tubes were less likely to elongate toward the opposite direction of light, although they elongated with no directional trend in darkness. These results suggest that light has some effect on the factors determining the direction of pollen tube elongation, but not on the factors controlling pollen germination.

The growth direction of a pollen tube is continuously reoriented by external signals and physical obstacles (Cheung and Wu, 2008). Without any chemotropic attractants or physical directional control caused by configuration of female tissue or gametophytes, pollen tubes elongated randomly *in vitro* (Horade *et al*., 2013). However, the pollen tubes did not show negative phototropism under LED lighting from only one direction in the present study. Therefore, it can be assumed that there is an integrative mechanism that causes unequal elongation of the pollen tube tip, e.g. perception of the light environment (Casal, 2013), transcription factors such as phytochrome interacting factors (PIFs) (Chen and Chory, 2011), or hormones and growth-related genes such as for hypocotyl cells (Braidwood *et al*., 2014).

In a unique manner, the pollen tubes did not elongate toward light from 735 nm peak wavelength LEDs. This result was consistent with previous studies that examined the effect of different light wavelengths in combination with hormones on pollen tube growth in *Arachis hypogaea* (Chhabra and Malik, 1978) and *Pinus roxburghii* (Dhawan and Malik, 1981). Considering the red:far-red ratio, phytochrome undergoes a conformational change from the active far-red form into the red-light-absorbing, active red form, and derepresses PIF activity, resulting in an increase in cell elongation. If the phytochrome exists in pollen tube tips and causes local cell elongation at the point irradiated with far-red light, the phenomenon of pollen tube elongation away from light may be explained.

In conclusion, our results demonstrate that the different wavelengths of LED light affected the direction of pollen tube elongation, but not pollen germination, in an *in vitro* experimental system. Further studies are necessary to clarify the influence of light wavelength on determination of the direction of pollen tube elongation.

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Cross-protection against anthracnose with heat stress, antioxidative changes and proteomic analysis in mycorrhizal cyclamen

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Key words: *Colletotricum gloeosporioides*, DPPH, growth promotion, shock heat stress, SOD, symbiosis.

Abstract: Cross-protection against anthracnose with shock heat stress, antioxidative changes and proteomic analysis in mycorrhizal cyclamen were investigated. Eleven weeks after arbuscular mycorrhizal fungus (AMF, *Glomus fasciculatum*) inoculation, cyclamen (*Cyclamen persicum*, cv. Pastel) plants were acclimated under 20°C for 4 days, followed to 35°C (shock heat stress, SHS) for 7 days, and inoculated with *Colletrichum gloeosporioides* (CG) as anthracnose pathogen. Seven days after SHS treatment, dry weights of leaves, bulbs and roots increased in mycorrhizal plants, thus, growth promotion appeared. In addition, mycorrhizal plants showed higher resistance to anthracnose compared to non-mycorrhizal control plants. Regarding antioxidative activity, superoxide dismutase (SOD) activity increased in roots of mycorrhizal plants under 7d after SHS. DPPH radical scavenging activity increased in some parts of the mycorrhizal plants under SHS and CG-inoculated conditions. As for proteomic analysis, totally 29 spots changed in mycorrhizal plants through SHS and CG-inoculated conditions. In this case, the spot of 20.4kDa was detected only in mycorrhizal plots, furthermore, 4 spots intensively appeared in mycorrhizal plots through SHS and CG conditions. From these findings, AMF could alleviate heat shock stress with promoting host plant growth and induce resistance to anthracnose under heat stress. In addition, it supposed that antioxidative modification would have cross association with the resistance to heat shock and anthracnose, and the symbiosis-specific changes in some proteins might have concern with the cross protection.

1. Introduction

Cyclamen is an herbaceous perennial used as a flowering potted plant (Karlsson and Werner, 2001; Elmer and McGovern, 2004). In the genus *Cyclamen*, *Cyclamen persicum* is the major species used for commercial cultivation (Ishizaka *et al.*, 2002). It has a longer growing season and temperature is one of the most important environmental factors that affect the growth, development and distribution of this plant (Yesson and Culham, 2006). Cyclamen is highly susceptible to various temperature conditions and the recommended temperature for cyclamen production from seeding to appearance of flower bud is 20°C (Ball, 1991). Currently, due to global warming, summer temperatures are increasingly elevated. However, garden type cyclamen is intolerant to heat stress, which severely affects growth and development of this plant in southwest Japan during summer (Goto *et al.*, 2011). On the other hand, in summer, heat is not the only stress factor, but pathogens also pose a threat. Pathogenesis infection impacts commercial cyclamen production. Although various diseases are reported, anthracnose is especially destructive disease worldwide. Anthracnose is responsible for *Colletotricum gloeosporioides* (CG) and it causes extensive lesions on aerial plant parts.

Today, cost effective and eco-friendly control strategies against heat and anthracnose are needed. Arbuscular mycorrhizal fungus (AMF) has drawn attention by crop researchers for its benefits to host plants. Improving nutrient uptake, especially of phosphate, of the host plant is well known as one of the benefits of arbuscular mycorrhizal symbiosis (Marschner and Dell, 1994). Additionally, several investigators have reported a higher resistance of mycorrhizal plants to biotic and abiotic stresses (Garmendia *et al.*, 2006 ; Wu *et al.*, 2006; Li *et al.*, 2010). As for cyclamen, mycorrhizal plants showed growth promotion under heat stress (30°C) (Maya and Matsubara, 2013 a) and higher tolerance to anthracnose caused by CG (Maya and Matsubara, 2013 b). However, growth improvement under extreme shock heat stress, resistance to anthracnose under such heat stress and proteomic changes through heat stress and anthracnose in mycorrhizal plants are yet to be revealed.

When plants suffer to heat stress and pathogenesis infection, reactive oxygen species (ROS) generate in cells and the oxidative stress to intercellular structures they cause are major damaging factors in plants (Wahid *et*

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al., 2007). In order to cope with these toxic ROS, plants have antioxidants as detoxification factors (Kuzniak and Sklodowska, 2004; Wu *et al.*, 2006). Antioxidative defense mechanisms consist of antioxidative enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) and nonenzymatic antioxidants such as polyphenols and ascorbic acid (Asada, 1999). Enhancement of antioxidative activity in mycorrhizal plants has been already reported (Lambais *et al.*, 2003).

Physiological changes in plants due to heat stress have not yet been revealed. However, plants have shown different responses under heat acclimation and heat shock (Durand *et al.*, 2012). According to Li *et al.* (1984), heat shock caused stronger growth inhibition than heat acclimation. Although heat shock is thought to be a stronger heat stress treatment, some researchers reported that heat shock (around 45°C) induced tolerance against aboveground diseases such as anthracnose to host plants (Teruya *et al.*, 2012; Yoshino *et al.*, 2012). Therefore, heat shock could be considered as a trigger defense system toward pathogens. On the other hand, AMF symbiosis also causes alteration of gene expression in leaves and roots (Liu *et al.*, 2007). Campos *et al.* (2012) reported that expression of some defense related genes such as transcriptional factors, regulators of Ca^{2+} signaling pathway, MAPKs and NPR1 (nonexpressor of PR genes) etc. had been up-regulated in mycorrhizal rice leaves. Yet, the influence of AMF on protein expression in cyclamen under heat stress and CG inoculation have not been revealed.

The aim of this study was to determine cross-protection against heat stress and anthracnose in mycorrhizal cyclamen in association with antioxidative changes and proteomic analysis.

2. Materials and Methods

Plant material and mycorrhizal inoculation

Cyclamen (*Cyclamen persicum*, cv. Pastel) seedlings (three months of age) were replanted in plastic pots containing autoclaved (121°C, 1.2 kg/cm^2, 15 min) commercial potting media (Sogemix SM-2). In the meantime, plants were inoculated with AMF (*Glomus fasciculatum*) inoculum for mycorrhizal (AMF) plants and autoclaved inoculum for non-mycorrhizal (control) plants. In both cases, inocula (3 g/plant) were placed 4 cm below the bulbs. Commercial AMF inoculum supplied by Idemitsuagri Co. Ltd, Tokyo, Japan was used in this study: the spore density was unknown. Plants were transferred into poly silver pot (9 cm diameter) after 11 weeks of AMF inoculation. Plants were fertilized with slow release granular fertilizer (N:P:K= 5:10:15, Ube Industries Ltd.) once a month and raised in a greenhouse.

Treatment with shock heat stress (SHS)

Eleven weeks after AMF inoculation, the plants are transferred to a growth chamber (20°C constant, 12 hr daylength, RH 60%) for 4 days (d) as acclimation under optimum environmental conditions. After 4 days at 20°C, the temperature was suddenly increased to 35°C (constant) as shock heat stress treatment (SHS). The plants were grown for 7 d under SHS.

Inoculation of Colletrichum gloeosporioides

The isolate (MAFF744024) of *Colletrichum gloeosporioides* (CG) was collected from the Ministry of Agriculture, Forestry and Fisheries, Japan. It was grown in potato dextrose agar (PDA) medium and was incubated at 25°C for two weeks in dark conditions to prompt sporulation. The spore concentration was adjusted to 10^5 cfu using sterile distilled water. The shoots of each plant were sprayed at 7 d after SHS with 10 ml of CG suspension. Symptoms of anthracnose were evaluated 9 d after CG inoculation. A completely randomized design with five replicates was used.

Disease incidence and index of anthracnose

Disease symptoms were checked 9d after CG inoculation. The disease severity in individual plants was rated visually on a scale of 0-5.

0= no symptoms (healthy plants);
1= <20% disease symptoms (small discolored leaf lesions covering less than 20% of total leaves of a plant);
2= 20-40% disease symptoms (minor small discolored lesions covering 20-40% of leaves);
3= 40-60% disease symptoms (moderate brown lesions in 40-60% of leaves and 15% defoliation);
4= 60-80% disease symptoms (mild wilt discolouration covering 60-80% of leaves and more than 50% leaf defoliation in case of *Fusarium* wilt);
5= 80-100% disease symptoms (stems and leaves severely affected).

The disease index for CG was calculated using the following formula:

Disease index= [Σ (number of diseased plants x severity level)] x 100/ (total number of plants x maximum level of disease severity)

Plant growth and mycorrhizal colonization

Dry weight of leaves, bulbs and roots of five plants which were grown at 20°C for 4 d, under SHS for 2 d, 7 d and for 9 d after CG inoculation was determined after plant material was dried at 100°C for 24 h. The lateral roots of each plant were sampled and used to check the level of AMF colonization according to Phillips and Hayman (1970). Lateral roots were sampled in 70% ethanol and later washed with distilled water. They were then ground in 10% NaOH and autoclaved (121°C, 1.2 kg/cm^2, 10 min) and subsequently washed with distilled water and stained with trypan blue. The ratio of AM fungal colonization was checked in 1-cm segments of lateral roots and approximately 60 samples of 1-cm segments were checked per plant. The average was calculated from the values of five plants for each time.

Antioxidative analysis

Superoxide dismutase (SOD) as enzymatic antioxidative activity and DPPH radical scavenging activity as non-enzymatic antioxidative activity were analyzed. Plants which were sampled 4 d after 20°C, 2 d and 7 d after SHS, and 9 d after CG inoculation preserved with liquid nitrogen were used for each analysis. Total SOD activity was measured according to Beauchamp and Fridovich (1971). DPPH radical scavenging activity test was carried out as described by Burits and Bucar (2000). All experiments were replicated three times.

Proteomic analysis

Protein extraction. One g of frozen extended leaves was homogenized in liquid nitrogen with mortar and pestle. Powdered leaf sample was mixed with 2 ml of extraction buffer containing 7 M urea, 2 M thiourea, 4% CHAPS and 50 mM Tris (pH 7). The mixture was then centrifuged (14,000 rpm, 4°C, 60 min), and the collected supernatant was mixed with 400 ml of 50% TCA. This mixture was centrifuged (14,000 rpm, 4°C, 10 min) and supernatant was discarded. One ml of 100% acetone was added to the pellet and this mixture was centrifuged (14,000 rpm, 4°C, 10 min); the supernatant was discarded. These steps were repeated three times. After acetone was dried at RT, 200 µl of dissolution buffer (8 M urea, 2% nonidet p-40, 1% dithioslatol) was added to the pellet and centrifuged (14,000 rpm, 4°C, 5 min). The supernatant was sampled and mixed with 20 µl of 1M iodoacetamide.

Electrophoresis. The first dimension IEF was run using WSE 1500 (ATTO Corp.) according to the manufacture's instruction manual with some modifications (300V, 210 min). After electrophoresis, the gel was soaked in 100 ml of 2.5% TCA for 5 min. Then, it was washed with distilled water for 2 h. For the second dimension (SDS-PAGE), it was soaked in 100 ml of SDS equilibration buffer A (50 mM Tris-HCl, 2% SDS, 0.0001% BPB, 0.001% DTT), shaken for 10 min and subsequently shaken in SDS equilibration buffer B (50 mM Tris-HCl, 2% SDS, 0.0001% BPB, 0.001% iodoacetamide) for 10 min. SDS-PAGE was run on AE-6500 (ATTO Corp.) using 12.5% polyacrylamide gel (ePAGEL E-D12.5L, ATTO Corp.) according to the manufacture's instructions (100V, 20 mA, 150 min). Finally, proteins were visualized by CBB staining.

3. Results

Seven days after SHS treatment, dry weights of leaves, bulbs and roots increased in mycorrhizal plants (Fig. 1 and 2). In addition, leaf yellowing and browning caused by shock heat stress were alleviated in mycorrhizal plants compared to non-mycorrhizal control plants. The AMF colonization level reached around 50% through all the treatments and did not differ among the treatments (data not shown). With regard to anthracnose symptoms, greater

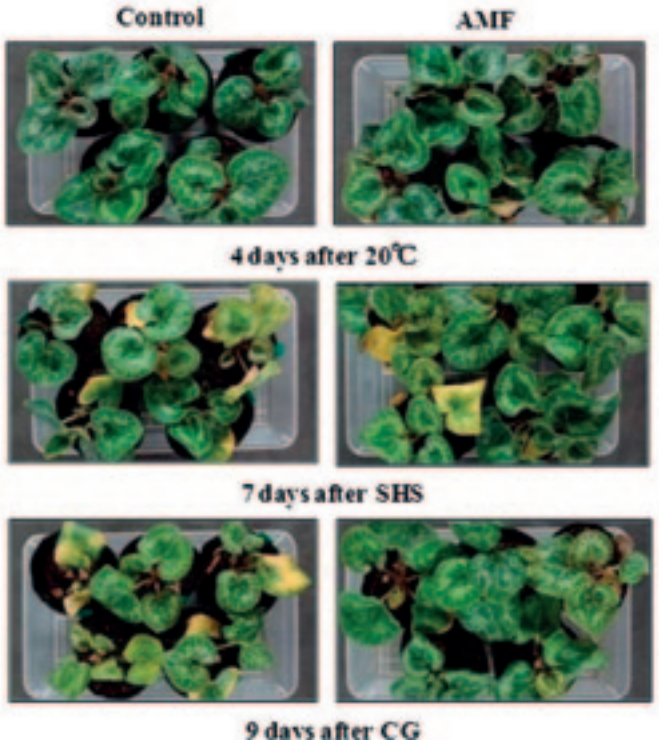

Fig. 1 - Growth of mycorrhizal cyclamen under shock heat stress (SHS) and *Colletotricum gloeosporioides* (CG) inoculation.

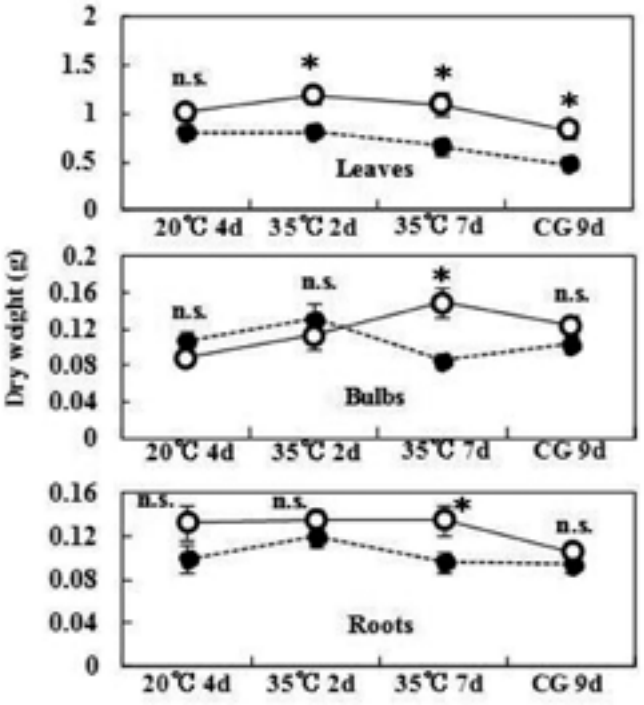

Fig. 2 - Dry weight of mycorrhizal cyclamen before and after SHS and CG inoculation. ● Control; O AMF (*Glomus fasciculatum*). SHS, CG, see Figure 1. 20°C 4d, 4 days after 20°C; 35°C 2d, 2 days after SHS; 35°C 7d, 7 days after SHS; CG 9d, 9 days after CG inoculation. Bars represent standard errors (n=5). NS, non-significant. *, significantly different between C and AMF by *t*-test (P<0.05).

severity appeared in non-mycorrizal plants and mycorrhizal plants showed less severity and lower indices (Fig. 3).

Differences in antioxidative activity occurred between AMF and control plants during the experimental period. As for SOD activity, significant change was not detected in leaves and bulbs but SOD activity was enhanced in roots at 20°C, 2 d and 7 d after SHS (Fig. 4A). However, no difference in SOD activity appeared under CG inoculation. DPPH radical scavenging activity as non-enzymatic antioxidative activity increased roots of mycorrhizal plants through the experimental period, and some parts of mycorrhizal plants also showed higher DPPH levels compared to control (Fig. 4B).

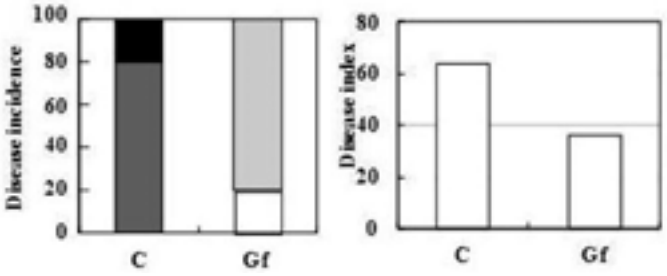

Fig. 3 - Disease incidence and index of anthracnose in mycorrhizal cyclamen 9 days after CG inoculation under SHS.
SHS, CG, C, AMF, see figure 2.
Ratio of diseased leaves to the total in each plant.
□ -20% ; ▒ 20-40% ; ▓ 40-60% ; ■ -80%.

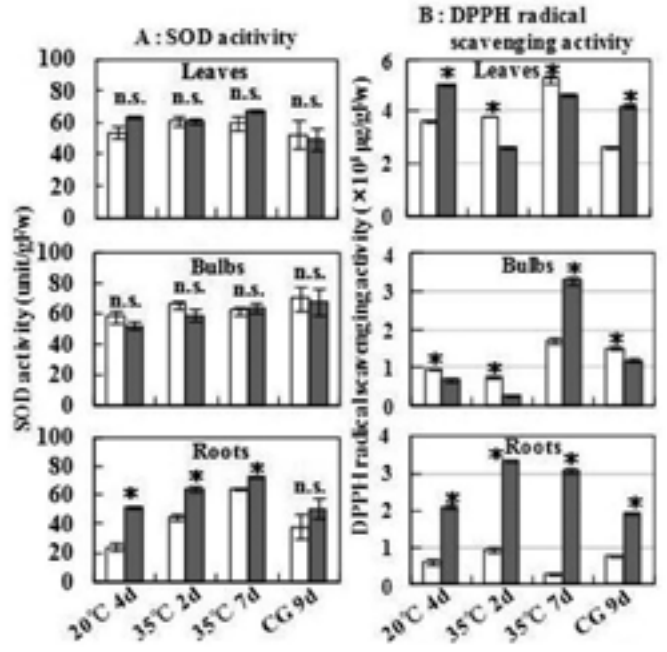

Fig. 4 - SOD activity, DPPH radical scavenging activity in mycorrhizal cyclamen before and after SHS and CG inoculation.
□ Control ; ▓ AMF. SHS, CG, see figure 2. Bars represent standard errors (n=3). NS, non-significant. *, significantly different between C and AMF by *t*-test (P<0.05).

As a result of proteomic analysis, overall the expressions of 29 spots were detected as different spots in mycorrhizal plants compared to control through SHS and CG inoculation (Table 1). Four days after 20°C, differences in expression of 11 spots between mycorrhizal and non-mycorrhizal plants appeared. In this case, although six increased spots and three newly appeared spots were detected in mycorrhizal plants, two spots slightly decreased in contrast to

Table 1 - Expression levels in 29 proteins in leaves of mycorrhizal cyclamen under SHS and CG inoculation

Spot ID	Estimated MW (kDa)	20°C 4 d		35°C 7 d		CG 9 d	
		C	AMF	C	AMF	C	AMF
A	68.0		+	+		- -	
B	68.0		+		+		+
C	87.1		++	++	++		+
D	33.4		+	++	+		++
E	29.9		-	+			- -
F	26.9		-		-		++
G	20.4		N		N		N
H	19.4		++	+	+		++
I	37.5		N	N	-	D	
J	36.7		N	N	-	D	
K	24.3		+				
L	27.3			+	+	+	
M	27.7				+		
N	19.7			+	+		++
O	19.5			+	+	+	
P	32.2			+	++		
Q	36.0			-		-	-
R	36.0			-		-	-
S	36.0			-		-	-
T	21.0				+		
U	27.0				++		
V	16.9			+	++	+	++
W	47.4			++	-	- -	-
X	20.7					- -	++
Y	36.3			+	++	+	++
Z	28.5					++	++
α	26.9					+	++
β	19.1					++	++
γ	19.1					++	++

SHS, CG, C, AMF, see Figure 2.
+ = slightly increased spot in expression;
++ = greatly increased;
- = slightly decreased;
-- = greatly decreased;
N= new spot;
D= disappeared spot.
In C plots, marks represent changes in expression level compared to former treatment.
In AMF plots, marks mean changes in comparison to C under same treatment.

control. Seven days after SHS, expression in 17 spots changed compared to 4 d after 20°C in control (Fig. 5A). As for mycorrhizal plants, 14 spots showed higher expression levels than non-mycorrrhizal plants at 7 d after SHS (Fig. 5B). After CG inoculation, expression levels of a total of 23 spots changed compared to 7 d SHS. In the control, eights spots increased in expression but six spots decreased and two spots disappeared compared to 7 d SHS (Fig. 5C). Regarding AMF, 13 spots increased in expression and two new spots appeared compared to control (Fig. 5D). Spot G (20.4kDa) was mycorrhizal-specific expression through SHS and CG treatments. The spots increased in expression by both SHS and CG were L (27.3kDa), O (19.5kDa), V (16.9kDa), Y (36.3kDa). In this case, especially, V and Y increased in mycorrhizal plants compared to the control.

4. Discussion and Conclusions

Mycorrhiza-induced growth promotion in cyclamen under heat stress (30°C) and anthracnose were reported in previous study (Maya and Matsubara, 2013 a, b). However, growth improvement under shock heat stress (35°C), resistance to anthracnose in light of cross-protection under heat stress, and proteomic changes through heat stress and

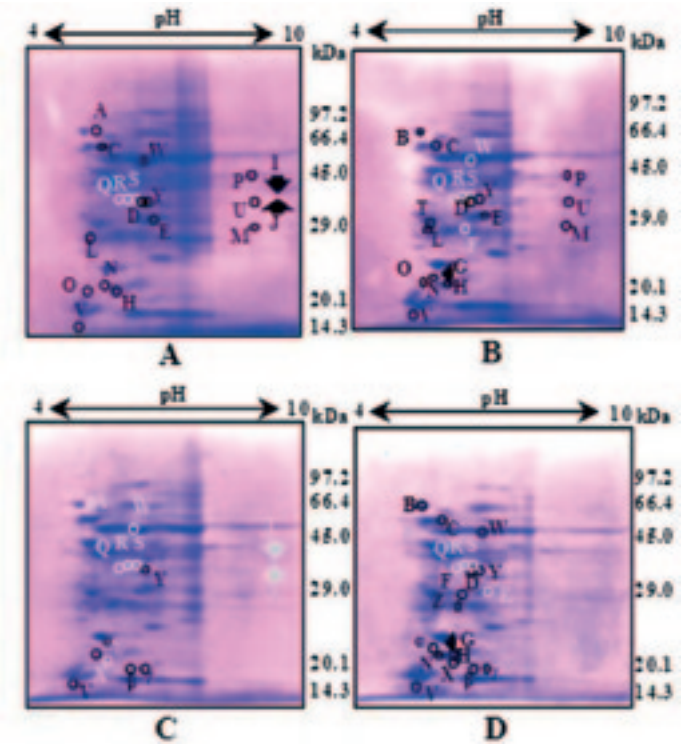

Fig. 5 - 2-D electrophoresis gel with leaf protein in mycorrhizal cyclamen 7 days after SHS and 9 days after CG inoculation. A (control), B (AMF) in 7 days after SHS. C (control), D (AMF) in 9 days after CG inoculation. SHS, CG, see figure 2. Letters show spot ID in Table 1. In A and C, black circle, expression-increased spots compared to former treatment; white circle, decreased spots; black arrows, newly appeared spots; white arrows, disappeared spots. In B and D, black circle, expression-increased spots compared to control under same treatment; white circle, decreased spots; black arrow, newly appeared spot.

disease in mycorrhizal plants have not been clarified. In the present study, growth of mycorrhizal cyclamen under SHS was greater than control, and AMF plants showed greater increases in dry weight of leaves than other parts. This phenomenon might be caused by an allocation of carbohydrate by AMF in shoots that was proportionally greater than tuberous tissues and below ground tissues (Shokri and Maddi, 2009). Generally, temperature influences mycorrhizal symbiosis and growth promotion (Zhu *et al.*, 2011) i.e. high temperature caused negative effect or no effect on mycorrhizal colonization (Li *et al.*, 2008; Compant *et al.*, 2010). In the present study, no significant differences occurred in AMF colonization levels, meaning heat stress might affect mycorrhizal symbiosis less.

Although many reports suggest that AMF had induced disease resistance to host plants, most of them refer to soil-borne disease interactions (Whipps, 2004; Li *et al.*, 2006). In contrast to soil-borne diseases, shoot disease resistance induced by AMF is rarely reported. However, some study have shown AMF alleviated shoot disease and induced systemic acquired resistance (SAR) (Campos *et al.*, 2012). As for anthracnose caused by *Colletotricum gloeosporioides*, one study demonstrated that AMF symbiosis suppressed anthracnose in cyclamen (Maya and Matsubara, 2013 b). In this experiment, mycorrhizal cyclamen plants also showed resistance to anthracnose even under the shock heat stress condition. Hence, it is supposed that AMF induced cross protection to heat stress and anthracnose in the cyclamen examined in this study.

Biotic (pathogen, nematode etc.) and abiotic stresses (heavy metal, drought, high temperature etc.) cause reactive oxygen species (ROS) production in plant tissues. Although ROS have a positive role as a signal which triggers stress responses, it causes destructive damage to organs in cells and a loss of homeostasis. In order to limit oxidative damage and remove ROS, plants have developed a detoxification system through changing antioxidative activity. Antioxidative activity includes enzymatic and non-enzymatic antioxidants where SOD is a key enzyme in the enzymatic antioxidation system, and non-enzymatic antioxidation is also an effective defense system, because it efficiently prevents accumulation of ROS under stress conditions. There are reports suggesting that antioxidative modifications in mycorrhizal plants refer to AMF-induced stress resistance (Nahiyan and Matsubara, 2012; Lambais *et al.*, 2003). However, as for antioxidative changes in host plants, both increases and decreases in activity has been reported (Roldan *et al.*, 2008). Generally, ROS is produced in roots under heat stress (Kolupaev *et al.*, 2013). Increases in SOD activity in roots of mycorrhizal cyclamen under heat stress have also observed (Maya and Matsubara, 2013 a). In this study, the same phenomenon occurred in mycorrhizal cyclamen. In addition, as for DPPH radical scavenging activity that means total activity of non-enzymatic antioxidants: ascorbic acid, polyphenols, flavonoid etc., DPPH radical scavenging activity increased in roots of mycorrhizal plants in this experiment. Some investiga-

tions have shown that AMF-induced increases in DPPH radical scavenging activity under salinity stress and pathogen infection (Hichem *et al.*, 2009; Nahiyan and Matsubara, 2012). Although decreases and increases occurred in leaves and bulbs in this experiment, the decrease might be caused by the resolution of ROS by such increased antioxidants.

Some investigators have reported that AMF has enhanced activity of various defense mechanisms as well as antioxidants. Campos *et al.* (2012) reported that various genes which regulate stress response in plants had were up-regulated in mycorrhizal rice (*Oryza Sativa* L.), and suggested that salicylic acid (SA)-dependent and jasmonic acid (JA)-dependent mechanisms associate with AMF-induced disease resistance. They also implied that systemic acquired resistance (SAR) is caused in mycorrhizal plants. In the present experiment, expression of many protein spots changed following heat stress and CG inoculation. Moreover, expression levels of some spots also changed by AMF. In this case, 4 d after 20°C, an intensive increase in six spots and three newly appeared spots (G, H and I) were recognized in AMF plants. These spots may supposed to be associated with growth promotion through symbiosis. Although these changes might be defense response to AMF colonization itself, which is caused temporary in initial phase of AMF colonization (Campos *et al.*, 2010). At 7 d after SHS, an intensive increase in 12 spots and decrease in three spots (36.0 kDa) was visible in control plants compared to 20°C. Spots I and J, which were visible in the AMF treatment at 20°C, were expressed by SHS treatment. In mycorrhizal plants, 13 spots increased and especially six spots showed greater increase; spot G (20.4 kDa) was still visible at SHS treatment. After CG inoculation, eight spots increased intensively in non-mycorrhizal plants. Compared to control plants, mycorrhizal plants showed considerable increase in 13 spots and spot G was still detected. In this study, proteins which were up-regulated under both SHS treatment and CG inoculation were L (27.3 kDa), O (19.5 kDa), V (16.9 kDa) and Y (36.3 kDa). Especially, V and W showed greater expression levels in mycorrhizal plants than in the control under both stress conditions. Yoshino *et al.* (2012) reported that heat shock treatment induced tolerance to disease and increased free SA. Hence, it is supposed that these four spots are related with both heat stress tolerance and anthracnose resistance induced by AMF.

In conclusion, AMF induced cross-protection to shock heat stress and anthracnose in this study, and the defense systems could include antioxidative modification and changes in some protein expressions associated with such stress tolerances.

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Stem blight resistance of *Asparagus kiusianus* and its hybrid with *A. officinalis*

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Key words: Asparagus kiusianus, Asparagus officinalis, disease resistance, interspecific hybrid, *Phomopsis asparagi,* stem blight.

Abstract Resistance of cultivated asparagus (*Asparagus officinalis*), wild *A. kiusianus* and their interspecific hybrids to stem blight caused by *Phomopsis asparagi* (Sacc.) Bubák was evaluated. *A. kiusianus* had higher resistance than *A. officinalis* although there were individual variations. The resistance of interspecific hybrids between *A. officinalis* and *A. kiusianus* was mostly intermediate, but some individuals had strong resistance equivalent to *A. kiusianus*. The results suggest that it is possible to introduce the resistance of *A. kiusianus* to stem blight into *A. officinalis* through interspecific hybridization for further *Asparagus* breeding.

1. Introduction

Asparagus (*Asparagus officinalis* L.), of the family Asparagaceae, is a perennial crop widely cultivated in the world. The crop is cultivated from northern to southern regions in Japan, and is an important vegetable. It is expected that production of the crop will increase due to its high profits in many places. The southwestern region of Japan has an advantage because spears emerge earlier in this region than in the northern region. Several diseases such as stem blight, leaf spot and brown spot cause serious problems in open field culture in warm regions. Therefore, rain protected culture is necessary (Kobayashi and Shinsu, 1990). Among these diseases, stem blight caused by *Phomopsis asparagi* (Sacc.) is the most serious disease in Japan, Korea, China and Southeast Asia and causes devastating damage to the crop in economically producing fields; the disease is also distributed in Australia, Greece, Italy, New Zealand and USA (Davis, 2001; Elena, 2006; Udayanga *et al*., 2011). Recently disease damage was also reported in northern parts of Japan (Sonoda, personal communication). The causal fungus first forms small lesions or spots on the lower part of the asparagus stem. The lesions continue to expand and produce yellowish-brown spindle shaped spots (Sonoda *et al.*, 1997). Pycnidia usually appear at the central part of a spot and become secondary sources of infection (Sakai *et al*., 1992). The symptoms are different with aging of stems, and result in systemic symptom, and finally stems are killed (Fukutomi, 1993). Thus, production of cultivars with strong resistance to stem blight has become an urgent issue. The genus *Asparagus* is composed of about 100-300 (Bailey, 1944; Dahlgren *et al*., 1985) species mainly distributed in arid regions of the Mediterranean, Africa and Asia (Dahlgren *et al*., 1985). The species are widely used not only as food source but also for medicinal or ornamental purposes. Sonoda *et al*. (1997, 2001) reported that there were varietal differences of stem blight resistance but there were no strong resistant cultivars in *A. officinalis*. They also reported that several *Asparagus* species had strong disease resistance, but it was impossible to produce hybrids by interspecific crosses with *A. officinalis*. Kunitake *et al*. (1996) produced somatic hybrids between *A. officinalis* (2n=2x=20) and *A. macowanii* (2n=2x=20) by protoplasts electrofusion for the purpose of introduction of disease resistance characteristics into asparagus. However, the hybrids failed to mature because of their abnormal genome composition. Marcellán and Camadro (1999) also performed interspecific hybridization between *A. officinalis* and *A. densiflorus* (Kunth) Jessop cv. Sprengeri (2n=6x=60), but the

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endosperm failed to develop normally, leading to subsequent embryo abortion.

Asparagus kiusianus (2n=2x=20) is a dioecious species native to the coastal region of the Japan Sea from Yamaguchi to northern Kyushu. It is, therefore, expected that the species is adapted with the climate of these regions with high disease or salt resistance. Ito *et al*. (2011) successfully produced interspecific hybrids between *A. officinalis* and *A. kiusianus* and also obtained backcross progenies.

The objective of this study is to demonstrate the usefulness of *A. kiusianus* for producing stem blight disease resistant asparagus cultivars, making cultivation in open field culture in southwest region of Japan as well as in other warm climate areas in the world possible. Evaluation of disease resistance was performed in *A. kiusianus*, cross compatibility between *A. officinalis* and *A. kiusianus* and disease resistance in the interspecific hybrids was surveyed.

2. Materials and Methods

Plant materials

Asparagus officinalis cv. UC157F$_1$, as the control cultivar (Sonoda *et al*., 1997), and *A. kiusianus* Keya line from Fukuoka, Japan were used for inoculation tests in 2010 to compare the resistance to stem blight in these two species (Table 1). UC157F$_1$ and *A. kiusianus* (Nijinomatsubara line from Saga, Japan) were used for crossing experiments. UC157F$_1$ and its intraspecific F$_1$ hybrids, *A. kiusianus* (Nijinomatsubara line from Saga, Keya line and Uminonakamichi line from Fukuoka, Japan) and interspecific F$_1$ hybrids of *A. officinalis* with *A. kiusianus* (OK) were used for inoculation tests in 2011 and 2012 (Table 1).

Inoculum

Phomopsis asparagi strain P1, preserved in Saga Prefectural Agricultural Experiment Station, was cultured on potato sucrose agar (PSA) medium at 25°C for one month. After sporulation, conidiospores were suspended in sterilized water and adjusted to the inoculum density of 2×10^6 spores/ml for use in the following tests.

Interspecific cross

Interspecific crosses between *A. officinalis* (female) and *A. kiusianus* (male) and intraspecific cross in *A. officinalis* cv. UC157F$_1$ were made by hand pollination in a greenhouse of Kyushu University Farm in 2010. Obtained seeds were germinated at 25°C in plastic petri dishes, and germinated seeds were sown in vermiculite in plastic cell trays in an incubator. They were transplanted in plastic pots six months after seed sowing and transferred. Genomic DNA was extracted from young cladodes of each plant by modified CTAB method (Stajner *et al*., 2002). Hybridity of interspecific progenies was confirmed by three SSR markers (Caruso *et al*., 2008), AG2, AG7 and AG10, followed by Takeuchi *et al*. (2012).

Evaluation of stem blight resistance for inoculation test

Inoculation tests were performed three times from November 2010 to January 2011 with UC157F$_1$ and *A. kiusianus*, Keya line grown in plastic pots. Seven to 10 plants were used for each test. The plants were inoculated with the vinyl tape and cotton method by reference to the protocols previously described by Sonoda *et al*. (1997, 1999, 2001). Absorbent cotton moistened with spore suspension was put around the basal part (2-5 cm below the lowest branching nodes) of the stem, and covered with vinyl tape. The plants were maintained at 25°C with 90% humidity for three days, followed by incubation at 25°C and at 60-70% humidity in a growth chamber. The spears, which emerged two to three weeks after cutting the areal stems of the seedlings, were examined. Disease severity was determined by scoring 0-4 grades for each plant weekly until five weeks after inoculation. The disease severity grade (DSG) was: 0 = no lesion; 1 = small-sized lesion (<1 cm in length); 2 = spread lesion; 3 = large-sized lesion (>half of the aerial stem) or defoliation; 4 = aerial part death. Percentage of infected plants and disease indices were calculated, as follows:

$$\text{Percentage of infected plants} = \frac{\text{Number of infected plants}}{\text{Total number of plants employed}} \times 100$$

$$\text{Disease index (DI)} = \frac{\sum(\text{Number of plants classified into each grade} \times \text{DSG number})}{\text{Total number of plants employed} \times 4} \times 100$$

The degree of pycnidia formation was also surveyed. Resistance in UC157F$_1$ and its intraspecific F$_1$ hybrids, *A. kiusianus* (Keya line, Nijinomatsubara line and Uminonakamichi line) and interspecific F$_1$ hybrids between them was evaluated by inoculation test three times from August to September and from November to December in 2011 and from October to November in 2012. Assessments were performed according to the procedure as described above.

Table 1 - Plant materials used for inoculation tests

Species	Cultivar and line	Tested year	No. of individuals investigated
A. officinalis	UC157F$_1$	2010	14
A. officinalis	UC157F$_1$, F$_1$ progenies of ' UC157F$_1$'	2011, 2012	25
A. kiusianus	Keya line	2010	15
A. kiusianus	Keya line, Uminonakamichi line, Nijinomatsubara line	2011, 2012	21
Interspecific hybrid	Hybrids between *A. officinalis* and *A. kiusianus*	2011, 2012	25

3. Results

Evaluation of the resistance to stem blight in A. officinalis *and* A. kiusianus

Most of the plants in both species showed pathogenic symptoms on the stems (Table 2). All the inoculated asparagus cultivars began to show blight by one week after inoculation and the aerial parts of all plants were dead within five weeks. In contrast, *A. kiusianus* showed lower DI than *A. officinalis* and more than half of the plants survived five weeks after inoculation although they exhibited symptoms. There were differences of susceptibility among *A. kiusianus* accessions, whereas all *A. officinalis* accessions showed high susceptibility with severe symptoms. Pycnidia formation was frequently observed in *A. officinalis* from two to four weeks after inoculation (data are not shown). A few accessions showed pycnidia formation in *A. kiusianus*, whereas some other individuals showed no symptoms possessing strong resistance.

Interspecific crosses between A. officinalis *and* A. kiusianus

Fruit set rate and number of seeds in interspecific crosses between *A. officinalis* (female) and *A. kiusianus* (male) were lower than those in intraspecific crosses among *A. officinalis* (Table 3). Germination rate of the interspecific hybrid was high (76.1%) at 25°C. Hybridity of the progenies was confirmed by three SSR markers (Caruso *et al*. 2008), AG2, AG7 and AG10 (data are not shown). The hybrid plants showed intermediate morphology between the parents.

Evaluation of the resistance to stem blight in the interspecific hybrids

Most of the hybrid plants and their parents showed pathogenic symptoms (Fig. 1, Table 4). The aerial stems of all inoculated *A. officinalis* showed blight by one week after inoculation and most of them faded within four weeks (Fig. 2 D). In contrast, the aerial stems of *A. kiusianus* and the interspecific hybrids survived even four weeks after

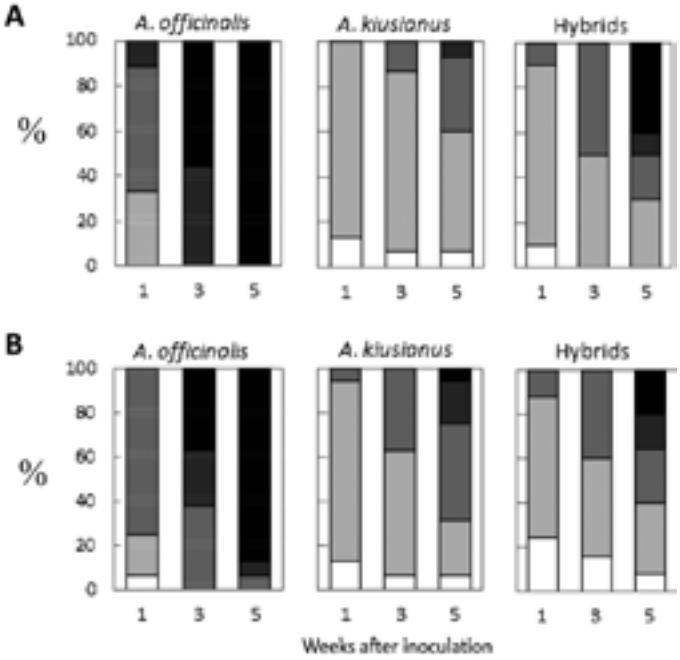

Fig. 1 - Frequency of individuals with their disease severity grades (DSG) in *A. officinalis*, *A. kiusianus* and F_1 hybrids screened for resistance to stem blight. Tests were performed in August (A) and November (B) in 2011. □ 0 = no lesion; ■ 1 = small-sized lesion (<1 cm); ■ 2 = spread lesion; ■ 3 = large-sized lesion (>half the size of plant) or defoliation; ■ 4 = aerial part death.

Table 2 - Development of disease symptoms in *A. officinalis* and *A. kiusianus* inoculated with *P. asparagi*

Species	Percentage of infected plants					Disease index				
	1 [z]	2	3	4	5	1	2	3	4	5
A. officinalis	100 a	100 a	100 a	100 a	100 a	45 a	54 a	88 a	98 a	100 a
A. kiusianus	77 a	85 a	93 a	93 a	93 a	25 b	38 b	54 b	63 b	69 b

[z] Weeks after inoculation.
Different letters in a column indicate significant difference according to student's *t*-test (P≦0.05).

Table 3 - Result of interspecific crosses between *A. officinalis* and *A. kiusianus*

Seed parent	Pollen parent	No. of pollinated flowers	No. of obtained fruits (%)[z]	No. of obtained seeds (n) [y]	No. of germinated seeds (%)[x]	No. of obtained seedlings
A. officinalis × *A. kiusianus*		301	27 (9.0)	67 (2.5)	51 (76.1)	42
A. officinalis × *A. officinalis*		46	25 (54.3)	119 (4.8)	104 (87.4)	45

[z] No. of obtained fruits/No. of pollinated flowers)×100.
[y] No. of obtained seeds/No. of obtained fruits.
[x] (No. of germinated seeds/No. of obtained seeds)×100.

Table 4 - Development of disease symptoms in *A. officinalis*, *A. kiusianus* and their hybrids inoculated with *P. asparagi*

Species	Percentage of infected plants					Disease index				
	1 [z]	2	3	4	5	1	2	3	4	5
A. officinalis	95 a	100 a	100 a	100 a	100 a	30 a	35 a	60 a	90 a	100 a
A. kiusianus	81 a	88 a	88 a	88 a	88 a	27 a	31 a	34 b	37 b	40 b
Hybrids	86 a	92 a	92 a	92 a	95 a	29 a	36 a	40 ab	47 b	59 b

[z] Weeks after inoculation.
Different letters in a column indicate significant difference according to Turkey's multiple range test (P≦0.05).

the inoculation (Fig. 2 E, F). The DI's in *A. kiusianus* and the interspecific hybrids were lower than those in *A. officinalis* although lesion was formed in most of the plants (Table 4). The disease severity grade of *A. officinalis* was high compared with that of *A. kiusianus* and the interspecific hybrids (Fig. 1). Most *A. kiusianus* showed lower grades even five weeks later. The interspecific hybrids showed varied degrees of symptoms and the distribution of DSG was intermediate between the parental species. Diseased lesions were observed in all the stems of *A. officinalis* and numbers of pycnidia were frequently formed four weeks after inoculation (Figs. 3, 4). There was little pycnidia formation in *A. kiusianus* and interspecific hybrids, while lesions were observed in the stems of most plants of *A. kiusianus* and the interspecific hybrids. The hybrids showed intermediate susceptibility between their parent species and their pycnidia formation rates were as low as the degree of *A. kiusianus* (Fig. 4).

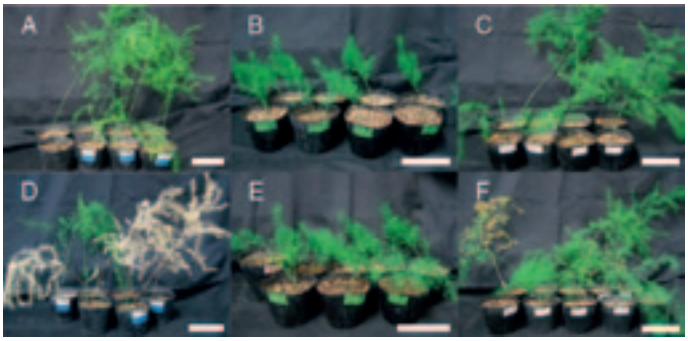

Fig. 2 - Disease symptoms of *A. officinalis* (A, D), *A. kiusianus* (B, E) and F_1 hybrids (C, F) inoculated with *P. asparagi*. A-C: 1 week after inoculation, D-F: 4 weeks after inoculation. *Scale bars* 9 cm.

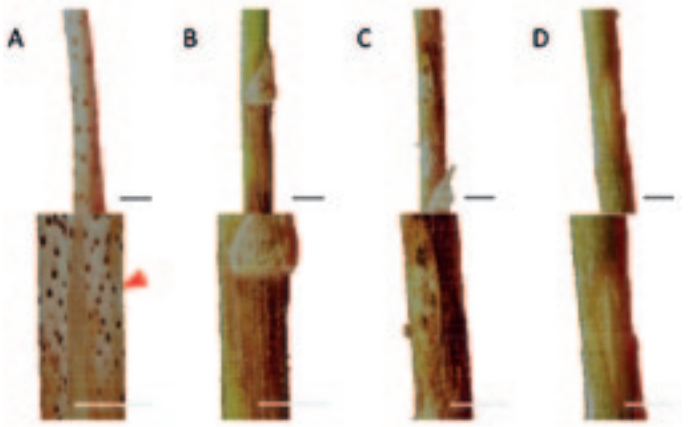

Fig. 3 - Lesion on stems of *A. officinalis* (A), *A. kiusianus* (B) and interspecific hybrids (C, D) inoculated with *P. asparagi*. Arrows indicate pycnidia. *Scale bars* 1 mm.

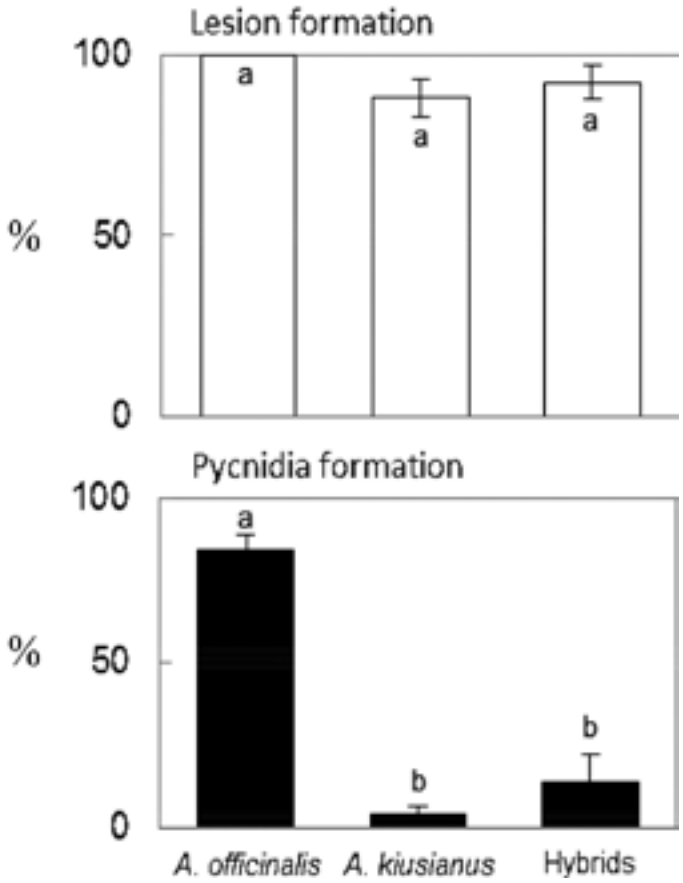

Fig. 4 - Rates of lesion and pycnidia formation of *A. officinalis*, *A. kiusianus* and interspecific hybrids four weeks after inoculation with *P. asparagi*. Bars represent SE (n=3). Different letters indicate significant difference according to Turkey's multiple range test (P≦0.05).

4. Discussion and Conclusions

The symptoms were severer in this study than those reported by Sonoda *et al*. (1997, 1999, 2001). DI was nearly 100 in *A. officinalis* four weeks after inoculation in this study, while it ranged from 42 to 79 in another investigation (Sonoda *et al*., 1997). This might be due to aggressive pathogenicity of the *phomopsis* strain used or differences in environmental conditions from the previous study (Sonoda *et al*., 1997). The results of the present study show that *A. kiusianus* had higher resistance than *A. officinalis* with various degrees of resistance among individuals. Although the lesions were observed in most *A. kiusianus* plants, the resistant accessions displayed reduced disease development and varying levels of resistance. Pycnidia were infrequently formed on the stems of *A. kiusianus*, restricting the spread the pathogen. It is thought of as incomplete (or partial) resistance (Poland *et al*., 2009). The rate-reducing resistance or partial resistance is believed to be effective against a large number of pathogen genotypes and durable resistance since it is non-race specific (Niks and Rubiales, 2002; Poland *et al*., 2009). Therefore, the resistance identified in *A. kiusianus* will be useful for asparagus breeding programs.

There were differences in disease resistance among *Asparagus* species (Sonoda *et al*., 2001). *Asparagus densiflorus*, *A. virugatus*, *A. asparagoides* and *A. macowanii* were highly resistant. They are, however, genetically distant to *A. officinalis* and extremely difficult to obtain viable interspecific hybrids. In contrast, *A. kiusianus* is genetically a closer species to *A. officinalis* than other *Asparagus* species based on the analysis of non-coding cpDNA regions (Fukuda *et al*., 2005, 2011; Kubota *et al*., 2011), although *A. kiusianus* is endemic to Japan, far from of the habitat of *A. officinalis* which is the Mediterranean. Ito *et al*. (2011) obtained interspecific crosses between *A. officinalis* and *A. kiusianus* and their backcross progenies, suggesting the possibility of producing disease resistant asparagus cultivars. It was indicated in this study that the resistance derived from *A. kiusianus* was inherited in the interspecific hybrids, although it was variable among individuals.

Further study with these hybrids and their backcross progenies are needed in order to screen the genotypes with a higher level of resistance to stem blight for breeding disease-resistant asparagus cultivars.

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Black root rot caused by *Diaporthe sclerotioides* threatens cucurbit cultivation in Japan

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Key words: Black root rot, cucurbitaceae, *Diaporthe sclerotioides,*

Abstract: In Japan, since black root rot of cucurbitaceous crops was found more than 30 years ago, the disease has caused severe economic losses to the cucurbit crop industry. Subsequent to the pathogen being correctly identified as *Diaporthe sclerotioides* based on morphology and DNA sequence, knowledge about DNA sequences has developed making technical tools to detect and quantify the pathogen in natural samples of plants and soils available. In addition to chemical soil disinfectants, solarization and biological soil disinfestation have been developed as environment-friendly methods to effectively control this disease. Although it is difficult to apply such temperature-dependent methods in open-fields especially under cool climate conditions, an alternative approach, which changes soil pH to weak alkaline with amending steel converter slag, has also proved effective against the disease. In this mini-review, the process of *D. sclerotioides* identification, detection and quantification methods developed for this fungus, host specificity, and disease control measures available and practiced in Japan are discussed concisely.

1. Introduction

Black root rot of cucurbitaceous crops causes severe root destruction leading to growth depression, non-vascular wilt and premature death of plants. The disease was originally described in gherkin (*Cucumis sativus*) roots in the Netherlands by Van Kesteren, who classified its causal agent as *Phomopsis sclerotioides* Kesteren (Van Kesteren, 1966). It has since spread to other European regions, including U.K., Germany, Denmark, Norway, France (Ebben and Last, 1973), and Italy (Cappelli *et al.*, 2004). In Japan, the disease was first reported in 1985 in squash root which was the rootstock of a cucumber (*Cucumis sativa*) (Hashimoto and Yoshino, 1985). Since then, the disease has also been found in melon (*Cucumis melo*), watermelon (*Citrullus lanatus*), pumpkin (*Cucurbita maxima*), and other cucurbits, threatening the production of these major cucurbit crops with severe economic damage. Shishido *et al.* (2006) identified the causal agent of this disease as *P. sclerotioides* Kesteren through both morphological and phylogenetical analyses. Because of recent development in DNA sequence comparisons, asexual state of genus, *Phomopsis*, has connected to sexual state, *Diaporthe*, which has priority over *Phomopsis*, and should be the generic name adopted for these taxa (Udayanga *et al.,* 2012; Gomes *et al.*, 2013), and thus I use *Diaporthe sclerotioides* as the name of the pathogen of this disease in this article.

To control black root rot of cucurbits, solarization with a combination of soil fumigants such as chloropicrin has been widely applied in greenhouses, especially in warmer climate regions (Kobayashi *et al.,* 1997). However, the disease has been consistently spreading in cucumber production areas in northern parts of Japan, where climate conditions are not warm enough to apply such temperature-dependent measures. In addition, the disease has not yet been contained in the southern parts of Japan even though the incidence is sporadic and inconsistent (Shishido, 2006). The present paper offers a concise review of recent developments in knowledge about black root of cucurbit crops as well as control measures against this disease in Japan.

2. Taxonomy of *Diaporthe sclerotioides*

Morphology

The telemorphic stage of *D. sclerotioides* has not yet been discovered, while anomorphic stages of *Diaporthe* species are often characterized with specific pycinidia. *D. sclerotioides* forms pycinidium which are mostly subglobose or spherical under the epidermis (Van Kesteren, 1966). In our experiment, pycnidia were found submersed in sterilized beanpods after three weeks of incubation, mostly globose and varying in size (200-500 μm in diameter) (Fig. 1A). The internal cavity of a pycnidium was often divided by protrusions of the proliferous layer (Fig.

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1B). Numerous pycnospores (Fig. 1C), mostly ellipsoidal to ovoid, hyaline, 7-12 × 3-6 µm in size, usually with two guttules, were observed in the pycnidium (Fig. 1D) and apparently were produced on conidiogenous cells (Fig. 1E). Conidiophores filiform, hyaline, septate at the base, rarely branched, up to 25 µm, were formed from the inner cells of the locular walls. These only α-type conidiospores were unlike other *Diaporthe* species and no β-type were observed. Although we found that an isolate produced pycnidia and conidiospores, the event was very rare and no such organs have been reported in the natural environment. Therefore, we suspect that conidiospores are not the primary source of inoculum of this pathogen.

On the other hand, *D. sclerotioides* easily forms dull, grayish-brown mycelial mats on common agar media including potato dextrose agar (Shishido *et al.*, 2006). The mycelium consisted of thin hyaline hyphae (2-5 µm in diameter) and thick hyaline to dark brown hyphae (10-20 µm in diameter) (Fig. 1F). A layer of the thick hyphae and dark brown, thick-walled cells formed a small sclerotium (Fig. 1G), and later became superficial or submersed pseudo-sclerotia, mostly flattened, of various sizes (Fig. 1H). Because these pseudo-sclerotia are commonly observed on diseased roots as well, they are likely the primary inocula of black root rot of cucurbit crops.

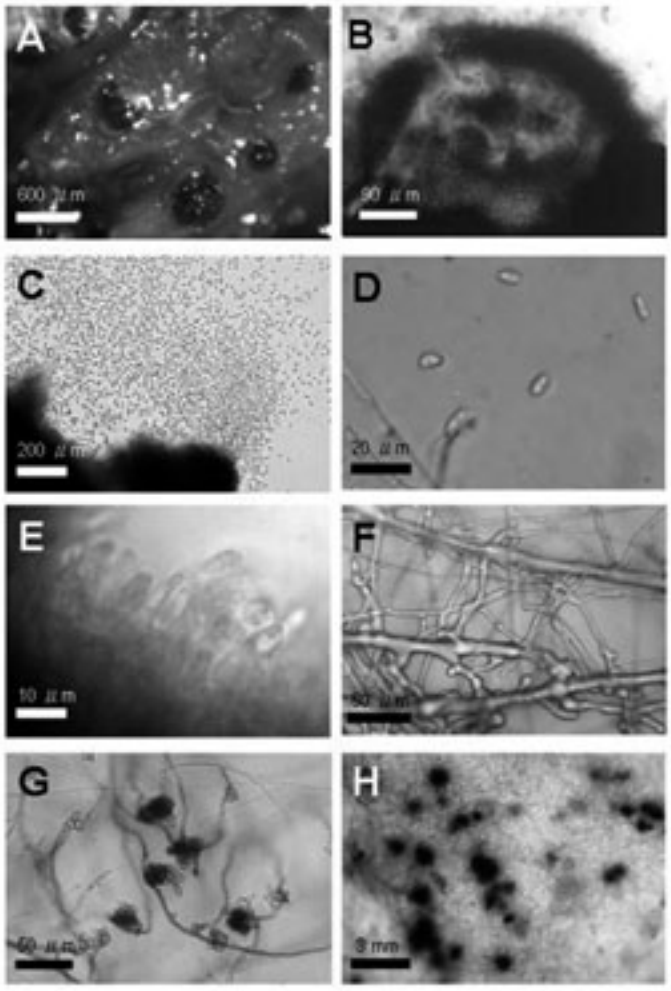

Fig. 1 - Typical features of *Diaporthe sclerotioides* grown under gnotobiotic conditions. A, Pycnidia on a sterilized beanpod. B, Vertical section of pycnidium. C, Numerous pycnospores mechanically released from pycnidium. D, Pycnospores. E, Pycnophores on the internal wall of pycnidium. F, Thick-walled, crenate aerial mycelium commonly found in various agar cultures. G, Masses of dark-colored mycelium, later become pseudomicrosclerotia. H, Small, brownish-black pseudomicrosclerotia on a boiled cucumber leaf. Reprinted from reference Shishido *et al.* (2006) with permission from the publisher.

Molecular phylogeny

Phylogenic analyses of the genus *Diaporthe* have been conducted in a number of studies (Rehner and Uechker, 1994; Zhang *et al.*, 1998; Kanematsu *et al.,* 2000; Farr *et al.*, 2002; Murali *et al.,* 2006; Shishido *et al.*, 2006; Santos *et al.,* 2010; Udayanga *et al.*, 2012; Gomes *et al.,* 2013). DNA sequences of the ITS regions of *D. sclerotioides* isolated from various parts of Japan formed a single distinct clade without differing from the ex-type *D. sclerotioides* (CBS 296.67) by even a single nucleotide (Shishido *et al.,* 2006). The close kin species of *D. sclerotioides* were *D. columnaris* (Farr *et al.,* 2002) and *D. strumella* var. *longispora* (Udayanga *et al.,* 2012). These species differ 11 and 26 bases of the DNA sequences in their ITS regions out of 329 bases in total (ITS 1 and 2). The relationships detected with the DNA sequences of the ITS region were also found in other loci including elongation factor 1-α (Udayanga *et al.,* 2012) and a mating type gene, *MAT1-1-1* (Santos *et al.*, 2010).

Interestingly, we found no host-specific DNA sequences within the ITS regions among the Japanese isolates of *D. sclerotioides* that originated from four different host species: melon, watermelon grafted on bottlegourd, pumpkin, and cucumber. Rehner and Uecker (1994) argued that the host-based species concept was not reliable for *Diaporthe* because of the genetic diversity among isolates of this genus from various hosts. Kanematsu *et al.* (2000) supported this hypothesis by demonstrating that the phenotypic divergence of *Phomopsis* species, i.e. W type (mainly white colonies, weakly virulent, bearing both α- and β-type conidia) and G type (mainly gray colonies, highly virulent, bearing only α-type conidia) was dependent on their ITS sequences rather than on the host species. Since the Japanese *D. sclerotioides* isolates of black root rot are all G type, it may not be surprising that these isolates create a single clade in the phylogenetic tree.

3. Methods for detection and quantification of *Diaporthe sclerotioides*

Plant pathogenic fungi are usually detected by their growth on selective media or by biochemical, chemical, and immunological analyses. However, none of these conventional techniques are available for *D. sclerotioides*. Moreover, morphological identification of these fungi on nonselective media is time-consuming and requires expert knowledge of classical taxonomy. In recent years, PCR-

based molecular techniques have contributed to the detection and identification of various types of plant pathogens. Real-time PCR techniques combine the sensitivity of conventional PCR with the generation of a specific fluorescent signal. This signal can be measured throughout the procedure, providing real-time analysis of the reaction kinetics, and thereby allowing for quantification of specific DNA targets (Schena *et al.*, 2004).

Shishido *et al.* (2010) constructed conventional and real-time PCR primers to detect *D. sclerotioides*, utilizing a DNA sequence in the ITS region specific to this fungus. The designed primers, CPs-1 (forward) and CPs-2 (reverse), successfully detected the fungus in soil and plant samples collected from fields naturally infested with the disease. Furthermore, the CPs-1/CPs-2 primer pair was applied to a real-time PCR assay with SYBR Green I, and the protocol achieved a linear response with a high correlation coefficient between input DNA and cycle threshold. However, because SYBR Green I is a DNA-intercalating dye without sequence specificity (Wittwer *et al.,* 1997), and because DNA in natural samples usually contains unknown sequences, the amount of DNA will only be accurate if no presence of other DNA similar to this fungus is confirmed in the sample. This problem was solved using a TaqMan probe-based real-time PCR assay, which is highly specific, sensitive and quantitative (Shishido *et al.,* 2013). In addition, the TaqMan probe-based protocol allows multiplex real-time PCR, and thus using internal standard DNA such as GFP for soil samples (Klerks *et al.,* 2004) and COX for plant samples (Weller *et al.,* 2000), quantification of the fungal DNA should be more accurate than the mono-plex counterparts. Table 1 summarizes primer sequences that can be used for detecting and quantifying *D. sclerotioides* DNA in natural samples.

4. Host range and specificity of *Diaporthe sclerotioides*

In general, it is important in breeding programs to know if there are specific interactions between pathogenic microorganisms and host species. Although *D. sclerotioides* can cause black root rot only in cucurbit species, until recently little has been known about the degrees of either the host susceptibility to this disease or host specificity of the pathogen. Shishido *et al.* (2014) hypothesized that *D. sclerotioides* isolates were more infective and virulent to the cucurbit species from which the pathogens were originally isolated than to other host species. They conducted cross-inoculation experiments using cucumbers, melons, pumpkins, watermelons, and bottlegourd (*Lagenaria siceraria* var. *gourda*), by inoculating 12 *D. sclerotioides* isolates from these cucurbit species. The virulence of the isolates was evaluated as the area under the disease progress curve (AUDPC). All cucurbit species were susceptible to each isolate, but AUDPCs were significantly different among the hosts as melon > cucumber ≥ watermelon ≥ bottlegourd ≥ pumpkin.

The infectiveness of isolates, on the other hand, was assessed as the quantity of *D. sclerotioides* DNA detected in the hypocotyls of seedlings two weeks after inoculation using the TaqMan-based real-time PCR protocol described above. The fungal DNA quantities varied among the species in the same order as the AUDPCs. Orthogonal contrasts indicated no specificity in either the fungal virulence or infectiveness between *D. sclerotioides* isolates and the cucurbit hosts from which these isolates originated (Fig. 2). Based on these results, the original hypothesis was refuted and they concluded that though host susceptibility to black root rot varies among cucurbit species, *D. sclerotioides* isolates are unlikely to have specificity to the host

Table 1 - PCR protocols for detecting and quantifying *Diaporthe sclerotioides* in natural samples

Detection of *D. sclerotioides* (Shishido *et al*., 2010)	
CPs-1 (forward) CPs-2 (reverse)	5´-GCCTCGGCGCAGGCCGGCCTCACC-3´ 5´-GGGGCCTTCCAGAACGAAATATAATTT-3´
Note: Not recommended for real-time PCR, Expected amplicon size: 392 bp	
Detection and quantification of *D. sclerotioides* (Shishido *et al*., 2013)	
CPs2f (forward) CPs2r1 (reverse) CPs2t (probe)	5´-ACTGCTTGGTGTTGGGGCACC-3´ 5´-TCCAGAACGAAATATAATTTACTACGCT-3´ 5´- [FAM]-AAAGGGCGGGCCCTGAAATCTAGTGGCGA-[TAMRA] -3´
Note: Applicable with SYBR Green I instead of TaqMan probe, Expected amplicon size: 101 bp	
Internal standard of soil samples (Klerks *et al*., 2004)	
FPGFP (forward) RPGFP (reverse) PYYGFP (probe)	5´-TGGCCCTGTCCTTTTACCAG-3´ 5´-TTTTCGTTGGGATCTTTCGAA-3´ 5´- [VIC]-AACCATTACCTGTCCACACAATCTGCCC-[TAMRA] -3´
Note: Applicable with SYBR Green I instead of TaqMan probe as an external standard.	
Internal standard of plant samples (Weller *et al*., 2000)	
COX-F (forward) COX-R (reverse) COX-P (probe)	5´-CGTCGCATTCCAGATTATCCA-3´ 5´-CAACTACGGATATATAAGAGCCAAAACTG-3´ 5´- [VIC]-AGGGCATTCCATCCAGCGTAAGCA-[TAMRA] -3´
Note: Applicable with SYBR Green I instead of TaqMan probe as an external standard.	

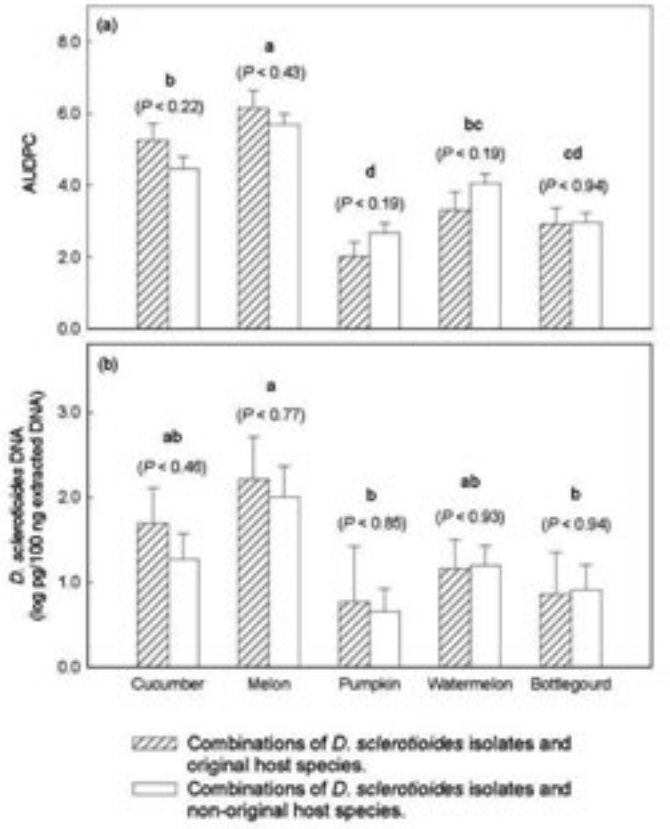

Fig. 2 - The area under the disease progress curve (AUDPC) for black root rot in cucumber, melon, pumpkin, watermelon, and bottlegourd plants (a), and the quantity of *Diaporthe sclerotioides* DNA detected in the hypocotyls of these plants (b) after root inoculation with 12 isolates of *D. sclerotioides*. In both charts, orthogonal contrasts were tested between original host-fungal isolate combinations and non-original host-fungal isolate combinations with *P* values showing type I error probabilities of null hypotheses. The same letters indicate no significant difference between plant species as determined by Tukey's HSD test ($P < 0.05$). Error bars denote the standard error of the mean. Reprinted from reference Shishido *et al.* (2014) with permission from the publisher.

species in terms of either virulence or infectiveness. This research implies that in practice seedlings and soils from infested areas should be handled carefully because the pathogen may spread to various cucurbit species irrespective of the original host.

Interestingly, the relationship between the AUDPC and *D. sclerotioides* DNA quantity in hypocotyls provided evidence that the virulence of this pathogen was not highly correlated with its infectiveness, although some degree of fungal invasion was obviously required for disease development (Fig. 3). The correlation analyses also showed that, although for most of the cucurbit species there were statistically significant correlations between AUDPCs and *D. sclerotioides* DNA quantities in the hypocotyls, their relatively low coefficients of determination indicated limited associations for these variables, therefore suggesting that the virulence of *D. sclerotioides* may be due to factors in addition to infectiveness.

5. Control measures against black root rot of cucurbit crops

Van Kesteren (1966) indicated that *Cucurbita ficifolia* was tolerant to the disease despite apparent root infection by *D. sclerotioides*. However, no true resistant variety or *R*-genes as such has been reported to black root rot. It is possible to use *C. ficifolia* for rootstocks of cucumber, and in fact it used to be a common practice in cucumber production in Japan. Unfortunately, the rootstock of *C. ficifolia* causes a fine white powder on the surface of the cucumber fruits, called "bloom", primarily composed of silica (Mitani *et al.*, 2011). In the late 1980s, cucumber without any bloom (bloomless cucumber) became popular in Japan because of its more attractive and distinctly shiny appearance; and thus *C. ficifolia* has no longer be used as rootstock of cucumber in the commercial production.

Because *D. sclerotioides* is a soil-borne pathogen, soil disinfestation is a common control measure against black root rot of cucurbits. Although chloropicrin appears to be the most effective among disinfectant chemicals, its high toxicity to humans and unpleasant odor limits its popularity in practical applications (Shishido and Takeuchi,

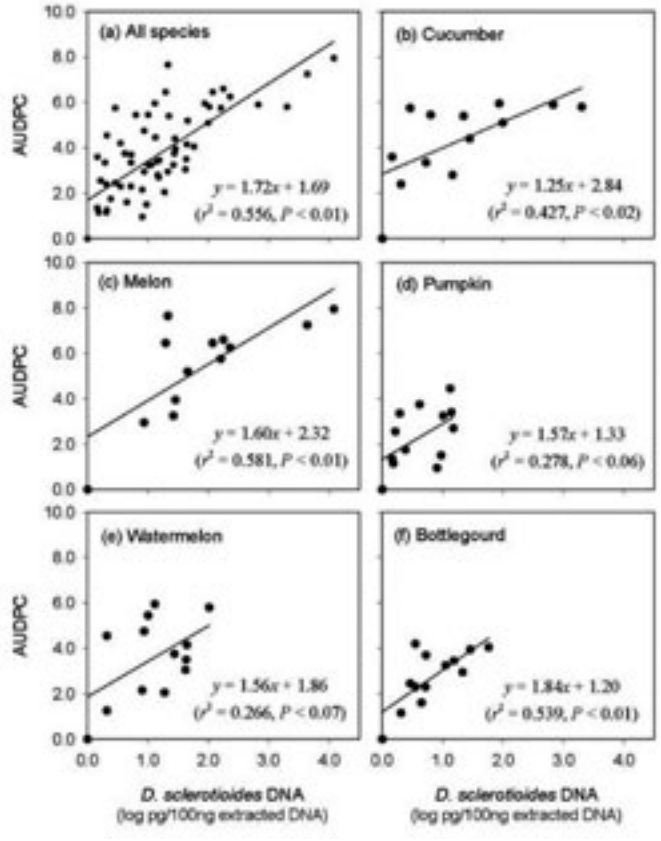

Fig. 3 - Correlations between the area under the disease progress curve (AUDPC) and the quantity of *Diaporthe sclerotioides* DNA detected in the hypocotyls of all the cucurbit species combined (a), cucumber (b), melon (c), pumpkin (d), watermelon (e), and bottlegourd (f) after root inoculation with 12 isolates of *D. sclerotioides*. Reprinted from reference Shishido *et al.* (2014) with permission from the publisher.

2005). Soil solarization is an alternative and environmentally-healthy measure against the disease since the pathogen is known to be heat-sensitive (Kobayashi *et al.,* 1997). One of the problems in soil solarization is to maintain high temperatures (37.5°C for 2 days or 35.0°C for 6 days) throughout the root range of soil depth, *ca.* 30 cm. In recent years, biological soil disinfestation (BSD), or anaerobic soil disinfestation (ASD) has been successfully applied to control some soil-borne pathogens and pests including *Fusarium oxysporum*, *Ralstonia solanacearum*, and parasitic nematodes (Momma *et al.,* 2006; Lamers *et al.,* 2010). BSD increases the effect of solarization by amending organic substances such as wheat bran or green manure crops under anaerobic conditions. Yokoyama *et al.* (2012) demonstrated that BSD with a low concentration of ethanol (0.5%-1.0%) was sufficiently effective to control black root rot of cucumber. They recommend a low concentration of ethanol instead of wheat bran for the soil amendment because the former produces almost the same level of disease control efficacy to the latter with little unpleasant odor during the anaerobic process.

Although BSD may be a promising control measure against black root rot, the practice may only be applicable in protected facilities such as greenhouses and walk-in tunnels in relatively warm climate regions. Therefore, other control measures are needed that are applicable in vast, unprotected fields especially in cool climate regions. Iwadate (2012) demonstrated that changing soil pH to weak alkaline, i.e. pH=7.5, with amending steel converter slag, significantly reduced the disease severity of cucumber in unprotected fields. All of these cucumber fields were in Iwate Prefecture, located in the north-eastern part of Japan, an area known for its cool climate. Although the mechanisms of disease suppression of this method have not yet been elucidated, it is certainly interesting since steel converter slag is easily available at a low cost as a byproduct of steel manufacturing.

To date, only a few studies of biological control have been conducted regarding black root rot, e.g. *Gliocladium roseum* by Moody and Gindrat (1977) and *Pseudomonas* sp. by Fuchs and Defago (1991). Nonetheless, because *D. sclerotioides* is a slow growing fungus compared with other major soil-borne fungal pathogens such as *Fusarium*, *Rhizoctonia*, and *Pythium*, the fungus may be less competitive in searching for nutrients and habitats in soil. Interestingly, hypovirulent elements such as double-stranded RNA are also known to infect *Diaporthe* species (Ghabrial, 2013). Although the detailed mechanism has not been elucidated, some isolates of *D. sclerotioides* significantly reduce in virulence (Shishido *et al.,* 2014). Therefore, biological control including hypoviruses may have potential for controlling black root rot of cucurbit crops in future.

6. Conclusions

In Japan, more than 30 years have passed since black root rot was first reported in cucumber. Since then, the fungal isolates were correctly identified as *Diaporthe sclerotioides* based on their morphology and DNA sequence. In addition, the knowledge of DNA sequence has developed technical tools for detecting and quantifying the pathogen in natural samples of plants and soils. On the other hand, aside from chemical soil disinfestation, environment-friendly control measures have also been developed by applying solarization as well as biological soil disinfestation. Although such temperature-dependent methods may not properly be applicable to out-fields especially in cool climate regions, an alternative measure by changing soil pH to weak alkaline with amending steel converter slag has proved effective to control this disease. Nonetheless, the area of its infestation is still expanding to northern parts of Japan. To prevent further damage to cucurbit production from black root rot and reducing the infestation of *D. sclerotioides*, studies are needed especially in the area of breeding of resistant varieties and understanding the ecology of the pathogen to develop more promising and effective measures to control of this disease.

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Wild grape germplasms in Japan

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Key words: anthocyanins, breeding, classification, geographic distribution, growth cycle, *Vitis.*

Abstract: In Japan, seven species and eight varieties of wild grapes were identified, among which the main species are *Vitis coignetiae* Pulliat, *V. flexuos*a Thunb., and *V. ficifolia* Bunge var. *lobata* (Regel) Nakai (syn. *V. thunbergii* Sieb. et Zucc.). This paper summarizes the identification and classification of wild grapes native to Japan based on the past reports. Their distributions in Japan and physiological and ecological traits are also reviewed for effective practical use for grape breeding in the future.

1. Introduction

It is thought that ancestors of grape (genus *Vitis*) appeared during the first half of the Cretaceous period. They then spread around the world according to environmental and anthropogenic influences, and now comprise three major groups of species: European, North American, and East Asian species, which differ in their physiological and ecological characteristics (Horiuchi and Matsui, 1996). Wild grapes native to Japan belong to the group comprising the East Asian species. Only a few reports on wild grape species, including classification, physiological, and ecological characterizations, have been published so far (Horiuchi and Matsui, 1996). Nevertheless, the importance of wild grapes as genetic resource for grape breeding has gradually been recognized because some wild grapes show superior traits towards global warming in terms of sustainable berry production under hot and humid conditions.

This paper describes the identification and classification of wild grapes native to Japan. Their physiological and ecological traits, as well as their utilization are also reviewed by focusing on the latest research findings regarding wild grapes native to Japan.

2. Geographical distribution

This, and previous studies, found that seven *Vitis* species and eight varieties are distributed throughout Japan, from Hokkaido (northern region) to Okinawa (southern region) (Table 1) (Nakagawa *et al*., 1991). Of these, Yamabudo, Ebizuru, and Sankakuzuru are the three main species found in Japan. Many other species exist locally in limited areas. In addition, researchers from Osaka Prefecture University discovered Shiohitashibudou (tentative name) (Nakagawa *et al*., 1991). The geographical distribution of the wild grapes native to Japan are shown in figures 1-4. These figures were created from a site survey from Hokkaido to Okinawa starting in 1973, and were made based on past records and reports using conserved (pressed) leaf specimens from Hokkaido University, Tokyo Metropolitan University, Kyoto University, Niigata University, Kumamoto University (Japan), and Taiwan University (Taiwan).

Yamabudou, Vitis coingnetiae *Pulliat (Fig. 1)*

This species is widely distributed from level ground to the lowest mountain areas in Hokkaido; from the lowest areas in the mountains to the mountain zone in the Tohoku district (northeastern region of Japan); from the mountain zone to the alpine region in the Chubu district (central region of Japan); and in the alpine regions of the Kinki, Chugoku, and Shikoku districts. It is thought that this species is also present in a limited area of the alpine regions in the Kyushu district, but it has not yet been discovered around Mt. Aso, which is consistent with the fact that we could not find any pressed leaf specimen in the universities located in the Kyushu distinct. It is noteworthy that this species is not distributed in South Korea, China, and neighboring countries; including Far Eastern Russia. However, it has been confirmed that Yamabudou grows naturally in the South Chishima and Sakhalin districts (Horikawa, 1972).

Sankakuzuru (Gyojanomizu), V. flexuosa *Thunb. (Fig. 2)*

This species is distributed in the mid regions of the Yamabudou (Fig. 1) and Ebizuru (Fig. 3) ranges, overlap-

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Table 1 - Systematic and geographical distribution of wild grapes native to Japan (Nakagawa *et al*., 1991)

Species or varieties	Japanese name	Locality where grown
Vitis coignetiae Pulliat	Yamabudou	Hokkaido, Honshu, Shikoku
Vitis coignetiae Pulliat var. *glabrescens* Hara	Takeshimayamabudou	Hokkaido, Honshu
Vitis flexuosa Thunb.	Sankakuzuru (Gyojanomizu)	Honshu, Shikoku, Kyusyu
Vitis flexuosa Thunb. var. *rufo-tomentosa* Makino	Kesankakuzuru	Southern Honshu, Shikoku
Vitis flexuosa Thunb. var. *tsukubana* Makino	Usugesankakuzuru	Northern Honshu
Vitis flexuosa Thunb. var. *crassifolia* Hara	Atsubasankakuzuru	Shikoku
Vitis saccharifera Makino	Amazuru (Otokobudou)	Southern Honshu, Shikoku
Vitis yokogurana Makino	Yokogurabudou	Shikoku (Kochi Pref.)
Vitis ficifolia Bunge var. *lobata* (Regel) Nakai (*Vitis thunbergii* Sieb. et Zucc.)	Ebizuru [(z)]	All over Japan
Vitis ficifolia Bunge var. *izu-insularis* Hara	Shititouebizuru	Izu Islands
Vitis ficifolia Bunge var. *sinuata* Hara	Kikubaebizuru	Southern Honshu, Shikoku, Kyusyu
Vitis ficifolia Bunge var. *ganebu* Hatusima	Ryuukyuuganebu	Amami Islands, Okinawa Islands, Yaeyama Islands
Vitis austrokoreana Hatusima	Kenashiebizuru	Tsushima Islands
Vitis kiusiana Momiyama	Kumagawabudou	Kyushyu (Kumamoto pref., Kagoshima pref.)
Vitis shiragai Makino	Shiragabudou	Honshu (Okayama pref.)
Vitis sp.	Shiohitashibudou (tentative)	Kyusyu (Kagoshima pref.)

[(z)] used in some classifications as a species (*thunbergii*).

Fig. 1 - Geographic distribution of *Vitis coignetiae* Pulliat (Nakagawa *et al.*, 1986).

Fig. 2 - Geographic distribution of *Vitis flexuosa* Thunb. (Nakagawa *et al*., 1986).

ping with the two species, and is found in slightly lower altitude areas than Yamabudou (Fig. 1). We can usually find this species from the lowlands to the mountainous area of the Tohoku district or the Chubu district; it does not grow naturally in Hokkaido.

Ebizuru, V. ficifolia *Bunge var.* lobata *(Regel) Nakai (Fig. 3)*

This species is one of the most widespread *Vitis* species in Japan. Its distribution extends from the southern Hokkaido region to the flatlands and mountainous terrain in the Okinawa district; it can be found in a wide variety of habitats, including both the seashore and urban districts. This species is considered to be highly adaptable to the environment, thus it has a wide distribution compared with other *Vitis* species. As a result, many variants of morphological and physiological traits are found in this species as a result of adaptation to local climates. Ryuukyuuganebu, Shichitouebizuru, and Kikubaebizuru are varieties belonging to *V. ficifolia*. Kenashiebizuru is also closely related to *V. ficifolia*, although its scientific name is given as *V. auskoreana* Hatusima. These grapes are generally included in the *V. ficifolia* group.

Other species (Fig. 4)

As shown in Table 1 and figure 4, in addition to these, many species and varieties are spread in various districts of Japan. Takeshimayamabudou, a variety of Yamabudou (*V. coingnetiae*), was discovered in Hokkaido (around Lake Akan) and Nagano prefecture. It has no hairiness on the lower leaf surface and a thinner leaf compared to Yamabudou. Kumagawabudou has prickly shoots and ovoid leaves; one wild grape (*V. davidii*) with prickly shoots grows naturally in China, however, it differs significantly from Kumagawabudou in its morphological characteristics. Shichitouebizuru grows in seaside areas of seven islands of Izu. Moreover, its fruit-set is the highest among all wild grapes native to Japan.

3. The classification of Japanese wild grapes

As genus *Vitis* is mainly classified by morphology, some taxa may be taken as different classifications even if they are the same grape. For example, Shiragabudou has two scientific names, *Vitis shiragai* Makino (Makino, 1918) and *Vitis amurensis* Rupr. (Ohwi, 1953). In this section, wild grapes native to Japan are classified concisely based on chemical, biochemical and genetic knowledge.

Morphological classifications

Classification by leaf structure. Galet (1979) tried to classify genus *Vitis* through ampelographic measurements

Fig. 3 - Geographic distribution of *Vitis ficifolia* Bunge var. *lobata* (Regel) Nakai (Nakagawa *et al*., 1986).

Fig. 4 - Geographic distribution of wild grapes native to Japan (Nakagawa *et al*., 1986).

of the leaf (Fig. 5, Table 2). Nakagawa *et al*. (1991) coded the characteristics of grape leaves and the result is presented in Table 3. Code numbers of vein length rations (ABC) for the five basic leaf shapes are as follows (Galet, 1979):

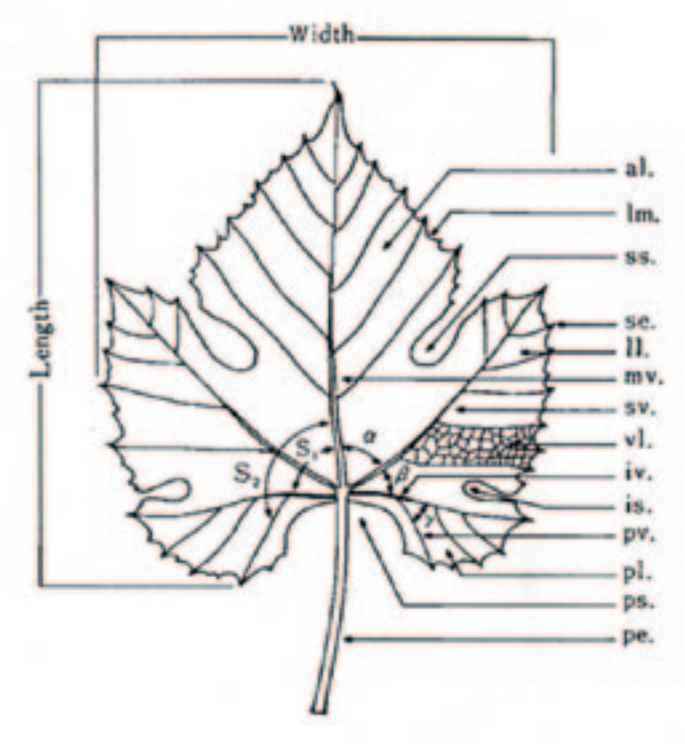

Fig. 5 - The general morphology of a mature grape leaf. al.= Apical lobe; II= Lateral lobe; pl.= Proximal lobe; ss.= Superior sinus; is.= inferior sinus; ps. Petiolar sinus; pe.= Petiol; mv.= Mid-vein; sv.= Superior lateral vein; iv.= Inferior lateral vein; pv.= petiolar vein; vl.= Veinlet; se.= Serration; lm= Leaf margin. (Nakagawa *et al*., 1991).

Table 2 - The code number of the Galet (1979) ruler for the values of A, B, C, r, S_1 and S_2

Code number	Vales of A, B and C	Vales of r	Vales of S_1	Values of S_2
0	0.91~1.00	≤0.80	≤ 70°	≤ 100°
1	0.81~0.90	0.81~0.90	71°~80°	101°~110°
2	0.71~0.80	0.91~1.00	81°~90°	111°~120°
3	0.61~0.70	1.01~1.10	91°~100°	121°~130°
4	051~0.60	1.11~1.20	101°~110°	131°~140°
5	0.41~0.50	1.21~1.30	111°~120°	141°~150°
6	0.31~0.40	1.31~1.40	121°~130°	151°~160°
7	0.21~0.30	1.41~1.50	131°~140°	161°~170°
8	0.11~0.20		141°~150°	171°~180°
9	0.00~0.10		≥151°	≥181°

A= L_2 length/L_1 length; B= L_3 length/L_1 length; C= L_4 length/L_1 length, where L_1= Midwein; L_2= Superior lateral vein and L_3= Inferior lateral vein; L_4= Petiolar vein.
r= Leaf length/leaf width.
$S_1 = \alpha + \beta$; $S_2 = \alpha + \beta + r$ where α= Angles between L_1 and L_2. β= Angles between L_2 and L_3. and r= Angles between L_3 and L_4.

Cordiform: 357 to 468, Cuneiform: 135 to 247, Truncate: 045 to 247, Orbicular: 015 to 136, Reniform: 014 to 136. According to this method, code numbers are relatively near for close species (e.g. *V. coignetiae* and *V. amurensis*) (Table 3).

Classification by pollen ultrastructure. Mochioka *et al*. (1993) observed ultrastructures of mature pollen grains of wild grapes native to Japan, Korea and China using a scanning electron microscope and reported that the pollen could be classified as one of three types by the lumina forms in muri (Fig. 6). They also reported the

Table 3 - The code number of various wild grapes obtained by using the Galet's method (Nakagawa *et al.*, 1991)

Species	ABC-r-S_1S_2
Vitis coignetiae	146-4-24
Vitis amurensis	146-3-24
Vitis flexuosa	357-7-01
Vitis ficifolia var. *lobata*	246-3-13
Vitis shiragai	136-3-13
Vitis sp. (Daisankakuzuru)	146-3-12
Vitis ficifolia Bunge var. ganebu	135-3-24
Vitis sp. (Shiohitashibudo)	257-5-02
Vitis ficifolia var. *izu-insularis*	146-3-01
Vitis kiusiana	368-7-01

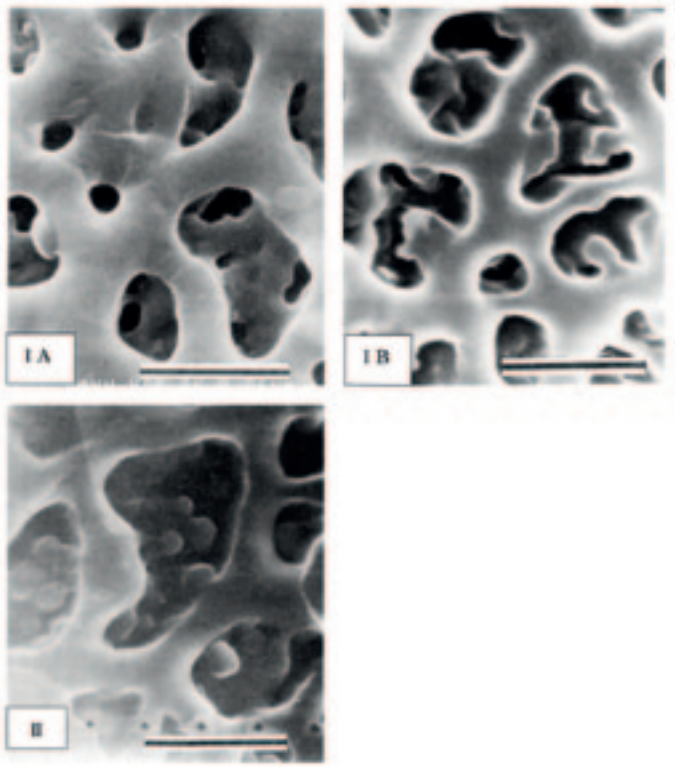

Fig. 6 - Scanning electron microphotographs of grape exine. Scale bars= 1 µm. Type-1 A pollen has perforations in the lumina; type-1 B pollen has perforations and corrugation in the lumina, and type-II pollen has granules in the lumina (Mochioka *et al*., 1993).

pollen ultrastructures of related species belonged to the same type (Table 4).

Chemotaxonomic classifications

Classification by anthocyanins in grape skin. Mochioka *et al*. (1995) analyzed by HPLC anthocyanins in the berry skin of 10 wild grapes (four species, five varieties and one unidentified type) native to Japan. The dendrogram, showing phylogenetic relationships, was drawn from the pairwise comparison of matching coefficients based on anthocyanin

Table 4 - Morphological characteristics of pollen grains of the wild grapes native to Japan, Korea and China (Mochioka *et al*., 1993)

Species or cultivars		Pollen size (μm)		L/W ratio	No. of colpi	Pollen exine type
		Length (L)	Width (W)			
Japan						
Vitis coignetiae Pulliat	♂	20.0±0.2 [z]	19.4±0.2	1.03±0.01	3	I A
	♀	22.0±0.2	20.7±0.2	1.06±0.01	0	I A
Vitis flexuosa Thunb.	♂	20.3±0.5	19.6±0.3	1.04±0.03	3	I A
	♀	20.6±0.2	20.1±0.2	1.03±0.01	0	I B, II
Vitis ficifolia Bunge var. *lobata* (Regel) Nakai	♂	20.0±0.2	19.2±0.2	1.04±0.01	3	II
	♀	20.9±0.2	20.0±0.2	1.05±0.01	0	II
Vitis ficifolia Bunge var. *izu-insularis* Hara	♂	20.9±0.3	20.1±0.4	1.04±0.01	3	II
	♀	21.4±0.2	20.2±0.2	1.06±0.01	0	II
Vitis ficifolia Bunge var. *ganebu* Hatusima	♀	21.4±0.3	20.3±0.2	1.05±0.01	0	I A
Vitis shiragai Makino	♂	21.1±0.2	20.7±0.2	1.02±0.01	3	I A
	♀	22.6±0.2	21.1±0.3	1.08±0.01	0	I A
Vitis kiusiana Momiyama	♀	20.4±0.2	19.5±0.2	1.05±0.01	0	I B
Vitis sp. (provisional name: Shiohitashibudo)	♀	21.3±0.3	20.0±0.3	1.06±0.01	0	II
Korea						
Vitis amurensis Rupr.	♂	20.6±0.3	20.1±0.2	1.03±0.01	3	I A
	♀	23.4±0.2	22.1±0.2	1.06±0.01	0	I B
Vitis sp. (provisional name: Daisankakuzuru)	♀	21.4±0.2	20.3±0.2	1.06±0.01	0	I B
China						
Vitis amurensis Rupr.	♂♀	21.5±0.2	20.7±0.2	1.04±0.01	3	I B
Vitis flexuosa Thunb.	♂	19.8±0.3	19.0±0.3	1.04±0.01	3	I A
Vitis ficifolia Bunge	♂	20.6±0.3	19.0±0.3	1.04±0.01	3	II
Vitis adstricta Hance	♂	21.5±0.2	21.2±0.2	1.02±0.01	3	II
Vitis adstricta Hance var. *ternata* W.T. Wang	♂	22.3±0.3	21.0±0.4	1.06±0.01	3	II
Vitis bellula (Rehd.) W.T. Wang	♂	19.3±0.3	18.8±0.2	1.03±0.01	3	II
Vitis davidii (Roman.) Foëx	♂	23.1±0.2	22.4±0.2	1.03±0.01	3	I B
	♂♀	20.4±0.2	19.9±0.1	1.03±0.01	3	I B
Vitis pseudoreticulata W.T. Wang	♂	21.3±0.2	20.8±0.2	1.03±0.01	3	I A
Vitis hancokii Hance	♂	20.1±0.2	19.6±0.1	1.03±0.01	3	I B
Vitis chugii Metcalf.	♂	19.9±0.2	19.3±0.2	1.03±0.01	3	I B
Vitis chunganensis Hu	♂	19.9±0.2	19.7±0.2	1.01±0.01	3	I A
Cultivar						
Vitis vinifera L.						
'Muscat of Alexandria'	♂♀	23.2±0.4	22.4±0.4	1.03±0.01	3	I B
Vitis lambrusca L.						
'Concord'	♂♀	23.3±0.3	22.9±0.3	1.02±0.01	3	I B
Vitis lambruscana Bailey						
'Delaware'	♂♀	21.3±0.3	20.1±0.3	1.06±0.01	3	I B
'Campbell Early'	♂♀	24.6±0.4	23.4±0.4	1.05±0.01	3	II
'Kyoho' (tetraploid)	♂♀	28.2±0.3	26.9±0.3	1.05±0.01	3.4	I A

[z] Each valure represents the mean of 20 individual measurements ±SE.

components, and agrees well with the morphological taxonomy (Fig. 7, Table 5) (Mochioka *et al*., 1995).

In this study 19 anthocyanins were identified, and there were more kinds of anthocyanins in the berry skins of wild grapes distributed in southern regions than those of wild grapes distributed in northern regions (Table 5).

Classification by isozyme and DNA analysis. Species-specificity was observed in the alleles dominated by *Gpi-2* and *Pgm-2* gene loci (Fig. 8, Table 6) (Ohmi *et al*., 1991). The F band of *Gpi-2* and the A and the C bands of *Pgm-2* existed only in wild grapes native to East Asia (Table 6).

While restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) analyses were used to analyze the relationships among wild and cultivated grapes, a phenogram of RAPD data obtained showed a clear separation between wild and cultivated grapes (Goto-Yamamoto *et al*., 1998).

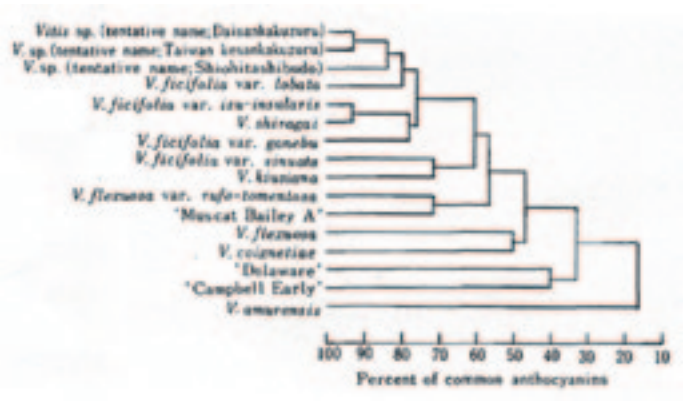

Fig. 7 - Dendrogram of berry skin anthocyanin phenotypes of wild and cultivated varieties (Mochioka *et al*., 1995).

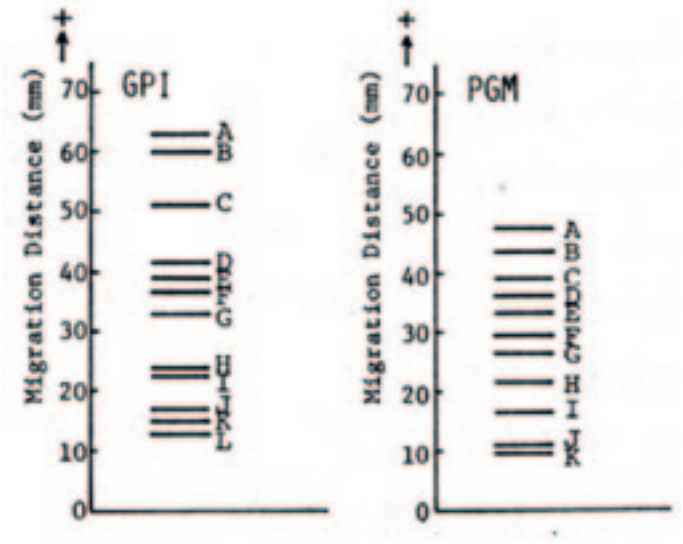

Fig. 8 - GPI and PGM isozymes bands coded by each alleles at *Gp1*-2 (left) and *Pgm*-2 (right) loci (Ohmi *et al*., 1991).

Table 5 - Percentage of anthocyanin composition in grape berry skins analyzed by HPLC [z] (Mochioka *et al*., 1995)

Species or cultivars	Peak No [y]																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Wild grape																			
Vitis coignetiae			1		4	66			1	4		2			1		18		2
V. amurensis	2		2			81													
V. flexuosa	5		4		4	57			7		4					1	12		2
V. flexuosa var. *rufo-tomentosa*			2		1	28			3			3	2	1	4	1	38	2	8
V. ficifolia var. *lobata*	6	2	10		6	44		2	2	1		7	5	10			12		1
V. ficifolia var. *izu-insularis*	15	3	17		5	27			3	1		3	12	5	2	2	4		
V. ficifolia var. *ganebu*	8	4	9	8	10	28		2	4			3	4	1	3	2	9		1
V. ficifolia var. *sinuata*	10	5	39		13	8	9	4	3		6		1		2	1			
V. *shiragai*	11	7	5	13	15	26			2	1		3	4	2	2	2	4		
V. kiusiana	13	10	28		19	11		1	1		6		3	3	2		1		
V. sp. (tentative name: Shiohitashibudou)	6	3	2	10	15	37		2	6	1	1	4	1	1		2	2		
V. sp. (tentative name: Daisankakuzuru)	5	3	28		17	12	7	7	4	3		2	3	2		2	3		
V. sp. (tentative name: Taiwan kesankakuzuru)	5	2	24		6	36		1	8	3		3	2	2		2	3		
Cultivars																			
'Delaware'					44			26					4	3				9	4
'Muscat Bailey A'			3		21		4	32	23				2	2	1	2	2	1	2
'Campbell Early'		6			6								12	46	6	2	7	6	

[z] Absorbance at 520 nm.

[y] Peak No. 3= delphinidia 3-monoglucoside, no. 5= cyaniding 3-monoglucoside; no. 9= petunidin 3-monoglucoside; no. 16= malvidin 3-monoglucoside.

Table 6 - Species-specific alleles at 2 *loci* in grape (Ohmi *et al*., 1991)

Locus	Allele	Species [z]
Gpi-2	A	Vin.
	B	Vin., Amur. (?)
	C	Lab., Rip. (?)
	D	Vin.
	E	Lab., Shir.
	F	Thun., Shir.
	G	Vin., Amur.
	H	Lab., Aest. (?), Vulp (?)
	I	Lab., Rup., Champ., Linc. (?), Coig., Shir.
	J	Bourq. and/or Lab.
	K	Champ.
	L	Vulp. and/or Lab.
Pgm-2	A	Shir.
	B	Vin.
	C	Amur., Coig., Thun., Shir.
	D	Vin.
	E	Vin.
	F	Lab. and/or Linc.
	G	Lab., Aest. (?)
	H	Vin., Rup., Champ., Lab., Coig., Aest. (?), Bourq. (?), Rip. (?), Vulp. (?)
	I	Vin.
	J	Rup.
	K	Champ.

[z] Abbreviations: Amur.= *Vitis amurensis*; Aest.= *V. aestivalis*; Bourq.= *V. aestivalis* var. *bourquiana*; Champ.= *V. champini*; Lab.= *V. labrusca*; Linc.= *V. lincecumii*; Rip.= *V. riparia*; Rup.= *V. rupestris*; Shir.= *V. shiragai*; Tun.= *V. thunbergii* (=*V. ficifolia* var. *lobata*); Vin.= *V. vinifera*; Vulp.= *V. vulpina*.

Classification by general judgments

Since the past horticultural plant classification was qualitatively performed considering a small number of characteristics, a different result for some researchers might be found (e.g. Shiragabudou). Therefore, using plural classification methods is desirable because just one method might induce the wrong result.

What follows is a brief discussion of judgments about some Japanese wild grapes with questionable taxonomic points.

Shiragabudou. Shiragabudou was discovered in Okayama prefecture, Honshu and was first named by Makino (1918). As the leaf shape of this wild grape resembles that of *V. amurensis* Rupr. Ohwi (1953) changed its scientific name to *Vitis amurensis* Rupr. The leaf morphology and pollen ultrastructures of these two wild grapes are in the same group (Table 3, 4), but anthocyanin composition in berry skins (Table 5) and species-specific alleles at 2 loci (Table 6) are apparently different. Furthermore, ecological differences exist between Shiragabudou and *V. amurensis.* Shiragabudou is distributed over the warm lowland from 20 to 240 m above sea level in Okayama while *V. amurensis* has a growth area in the cold districts at 40 to 50° N latitude. The cross section morphology of Shiragabudou shoots is hexagonal, while that of *V. amurensis* is circular.

These differences show that Shiragabudou and *V. amurensis* are not the same species, thus *Vitis shiragai* Makino should be used as the scientific name for Shiragabudou.

Ebizuru and its varieties. Ebizuru is distributed widely in Japan. There are a number of varieties and ecotypes in the Ebizuru group, and morphological differences are various. Even if they are the same species, there are several synonyms for this group. Even now, *Vitis ficilolia, V. ficifolia* var. *lobata*, and *V. thunbergii* are used as scientific names for Ebizuru. An isotype of *V. ficifolia* is the wild grape native to China. There are definitely differences in leaf morphology and bearing habit between Chinese *ficifolia* and Japanese Ebizuru.

*Natural hybrids***.** Different *Vitis* species can be hybridized easily with each other, so there are many natural hybrids. *V. yokogurana* is supposed to be a hybrid of *V. flexuosa* and *V. saccharifera* (Makino, 1918); *V. flexuosa* var. *tukubana* is supposed to be a hybrid of *V. flexuosa* and *V. ficifolia* var. *lobata* (Murata, 1971).

Yamabudou. Yamabudou (*V. coignetiae*) must be a species related to *V. amurensis*, but there is no report that both these two species are simultaneously distributed over the same regions in Japan or other countries.

Shiohitashibudou. (tentative name): Shiohitashibudou is an unidentified species, and it was discovered in Kagoshima prefecture, Kyushu (Nakagawa *et al*., 1991). Its leaf morphology is definitely different from that of other Japanese wild grapes. Shiohitashibudou might be a related species or natural hybrid of Ebizuru because its flowering habit is ever-bearing and the pollen ultrastructure is type II. Its bud endodormancy is deeper, and the soluble solid content of its juice is higher than other Japanese wild grapes (Mochioka, 1996).

Ryuukyuuganebu. Ryuukyuuganebu is distributed over Amami, Ryukyu and Yaeyama Islands, and is supposed to be a variety of *V. ficifolia*. However its leaf shape is different from *V. ficifolia* var. *lobata* (Fig. 9, Table 3), and its pollen ultrastructure is also different (Table 4). Ryuukyuuganebu is ever-green in its habitat.

4. Physiological and ecological traits of wild grapes native to Japan

Four species [*V. coignetiae*, *V. flexuosa*, *V. shiragai,* and Shiohitashibudou (tentative name. *Vitis* sp.)] and two varieties (*V. ficifolia* var. *lobata* and *V. ficifolia* var *ganebu*) of wild grapes native to Japan, and two species [Chosen Yamabudou (*V. amurensis*) and Daisankakuzuru (tentative name. *V.* sp.)] grown in Korea, and ‘Delaware’ (*V. labrus-*

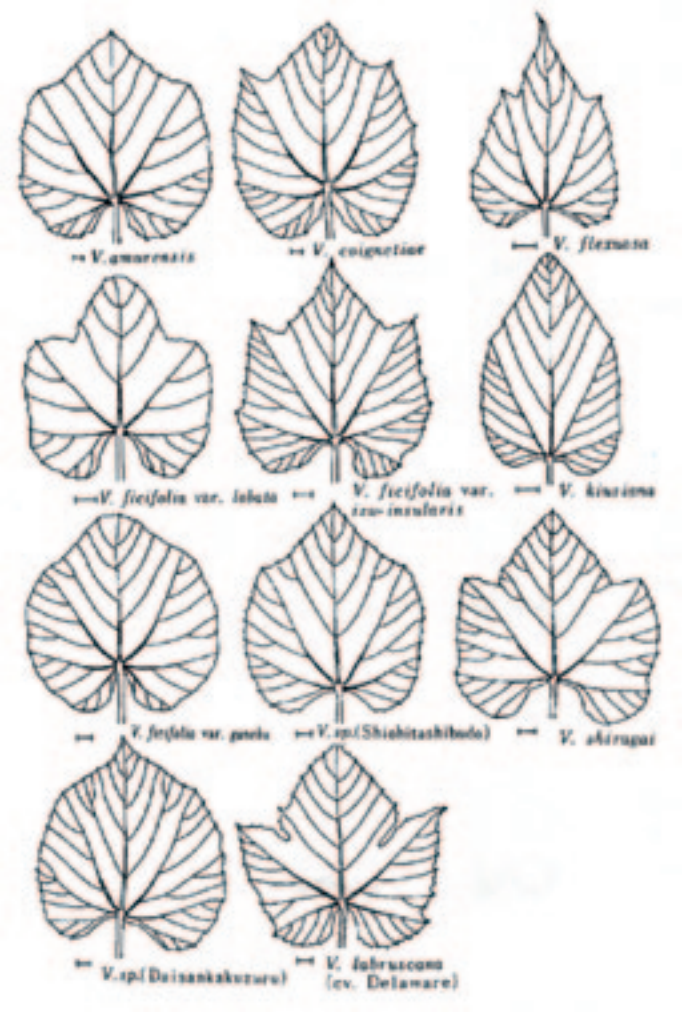

Fig. 9 - Standard design of mature leaf of various wild grapes (⊢⊣ 1 cm) (Nakagawa *et al*., 1991).

cana Bailey) (Table 7) were cultivated in the Horticultural Experiment Field at Osaka Prefecture University (Sakai, Osaka) and their physiological and ecological traits were compared (Nakagawa *et al*., 1986). Here, Chosen Yamabudou is a wild grape grown naturally in the northern and northeastern regions of China, northern region of Korea and southeastern region of the former Soviet Union. The results are illustrated below.

Growth cycle

Bud burst. Bud burst of Yamabudou and Chosen Yamabudou occurred earlier among the wild grapes, followed by Sankakuzuru, Ebizuru, and Daisankakuzuru. Shiohitashibudou showed the latest bud burst in this study (Table 7). 'Delaware' broke bud later than all wild grapes except for Shiohitashibudou.

Full bloom (50% cap off). In grape cultivation, early bud burst does not always mean early bloom. Indeed, the orders of bloom date differed from those of bud burst (from early to late bloom): Yamabudou, Sankakuzuru Ebizuru/Shiragabudou, Daisankakuzuru, Shiohitashibudou, and Ryuukyuuganebu. Here, Ebizuru blooms in the same period as Shiragabudou, and the bloom date of Sankakuzuru was comparable to that of 'Delaware' (Table 7).

Veraison (berry coloring begins). The skin color of Yamabudou changed earliest among the wild grapes. Changes in skin color in Chosen Yamabudou and 'Delaware' occurred on the same date. Skin color change occurred the latest in Daisankakuzuru, Shiohitashibudou, and Ryuukyuuganebu; and Sankakuzuru, Shiragabudou and Ebizuru were the next to latest (Table 7).

Maturity. Maturity is denoted when berry weights and soluble solids attain maximum maturation. 'Delaware' matured in mid-August, which was earlier than the studied wild grapes. Sankakuzuru and Ryuukyuuganebu matured in mid-September and Daisankakuzuru in early-October (Table 7). Thus, wild grapes tend to have medium to late maturation in Osaka.

Defoliation. Defoliation indicates the date when all leaves (from basal to tenth leaf) fall completely. Defoliation in Sankakuzuru and Daisankakuzuru took place in early-November, Yamabudou and Chosen Yamabudou shed their leaves in mid-November, and Shiohitashibudou, Ebizuru, and Shiragabudou in late-November. Interestingly, Ryuukyuuganebu, a subtropical grape, showed extremely late defoliation; in some cases, leaves did not fall until January.

Characteristics of organ

Shoot. Observations of shoot growth in summer enabled us to classify the wild grapes into three types: continuous, subcontinuous, and discontinuous. The continu-

Table 7 - Growth cycle of wild grapes native to Japan at Sakai Osaka (Nakagawa *et al*., 1986)

Species and varieties	Bud burst	Full bloom	Veraison	Harvest	Leaf fall
Chosen yamabudou (*V. amurensis* Rupr.)	3/27	5/14	8/1	9/29	11/14
Yamabudou (*V. coignetiae* Pulliat)	3/27	5/12	7/26	9/29	11/13
Sankakuzuru (*V. flexuosa* Thunb.)	3/29	5/26	8/6	9/14	11/7
Ebizuru [*V. ficifolia* Bunge var. *lobata* (Regel) Nakai]	3/29	6/4	8/11	9/11	11/27
Shiragabudou (*V. shiragai* Makino)	3/29	6/4	8/6	9/15	11/23
Daisankakuzuru (tentative *Vitis* sp.)	3/29	6/10	9/4	10/6	11/4
Shiohitashibudou (tentative *Vitis* sp.)	4/10	6/13	9/4	9/22	11/25
Ryuukyuuganebu (*V. ficifolia* Bunge var. *ganebu* Hatusima	4/3	6/19	8/31	9/20	after end of Dec.
Delaware (*V. labruscana* Bailey)	4/6	5/27	7/30	8/20	11/8

ous group had constant growth of some shoots in summer; Shiohitashibudou and Daisankakuzuru were included in this type. The subcontinuous type exhibited slight growth of some shoots in summer; Ryuukyuuganebu, Sankakuzuru, Ebizuru, and Shiragabudou corresponded to this type. Finally, the discontinuous group stopped shoot growth in summer, for example Yamabudou and Chosen Yamabudou in this study. Tendril placement of all *Vitis* species and varieties native to Japan is intermittent.

Inflorescence. Wild grapes native to Japan (seven species and eight varieties) are dioecious, meaning that they contain imperfect individual male and female plants. Three types of fruiting habits were found (Fig. 10). "A" type: as is the case of Yamabudou, Chosen Yamabudou, Sankakuzuru, Daisankakuzuru, Shiragabudou, and Kumagawabudou, with two to four inflorescences at the basal part on each shoot without any inflorescences on lateral shoots. "B" type: as seen in Shiohitashibudou, Shichitouebizuru, and some Shiragabudou, there are two to four inflorescences at the basal part on each shoot with some inflorescences on lateral shoots. "C" type: Ebizuru and Ryuukyuuganebu belong to this type, with two to six inflorescences at the basal part and the upper part of each shoot, having contiguous inflorescences from the base to the top of lateral shoots.

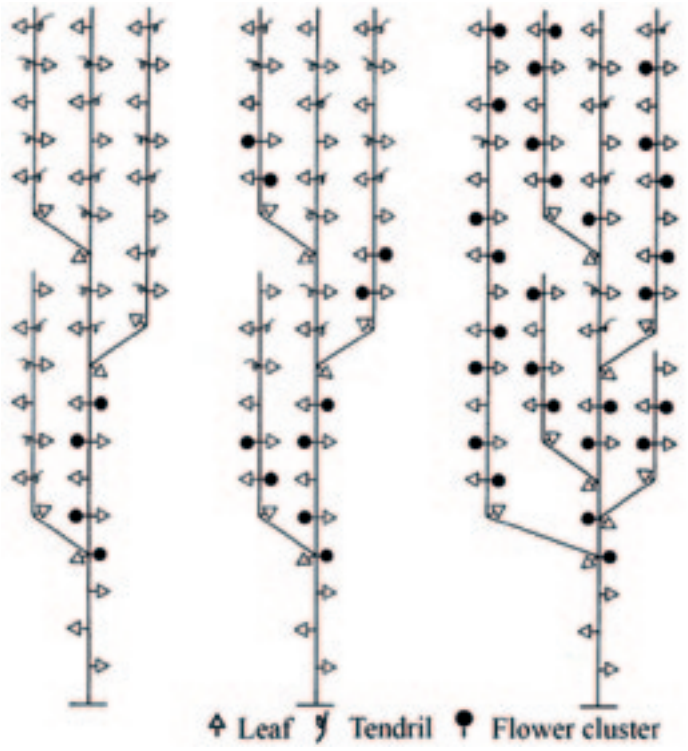

Fig. 10 - Fruiting habit of wild grapes native to Japan.

Grape comparison and fruit quality (Table 8)

Sugars. Shiohitashibudou attained a sugar concentration of 17.7%, which is the highest among the other species (measured as 12-14%) except Ebizuru and Ryuukyuuganebu, which had sugar concentrations of around 8%. Almost all studied species have high glucose content, especially Kumagawabudou, Shiohitashibudou, and Daisankakuzuru, which contained two to three fold more glucose than fructose.

Acids. Kumagawabudou and Daisankakuzuru contained about 0.7 and 0.8% of organic acids, while almost all other species contained about 0.5%; Shiragabudou showed the lowest level of organic acids (0.36%) (Table 8).

Amino acids. The concentration of amino acids varied widely from 50 mg% (Ebizuru) to 294 g% (Shiohitashibudou).

Anthocyanins. Northern species such as Yamabudou and Chosen Yamabudou showed small amounts of anthocyanin, while the southern species, Ryuukyuuganebu, contained a larger amount of this component (Table 8).

Dormancy and cold hardiness

Dormancy. Although all species have a dormancy trait, its intensity depends on the species. Ryuukyuuganebu showed a short dormancy period, while it was generally longer in Kumagawabudou and Shiohitashibudou (Nakagawa *et al*., 1986; Nakagawa, 1989).

Cold hardiness. Cold hardiness varied markedly among the wild grapes. For example, Ryuukyuuganebu and Kumagawabudou were very susceptible to severe damage at -3°C. On the contrary, Yamabudou, Ebizuru, Shiragabu-

Table 8 - Berry composition of wild grapes native to Japan (Nakagawa *et al*., 1986)

Species and varieties	Reducing sugars (%)	Glucose (%)	Fructose (%)	Glucose/ Fructose	Organic acid (%)	Amino acid (mg %)	Anthocuanin (OD 537 nm)
Chosen yamabudou (*V. amurensis* Rupr.)	12.7	9.2	3.5	2.6	0.50	191.2	0.12
Yamabudou (*V. coignetiae* Pulliat)	12.3	5.2	7.1	0.7	0.50	155.3	0.21
Sankakuzuru (*V. flexuosa* Thunb.)	14.2	8.0	6.2	1.3	0.52	214.0	0.30
Ebizuru [*V. ficifolia* Bunge var. *lobata* (Regel) Nakai]	8.1	4.1	4.0	1.0	0.51	50.7	0.30
Shiragabudou (*V. shiragai* Makino)	12.0	6.7	5.3	1.3	0.36	222.6	0.45
Daisankakuzuru (tentative *Vitis* sp.)	12.3	8.1	3.9	2.2	0.81	250.8	0.42
Kumagawabudou (*V. kiusiana* Momiyama)	12.0	9.2	2.8	3.3	0.72	180.5	0.49
Shiohitashibudou (tentative *Vitis* sp.)	17.7	12.7	5.0	2.5	0.48	294.5	0.41
Ryuukyuuganebu (*V. ficifolia* Bunge var. *ganebu* Hatusima	7.8	3.7	4.1	0.8	0.51	138.0	0.70
Delaware (*V. labruscana* Bailey)	16.8	7.5	9.3	0.8	0.77	220.8	0.05

dou, Shichitouebizuru, and Chosen Yamabudou showed moderate cold hardiness; their survival has even been reported at -10°C (Nakagawa, 1989).

5. Value and use of wild grape germplasms in Japan

Wine

Between the 1960s and 1980s, Yamabudou was successfully cultivated in commercial vineyards for wine-making in the town of Ikeda (Hokkaido); its cultivation has attracted attention as a means to revitalize towns in Japan. In China, *V. quinquangularis* is processed into an excellent wine (Li *et al*., 1992). Kumagawabudou, which is thought to be the same species as *V. quinquangularis* (Li *et al*., 1991), therefore, may be an important resource for wine making.

Breeding

The major cultivars, bred using wild grapes, are 'Sawanobori Waingurando' and 'Yama Sauvignon' in Japan, both of which are used for wine production. 'Sawanobori Waingurando' is a cross seedling of *V. amurensis* × (Seibel 13053 × Nakajima No.1, a strain of Yamabudou) and it was released in 1998. By contrast, 'Yama Sauvignon' is a progeny of Yamabudou × 'Cabernet Sauvignon', which was released in 1990 by Dr. Yoshihide Yamakawa at the University of Yamanashi. 'Yama Sauvignon' has the following superior characteristics: 1) no cracking of berry; 2) resistance to ripe rot, downy mildew, and gray mold; 3) adaptability to the prevailing weather conditions in Japan; 4) high productivity; and 5) high quality wine with typical aroma and taste (Yamakawa *et al*., 1989).

Considering the potential use of wild grapes as breeding material, these grapes have the following notable characteristics: drought resistance, cold hardiness, salt tolerance, water logging tolerance, heat tolerance, disease resistance, high concentration of important substances, ever bearing, and short dormancy. Four characteristics are especially promising: i) ever bearing, ii) short dormancy, iii) salt tolerance, and iv) heat tolerance. Therefore, we explain the usefulness of these characteristics for grape breeding programs in more detail.

i) Ever bearing

Strains of Ryuukyuuganebu and Ebizuru bloom and fruit as long as the growth of axillary buds continues. This trait makes it possible to carry out year-round culture and/or culture using a factory system, like some vegetables, using artificial light and controlled irrigation. However, the major gene related to this trait has not yet been identified.

ii) Short dormancy

Ryuukyuuganebu can be released from dormancy after being subjected to low temperature for extremely short periods. This trait may be profoundly related to ever bearing. Thus, it may be possible to force culture inside greenhouses due to the low cost, for year-round culture and/or culture using a factory system.

iii) Salt tolerance

Salt accumulation through the use of chemical fertilizers causes serious problems which sometimes result in the loss of plants. Shiohitashibudou is excellent in its resistance to salts and Ryuukyuuganebu grows naturally along the seashore. Although the mechanism underlying salt tolerance of this grape has not yet been fully elucidated, this trait could be useful not only for scion but also for root stock.

iv) Heat tolerance

As global warming progresses, fruit skin, including grape, shows poor coloration, which leads to a defective appearance and reduced commercial value. Poor coloration also affects wine production. Interestingly, the coloration of wild grapes native to Japan is very high, even under high temperature conditions during their ripening season. In grape, since MYB is involved in red skin coloration, it is valuable to compare MYB genes between wild grapes and primary Japanese cultivars such as 'Kyoho' and 'Aki Queen'. Thus, this trait could be useful for sustainable grape production with high quality and high adaptability in the production area.

6. Conclusions

Wild grapes native to Japan have been actively studied over a long period, but in recent years attention has declined. However, wild grapes native to Japan can offer many useful characteristics, such as short dormancy, ever bearing, heat and salt tolerance. These traits are very attractive, not only for their use as rootstock, but also in terms of breeding material or for genetic studies. We have recently begun a breeding study using Ryuukyuuganebu. It is expected that some novel grapes will be bred to withstand increasing global temperatures or for use in grape cultivation factories.

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Physiological effects of orange essential oil inhalation in humans

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Key words: aroma therapy, blood pressure, horticulture therapy, heart rate variability, people-plant interaction.

Abstract: This study was conducted to clarify the physiological and psychological effects of the odor of orange essential oil in humans. Thirteen healthy male university students (mean age 23.0±1.1 years) participated. The study was conducted in an artificial climate chamber with temperature 24°C, relative humidity 50%, and illumination 50 lux. The subjects randomly inhaled orange essential oil for 120 s. Fresh air inhalation was used as the control condition. Heart rate variability (HRV), blood pressure, and pulse rate were continuously measured before (resting time) and during inhalation of the experimental odor. In addition, sensory evaluation and subjective odor intensity were evaluated after inhalation. The high frequency component of HRV was significantly higher, systolic and diastolic blood pressure was significantly lower, and the subjective "feeling of comfort" was significantly greater during inhalation of the orange essential oil than during inhalation of fresh air. These findings indicate that inhalation of orange essential oil effectively induces relaxation in humans.

1. Introduction

Traditional horticultural science has focused on plant production. Nowadays, the interaction between the welfare of human beings and plants is receiving widespread interest (Relf and Lohr, 2003). People-plant interactions are defined as the wide array of human responses (mental, physical, and social) that occur as a result of both active and passive participation with plants (Relf, 1992). One example of a positive response to plants is observed in the postexposure recovery from attention fatigue (Kaplan and Kaplan, 1989; Tennessen and Cimprich, 1995; Lohr *et al*., 1996; Herzog *et al*., 1997; Wells, 2000). In addition, activities that involve direct contact with plants, such as gardening and horticulture, have been used as therapy for different groups of people in various settings to promote health, well-being, and social inclusion (Davies, 1998; Sempik *et al*., 2003).

The natural environment, which includes plants, affects humans via the five senses and provides stimulation via vision (scenery) and olfaction (aroma of the plant) (Tsunetsugu *et al.*, 2010). There are two areas of experimental methods to clarify the interactions between people and plants. The first and predominant area of study is field experiments, clarifying the effects of total environments. The second area is through indoor experiments that test each stimulation on the basis of the five senses; the results of indoor experiments can support the outcome of field experiments. Therefore, we focused on indoor experiments based on the physiological effects of plant odor stimulation.

The physiological and psychological effects of essential oils have been acknowledged in folk medicine and aromatherapy for a long time (Tisserand, 1988). Also, essential oils and their components are widely used as constituents of different medical products in the medicine industry, as flavoring additives in the food industry, and as cosmetics and fragrances (Cawan, 1999). Oranges are a favorite fruit worldwide. In a previous ambient odor study, exposure to orange essential oil in a dentist's waiting room decreased anxiety and improved mood in female patients (Lehrner *et al*., 2000).

The present paper reports an indoor experiment conducted in humans to determine the physiological effects of the odor of orange essential oil by measuring HRV, blood pressure, and pulse rate before and during inhalation of orange essential oil and comparing the values with those obtained before and during inhalation of fresh air (control).

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2. Materials and Methods

Subjects

Thirteen healthy male university students were recruited for the study (mean age 23±1.1 years), which was conducted according to the guidelines of the Institutional Review Committee of the Center for Environment, Health and Field Sciences, Chiba University, Chiba, Japan. Before beginning the experiment, the subjects provided written informed consent after a detailed description of the aim and experimental procedures were provided to them.

Experimental stimuli and odor delivery system

Orange essential oil (Ogawa & Co., Ltd., Japan) was used as an experimental stimulus. Fresh air without any odor was used as a control. The odor stimuli were controlled by an odor delivery system that comprised four parts: 1) a polypropylene odor bag, 2) a container, 3) an air pump with a gauge, and 4) a funnel with a plastic tube. The polypropylene odor bag was filled with 24 L of diluted orange essential oil. The odor of orange essential oil flowed from the plastic odor bag through an air pump set at 2.5 L/min and was delivered through a Teflon tube to a funnel located 15 cm from the subject's nostrils.

After inhalation of the odor stimuli, the subjects were asked to evaluate the subjective odor intensity on a 6-point scale, from an "insensible level" (0) to an "unbearable level" (6). The level of subjective intensity was controlled at an "easily sensible level."

Experimental design

The study was conducted in an artificial climate chamber with temperature 24°C, relative humidity 50%, and illumination 50 lux. The time schedule for the experiment is shown in figure 1. After the attachment of sensors for physiological measurement, the subjects were asked to close their eyes and rest. Blood pressure and pulse rates were monitored in real time. After 30 s in a stable state, the subjects were exposed to odor simulation for 120 s. Sensory evaluation was conducted after physiological measurements. The subjects were asked to evaluate their subjective feelings and subjective intensity.

Measurement

HRV, blood pressure, and pulse rates were measured as physiological indices. These indices are frequently employed to estimate changes in autonomic nervous activity (Tsunetsugu *et al*., 2010). The time interval between two consecutive R waves (R-R interval) on an electrocardiogram was measured using a portable electrocardiograph (Activtracer AC-301A, GMS, Japan) with three disk electrodes that were attached to the patient's chest; this data was then analyzed using the maximum entropy method (Memcalc/win; GMS, Japan). Two major spectral components of HRV were calculated: low frequency (LF: 0.04-0.15Hz) and high frequency (HF: 0.15-0.4 Hz) components (Task Force of the European Society

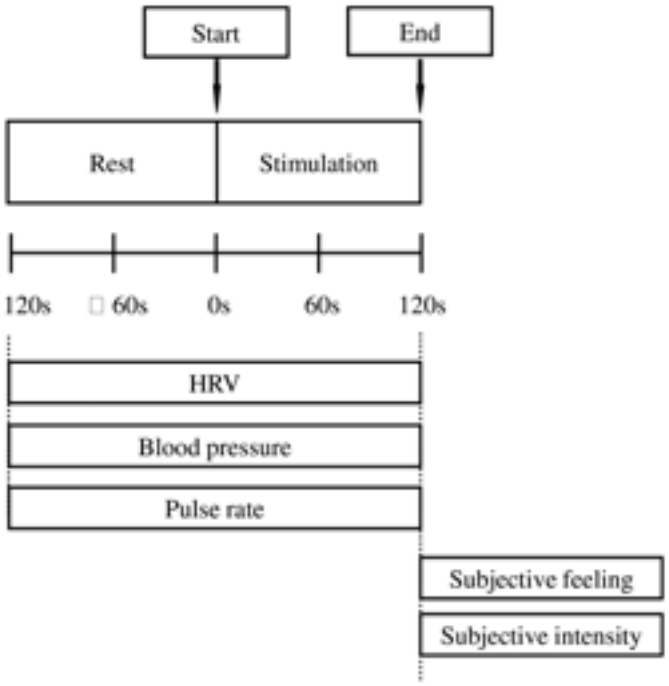

Fig. 1 - Time schedule for the experiment conducted to investigate the physiological indices of the olfactory effects of orange essential oil odor.

of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). HF is considered to reflect parasympathetic nervous activity (Cacioppo *et al*., 1994), which increases under relaxation conditions, while LF/(LF+HF) is considered to reflect sympathetic nervous activity (Weise and Heydenreich, 1989), which increases under stressful conditions.

Blood pressure and pulse rates were measured on the left middle finger (Finometer pro, FMS Ltd. Co., Netherland). This method is noninvasive, and data is available per second.

Sensory evaluation was conducted after odor inhalation. The subjects were asked to evaluate and rate their "feeling of comfort," "feeling of being soothed," and "feeling of naturalness" on a 13-point scale.

Statistical analysis

A paired t-test was used for comparison of HRV, blood pressure, and pulse rate measurements during inhalation of orange essential oil with those during inhalation of fresh air. The Wilcoxon signed-rank test was used to analyze the subjective feelings scores. Statistical analysis of physiological data was processed with EXCEL 2003 (Microsoft Inc., Japan). Each measured value is represented as mean ± SD. A P-value of <0.05 was considered statistically significant.

3. Results

The subjective odor intensity ratings revealed the orange essential oil to be at the "easily sensible level",

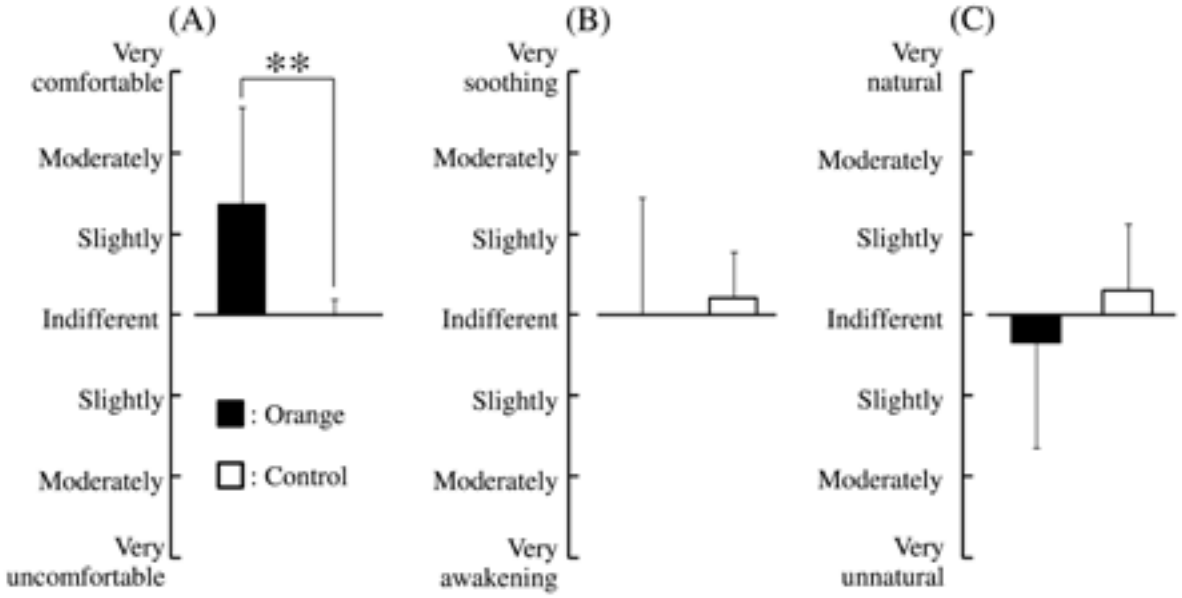

Fig. 2 - Changes in subjective feelings after inhalation of an orange essential oil or fresh air as a control. (A) feeling of comfort; (B) feeling of being soothed; (C) feeling of naturalness.
n= 13, mean ± standard deviation.
$^{**}P < 0.01$ as determined by the Wilcoxon signed-rank test.

which was significantly different from control (fresh air) inhalation. Figure 2 reports results of sensory evaluation of subjective feelings during orange essential oil inhalation. With regard to the "feeling of comfort," the subjects rated orange essential oil as "moderately comfortable," which was significantly different from control inhalation (Fig. 2 A). With regard to the "feeling of being soothed" (Fig. 2 B) and "feeling of naturalness" (Fig. 2 C), no significant differences were observed between the ratings for orange essential oil and those for fresh air.

Figure 3 shows the HF component of HRV recorded every 30 s during the 120 s of essential oil inhalation; this was significantly enhanced compared with that during control inhalation (Fig. 4).

The relative systolic blood pressure, measured every 30 s during exposure, tended to be lower with orange essen-

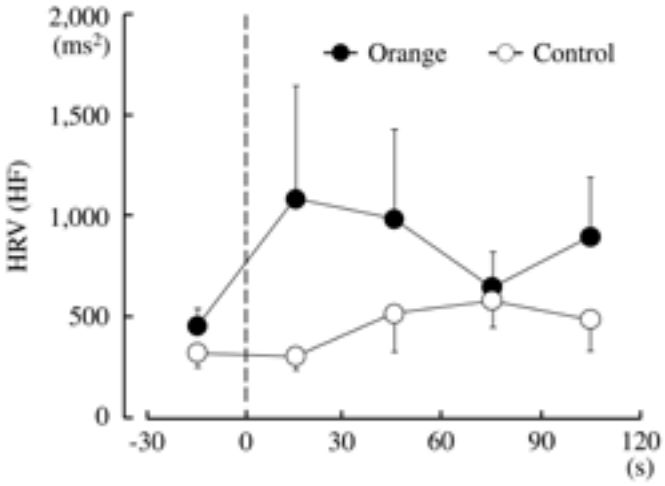

Fig. 3 - Changes in the high frequency component of heart rate variability during 120 s of inhaling an orange essential oil or fresh air as a control.
n= 13, mean ± standard deviation, determined by the paired *t*-test.

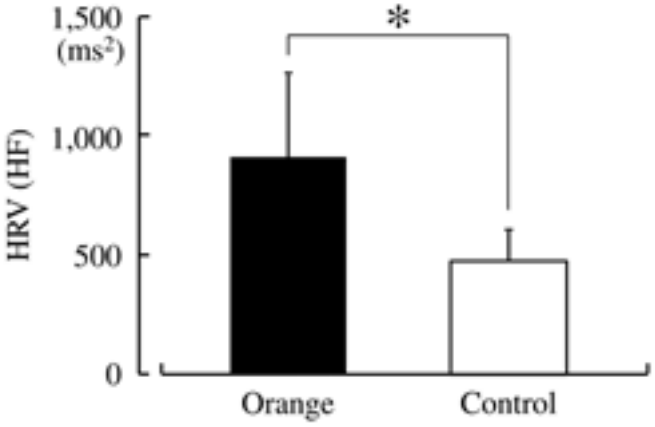

Fig. 4 - Changes in the average high frequency component of heart rate variability during 120 s of inhalation of an orange essential oil or fresh air as a control.
n= 13, mean ± standard deviation
$^{*}P < 0.05$ as determined by the paired *t*-test.

tial oil inhalation than during control inhalation (Fig. 5). The average relative systolic blood pressure (Fig. 6), relative diastolic blood pressure (Fig. 7), and average relative diastolic blood pressure showed similar results (Fig. 8).

4. Discussion and Conclusions

In this study, subjects exposed to the odor of orange essential oil for 120 s showed a significantly greater "feeling of comfort" compared with those who were exposed to fresh air without any odor. The results regarding psychological states indicated that inhalation of orange essential oil has beneficial effects. Inhalation of essential oil is believed to produce reliable and predictable effects on psychological state (Sanderson and Ruddle, 1992), and previous studies have investigated this possibility. The effects of orange essential oil have been demonstrated through relief from anxiety and tension and improvements in mood (Lehrner *et al*., 2000, 2005). Subjective relaxation effects have also been found for lavender essential oil (Diego *et al*., 1998; Motomura *et al*., 2001; Moss *et al*., 2003; Bur-

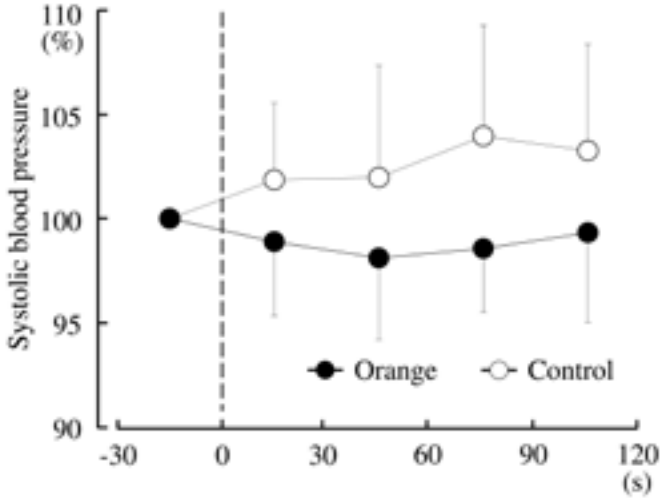

Fig. 5 - Changes in the relative systolic blood pressure during 120 s of inhalation of an orange essential oil or fresh air as a control.
n= 11, mean ± standard deviation, determined by the paired *t*-test.

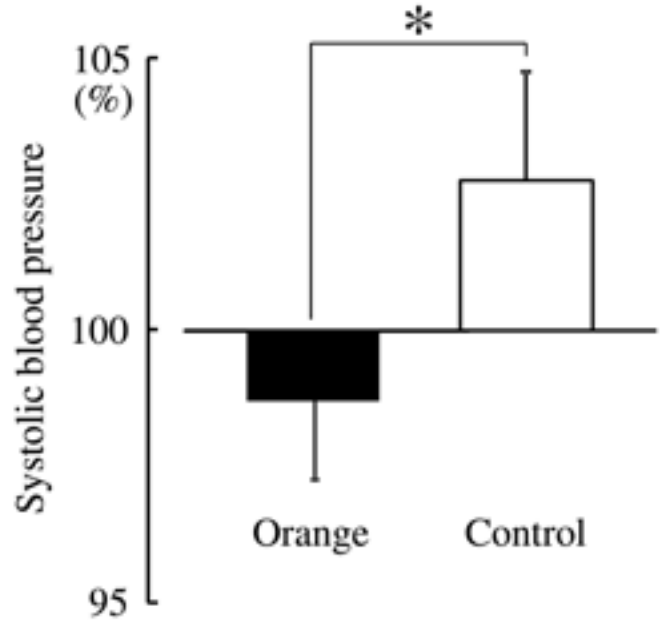

Fig. 6 - Changes in the relative average systolic blood pressure during 120 s of inhalation of an orange essential oil or fresh air as a control.
n= 11, mean ± standard deviation.
*$P < 0.05$ as determined by the paired *t*-test.

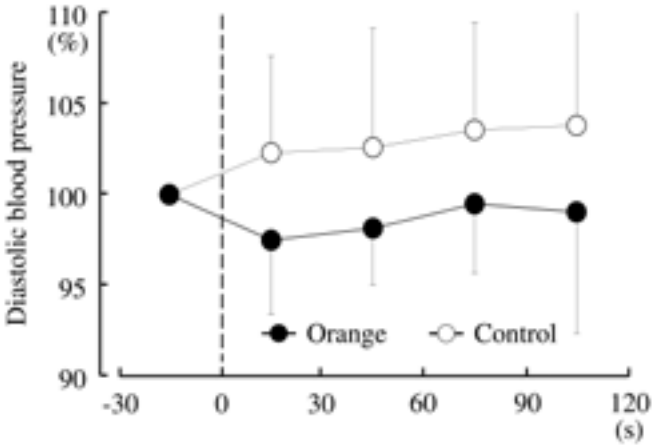

Fig. 7 - Changes in the relative diastolic blood pressure during 120 s of inhalation of an orange essential oil or fresh air as a control.
n= 11, mean ± standard deviation, determined by the paired *t*-test.

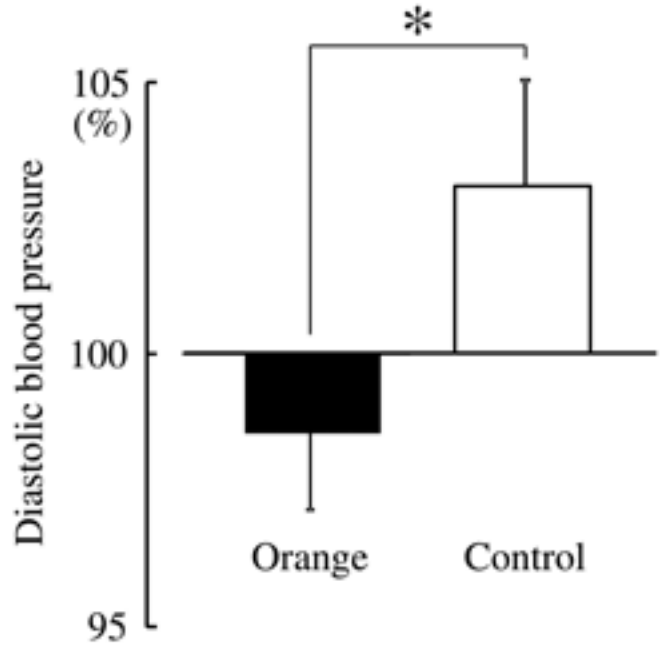

Fig. 8 - Changes in the relative average diastolic blood pressure during 120 s of inhalation of an orange essential oil or fresh air as a control.
n= 11, mean ± standard deviation.
*$P < 0.05$ as determined by the paired *t*-test,

nett *et al*., 2004) and peppermint essential oil (Ilmberger *et al*., 2001; Raudenbush *et al*., 2001, 2002).

The data obtained in this study suggest that the inhalation of orange essential oil has positive effects on autonomic nervous system activity. The test subjects showed a significant enhancement of parasympathetic nervous activity, which has a relationship with relaxation. The results from the present study are consistent with those of a previous study that investigated the HF component of HRV in healthy, young, adult university students (Park *et al*., 2007; Tsunetsugu *et al*., 2007; Park *et al*., 2008; Lee *et al*., 2009; Park *et al*., 2009, 2010; Lee *et al*., 2011).

During inhalation of orange essential oil for 120 s, both systolic and diastolic blood pressure were significantly lower than those during control inhalation, indicating that the inhalation of orange essential oil has a significant relaxing effect on the human body compared with the inhalation of fresh air.

The results of this study demonstrate that when subjects were exposed to an odor simulating the natural environment, they experienced the environment via the five senses, including olfaction. These findings strongly support the belief that plant odor, such as that of oranges, is a factor for inducing relaxation in humans during exposure to the natural environment.

In conclusion, we measured physiological and psychological indices for the olfactory effects of orange essential oil and found that the HF component of HRV was significantly higher, systolic and diastolic blood pressure were significantly lower, and the subjective "feeling of comfort" was significantly greater during inhalation of orange essential oil than during inhalation of fresh air.

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Effects of wound treatments to flower organs on fruit set, development, and quality in sweet cherry (*Prunus avium* L.)

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Key words: anthocyanin concentration, artificial removal, cultivar difference, physical damage.

Abstract: Flower organs are important elements for pollination and fruit production. Although several studies on fruit trees have shown that damage to certain flower organs affects the resultant fruit, the influences of damage to flower organs on fruit production are largely unknown for most fruit trees. Sweet cherry is one of the most commercially important fruit trees, the tender flowers of which are occasionally damaged by factors such as insects, diseases, and weather disasters. In this study, the flower organs of two different Japanese sweet cherry cultivars, 'Satohnishiki' and 'Benishuho,' were wounded at flowering time by petal removal, stamen removal, sepal removal, or peduncle wounding, and the effects on fruit set, development, and quality at harvest were examined. All wound types slightly increased the fruit drop in 'Satohnishiki,' whereas fruit drop in 'Benishuho' was promoted by petal removal and peduncle wounding, and was suppressed by sepal removal. The fruits resulting from the wounded flowers developed in a similar manner to those of control flowers in both cultivars. Fruit weight, total soluble solid concentration, and titratable acidity of the flesh juice were not affected. On the other hand, the anthocyanin concentration in fruit skins was differentially affected depending on the cultivar, increasing in 'Satohnishiki' fruits but decreasing in 'Benishuho' fruits.

1. Introduction

Flower organs are important in plant pollination, with petals and stamen attracting pollinators such as insects and bees (Proctor and Yeo, 1973; Westwood, 1978). Since pollination is generally indispensable for successful fertilization and setting, flower organs are important in fruit production as well. However, flowers are occasionally damaged by insects, diseases, and weather disasters such as strong wind and hail.

Several studies indicate that successful pollination and fruit quality are reduced in damaged flowers. In persimmon, the calyx robes play an essential role in fruit development after setting, and damage or removal of the robes results in inferior fruit growth (Nakamura, 1967; Yonemori *et al*., 1996). It was recently reported that removal of the calyx from apple blossoms at flowering time reduced fruit size and weight at harvest (Kitahara *et al*., 2013). However, the roles of flower organs in determining fruit set, development, and quality in most fruit trees are still largely unknown.

Sweet cherry is one of the most commercially important fruit trees. This tree has tender flowers that bloom from April to May in Japan, when hail still occurs in certain regions. In addition, flower organs such as petals and stamen, occasionally suffer damage from feeding insects. It was recently reported that artificial stamen removal (emasculation) at the balloon stage of flowering in sweet cherry trees suppressed ovule development and reduced fruit set (Hedhly *et al.*, 2009). Damage to other flower organs may also influence fruit set, development, and quality of sweet cherries.

In this study, artificial wounds were inflicted to petals, stamen, sepals, and peduncles of flowers in two Japanese cultivars of sweet cherry in order to examine the roles of these flower organs on fruit production.

2. Materials and Methods

Plant materials

Adult trees of the sweet cherry (*Prunus avium* L.) cultivars, 'Satohnishiki' and 'Benishuho,' planted at the experimental orchard of the Shonai Laboratory for Agricul-

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tural Production in Sakata City of Yamagata Prefecture were used in this study. Seventeen lateral branches were randomly selected from three trees per cultivar. A total of 150 flowers (seven to ten flowers per branch) were used for each treatment as described below.

Wound treatments

Four wound treatments were designed: (1) petal removal (all petals were removed), (2) stamen removal (all stamens were removed), (3) sepal removal (all sepals were removed), and (4) peduncle wounding (the middle part of peduncle was pierced using a sewing needle). Flowers without wounds were used as controls. Treatments were conducted either on the day of flowering or the following day ('Satohnishiki' 29 April-4 May 2013; 'Benishuho' 3-7 May 2013).

All flowers used in this study were artificially pollinated with the mixed pollen of 'Rockport Bigarreau,' 'Napoleon,' and 'Jabouley' sweet cherries immediately after wounding. Pollen source flowers were collected from the experimental orchard of the Faculty of Agriculture, Yamagata University in Tsuruoka City of Yamagata Prefecture. Fruit thinning was performed on 4 June (37 days after full bloom) in 'Satohnishiki' and on 18 June (46 days after full bloom) in 'Benishuho', according to the customary practices in Yamagata Prefecture, with minor modification. The fruits from the wounded flowers were retained, while all other fruits from the same branch were thinned so that one branch bore two or three pieces of fruit per bouquet spur.

Fruit drop and fruit development

Fruit drops were counted twice per week from flowering to harvest. The fruit drop rate was calculated as the number of fruits remaining compared to the number of wounded flowers.

Fruit development was observed by measuring fruit diameter twice per week from 29 May when fruit set was first established.

Measurements of fruit quality

All fruits were harvested at the optimum time according to each cultivar. The diameter and weight of the fruits, total concentration of soluble solids, titratable acidity of the flesh juice, and anthocyanin concentration of the fruit skin were examined.

The total soluble solids concentration was determined using a hand refractometer (ATC-1, ATAGO Co. Ltd., Tokyo). Titratable acidity was determined by titrating the juice with 0.1 N NaOH. The results are expressed as equivalents of malic acid.

Fruit skin discs were removed using a cork borer. Anthocyanin was extracted from the discs by incubation with 1% HCl-MeOH for 24 h under refrigeration. The anthocyanin concentration was determined based on the absorbance at 530 nm (UV-150-01 spectrophotometer, Shimadzu Co. Ltd., Kyoto) and calculated as equivalents of cyanidin-3-glucoside.

3. Results and Discussion

Fruit drop

Figures 1 and 2 show the time-course of fruit drop and cumulative fruit drop rate in the 'Satohnishiki' and 'Benishuho' cultivars, respectively. Compared to control fruits, wounding promoted fruit drop in both cultivars, except for sepal removal in 'Benishuho' which slightly depressed the fruit drop. The wound-induced fruit drop rate was higher in 'Benishuho' than in 'Satohnishiki'. Although fruit drops were normally heavier in 'Satohnishiki' than in 'Benishuho' (Takahashi and Arasawa, 2001), the latter was more sensitive to wounding, and thus led to larger fruit drops.

Two peaks in fruit drop were observed (Figs. 1 and 2). The first large peak was caused by fertilization failure, whereas the second smaller peak was caused by competition for nutrients among the developing fruits (Westwood, 1978; Fukai, 1995). Peduncle wounding commonly promoted fruit drop in both cultivars, but the peak within which the drops occurred differed between the two cultivars. More fruits dropped during the second peak in

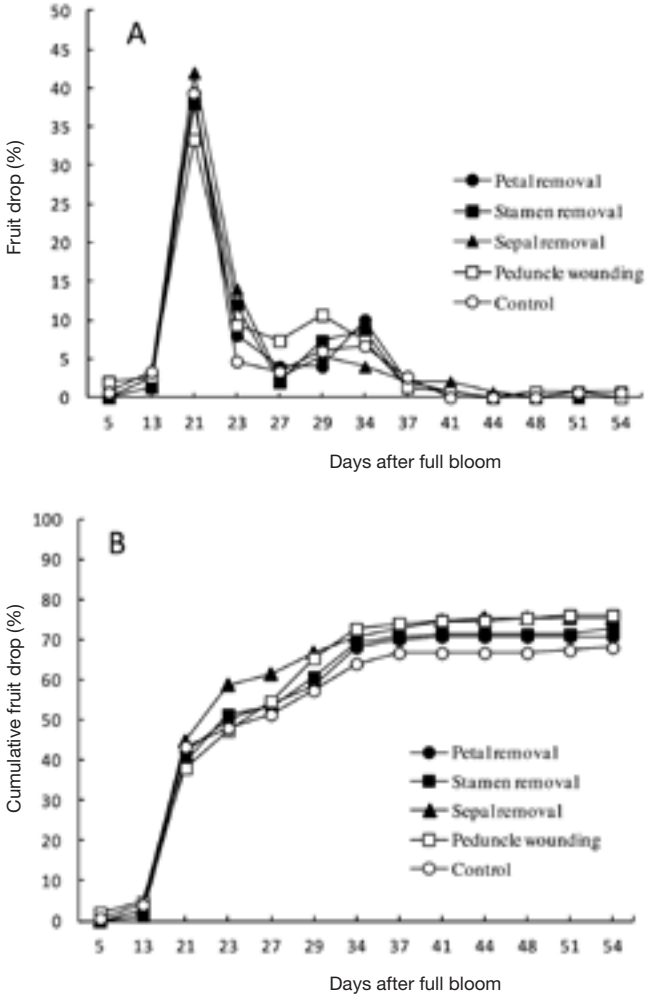

Fig. 1 - The effects of wounding of flower organs on fruit set in 'Satohnishiki' sweet cherry. A, fruit drop rate. B, cumulative fruit drop rate. N=150 flowers for each treatment and control.

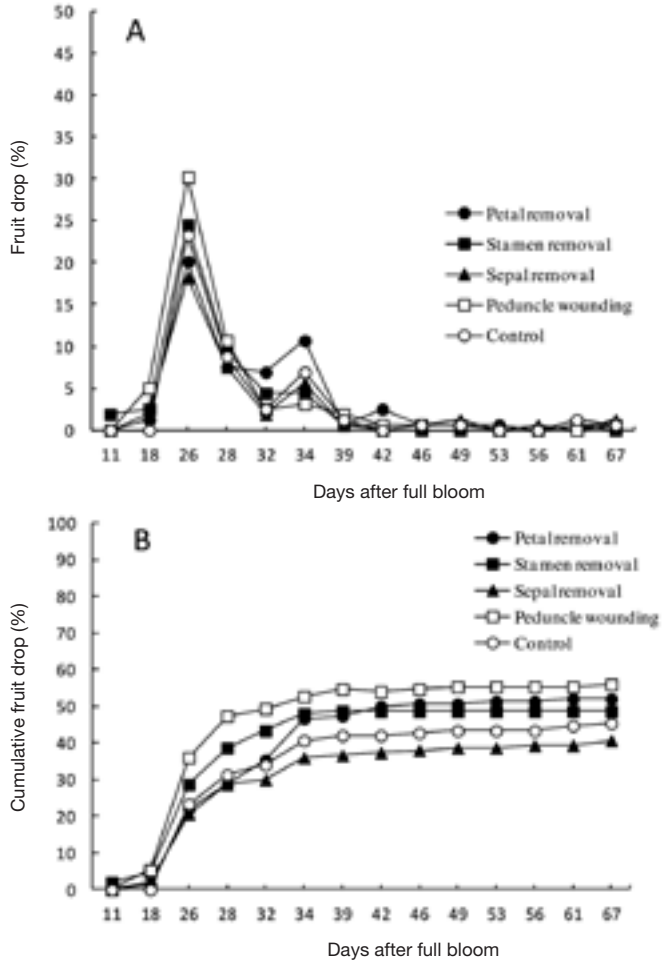

Fig. 2 - The effects of wounding of flower organs on fruit set in 'Benishuho' sweet cherry. A, fruit drop rate. B, cumulative fruit drop rate. N=150 flowers for each treatment and control.

'Satohnishiki,' while more fruits dropped during the first peak in 'Benishuho'. It is possible that peduncle wounding restricts the translocation of photosynthates from the leaves to the young fruits in 'Satohnishiki,' whereas it might also disturb fertilization in 'Benishuho'.

Wounding at flowering did not severely influence fruit set in this study. On the other hand, removal of the stamen at the balloon stage was reported to reduce fruit set in 'Vignola' and 'Sunburst' sweet cherry trees (Hedhly *et al.*, 2009). In addition, the removal of sepals at the pink bud stage in 'Cox's Orange Pippin' apples was reported to reduce fruit set to approximately 85% of normal (Vemmos and Goldwin, 1994). Taken together, these results suggest that wound stresses at immature stages of flower development are more detrimental to fruit set than wounds occurring at flowering. Further studies are necessary to determine whether wound stresses on flower organs at earlier stages of flower development influence fruit set in sweet cherry trees.

Fruit development

Figures 3 and 4 show the changes in diameter of 'Satohnishiki' and 'Benishuho' fruits, respectively. The growth patterns revealed double sigmoidal curves in both cultivars. Fruits from wounded flowers grew in a manner similar to those from non-wounded flowers in both cultivars. This result indicates that fruits from wounded flowers can continue to grow in a similar manner to normal ones.

Fruit quality

Fruit weight, total soluble solids concentration, and titratable acidity of the flesh juice at harvest were not af-

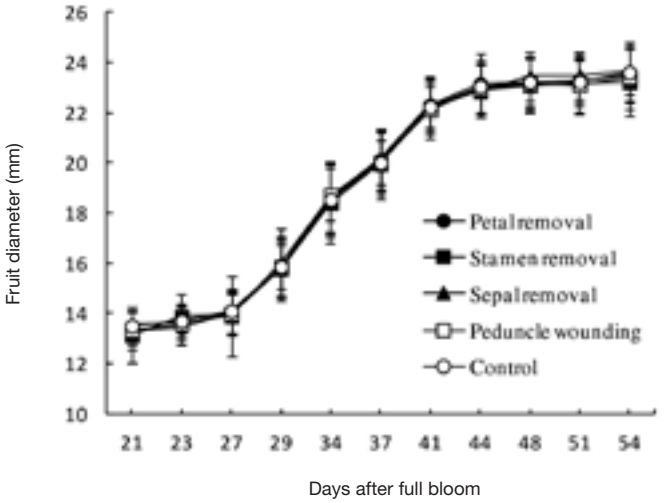

Fig. 3 - The effects of wounding of flower organs on fruit development in 'Satohnishiki' sweet cherry. Vertical bars represent ±SD (Petal removal, n = 48; Stamen removal, n = 43; Sepal removal, n = 41; Peduncle wounding, n = 43; Control, n = 54).

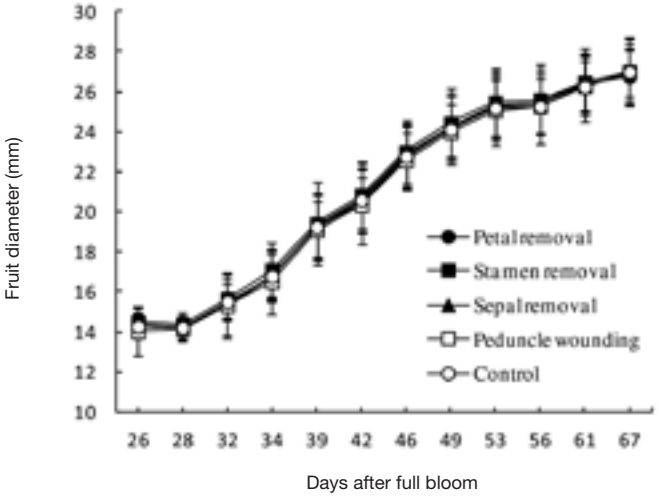

Fig. 4 - The effects of wounding of flower organs on fruit development in 'Benishuho' sweet cherry. Vertical bars represents ±SD (Petal removal, n = 72; Stamen removal, n = 83; Sepal removal, n = 93; Peduncle wounding, n = 72; Control, n = 82).

fected by wounding in 'Satohnishiki' (Table 1). None of these parameters were affected in 'Benishuho' either, except that stamen removal significantly increased the total soluble solids concentration (Table 2). On the other hand, anthocyanin concentration in the fruit skin was affected in both cultivars in different ways. In 'Satohnishiki', all wounds increased fruit skin anthocyanin concentration, with peduncle wounding causing a significant increase in anthocyanin concentration. Conversely, in 'Benishuho', all wound types decreased anthocyanin concentration, among which stamen and sepal removal significantly decreased anthocyanin concentration.

These results indicate that the fruit quality of these sweet cherry cultivars at harvest was minimally affected by flower wounding, with the exception of anthocyanin accumulation in the fruit skin. Interestingly, the effect on the anthocyanin concentration observed in 'Benishuho' appeared to be quite opposite to that in 'Satohnishiki'. The reason for this difference is unclear. Thus, such cultivar differences should be examined further, and the responses of other cultivars should be identified in the future.

Anthocyanin accumulation in fruit skin is controlled by both environmental and physiological factors (Westwood, 1978). For example, L-phenylalanine ammonialyase (PAL) is a key enzyme in anthocyanin biosynthesis in various kinds of fruits (Kataoka *et al.*, 1983; Farger and Chalmers, 1997). Increases in ethylene production induced by wounding of plant organs were reported to increase PAL activity and promote anthocyanin synthesis (Kataoka *et al.*, 1983; Arakawa, 1990). Further studies are required to clarify the effects of wounding on ethylene production and PAL activity, which is possibly related to anthocyanin accumulation in the fruit skin of sweet cherries.

4. Conclusions

The wounding of flower organs at the time of flowering slightly suppressed fruit set, but did not strongly affect fruit development or fruit quality at harvest in two Japanese cultivars of sweet cherry. On the other hand, anthocyanin concentrations increased in the skins of wounded 'Satohnishiki' fruits, but declined in wounded 'Benishuho' fruits. In the future, the effects of wound stresses at earlier stages of flower bud development and flowering need to be examined and it would be interesting to explore cultivar differences in response to such stresses.

Acknowledgements

We sincerely thank Mr. M. Ishiguro, Mr. S. Akashi, and the other staff members of the Shonai Laboratory for Agricultural Production of Yamagata Prefecture in Sakata City for their useful advice and technical assistance.

Table 1 - The effects of the wounding of flower organs on fruit quality at harvest in 'Satohnishiki' sweet cherry

Treatment	Number of fruits	Fruit weight (g)	Total soluble solids (° Brix)	Titratable acidity[z] (g/100 ml juice)	Anthocanin in fruit skin [y] (μg/cm^2)
Petal removal	48	6.97 a	20.2 a	0.75 a	24.6 ab
Stamen removal	43	6.80 a	20.4 a	0.83 b	24.4 ab
Sepal removal	41	7.04 a	20.1 a	0.77 ab	25.5 ab
Peduncle wounding	43	6.88 a	20.5 a	0.83 b	29.9 a
Control	54	6.88 a	19.9 a	0.81 ab	21.2 b

[z] as malic acid.
[y] as cyanidin-3-glucoside.
Letters following the means indicate statistical significance by Tukey's test, $p < 0.05$.

Table 2 - The effects of the wounding of flower organs on fruit quality at harvest in 'Benishuho' sweet cherry

Treatment	Number of fruits	Fruit weight (g)	Total soluble solids (° Brix)	Titratable acidity[z] (g/100 ml juice)	Anthocianin in fruit skin [y] (μg/cm^2)
Petal removal	72	9.81 a	17.2 ab	0.59 a	22.8 ab
Stamen removal	83	9.87 a	17.6 a	0.69 a	19.6 bc
Sepal removal	93	9.83 a	17.3 ab	0.62 a	17.7 c
Peduncle wounding	72	9.62 a	17.2 ab	0.62 a	22.0 abc
Control	82	9.62 a	16.9 b	0.57 a	26.4 a

[z] as malic acid.
[y] as cyanidin-3-glucoside.
Letters following the means indicate statistical significance by Tukey's test, $p < 0.05$.

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Diversity and breeding of flowering cherry in Japan

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Key words: genetic diversity, Hanami, horticulture, origin of 'Somei-yoshino,' *PolA1*, *Prunus.*

Abstract: In early spring, the flowering of cherry trees is taken as a good sign for farmers to initiate rice cultivation in Japan. In the era before calendars, the timing of cultivation was very important for growing rice plants in temperate regions. Nine native species of flowering cherry (*Prunus* subgenus *Cerasus*) are present in Japan and they are classified into three groups: Yamazakura, Miyamazakura, and Edohigan. More than 250 cultivars of Japanese flowering cherry have been selected or bred from these wild species. Two species, Oshimazakura and Edohigan, have specially contributed to the breeding of flowering cherry cultivars. While Edohigan is distributed in most areas of Japan, Oshimazakura (of the Yamazakura group) is an endemic species found around the Izu and Boso Peninsulas. 'Somei-yoshino,' *Prunus* × *yedoensis*, is the most popular cultivar and now comprises 80-90% of all flowering cherry trees planted in Japan. 'Somei-yoshino' was probably created through hybridization between Edohigan and Oshimazakura in the Edo era. In this paper, the diversity and breeding of Japanese flowering cherry, including the origin of 'Somei-yoshino,' are described along with the political and horticultural backgrounds.

1. Introduction

At the end of March, the flowering of Chinese plum has been taken as a sign to initiate the plowing of paddy fields for rice cultivation in China. Therefore, Chinese people take an interest in looking at plum flowers, which is the reason why many cultivars of Chinese plum have been developed. The Chinese plum blooms in early February in Japan. Japanese farmers pay attention to the flowering of flowering cherry because it blooms at the end of March (Fig. 1). As Japan is located at the northern limits of the rice cultivation area, without a calendar, the timing to start rice cultivation was very important to identify the cultivation period and secure the best yield for farmers. Rice cultivation started by plowing paddy fields, then about a month later sowing rice seeds on the paddy. Growth of rice plants was restricted to the period between May and October (*ca*. 160 days) both in China and Japan. Farmers had to harvest the rice grains before snow at the end of October. As the rice yield was the basis of Japanese hierarchy, everyone in Japan, not only farmers, has been interested in the flowering of flowering cherry. More than 250 cultivars of flowering cherry have been selected or bred from wild *Cerasus* species in Japan (Kawasaki, 1991).

Farmers both in China and Japan started rice cultivation by ploking paddy fields at the end of march (yellow circle).

'Somei-yoshino'

A single cultivar, *Prunus yedoensis* (Matsum.) A.V. Vassil. 'Somei-yoshino' (Iketani *et al*., 2006), comprises more than 80-90% of flowering cherry trees planted in parks, and along roads and rivers across Japan, except in Okinawa and Hokkaido Islands. As *Cerasus* species have complete self-incompatibility, 'Somei-yoshino' has been propagated as a clone using grafting techniques. This paper discusses three topics in terms of the origin of

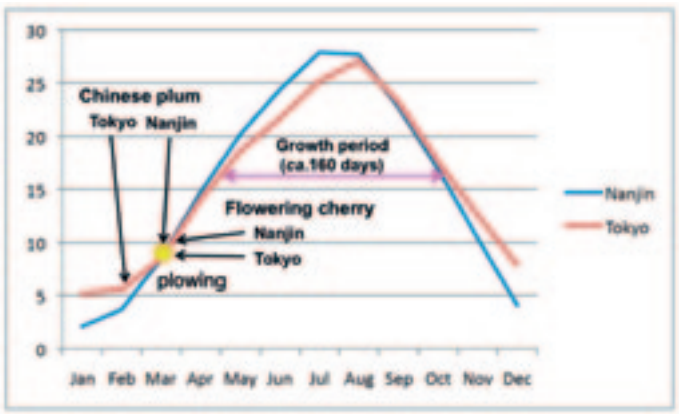

Fig. 1 - Changes of average temperature in Nanjin (China) and Tokyo (Japan).

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'Somei-yoshino': 1. Genetic variations of *Cerasus* species in Japan, 2. Political and horticultural backgrounds, 3. DNA analysis to reveal the origin of 'Somei-yoshino' (Fig. 2).

Fig. 2 - Flowering cherry 'Somei-yoshino'. Image credit: http://iko4wd.blog.so.net.ne.jp/2008-03-25.

2. Genetic variations of flowering cherry in Japan

Wild Cerasus *species in Japan*

The subgenus *Cerasus* of the genus *Prunus* includes more than 50 species, most of which are distributed in temperate areas in the Northern Hemisphere, especially in China, where 33 wild species occur (Yu and Li, 1986). In Japan, nine native species have been recorded (Table 1): Yamazakura (*P. jamasakura* Sieb. ex Koidz.), Oyamazakura (*P. sargentii* Rehder), Kasumizakura [*P. verecunda* (Koidz,) Koehne], Mamezakura (*P. incisa* Thumb. ex Murray), Takanezakura (*P. nipponica* Matsum.), Chojizakura [*P. apetala* (Sieb. et Zucc.) Fr. et Sav.], Oshimazakura [*P. lannesiana* (Carr.) Wilson var. *speciosa* (Koidz.) Makino], and Edohigan [*P. pendula* f. *ascendens* (Makino) Ohwi]. In addition, three wild species, Karamizakura (*P. pseudo-cerasus* Lindl.), Himarayazakura (*P. cerasoides* D. Don) and Kanhizakura (*P. campanulata* Maxim.), have been popularly cultivated since their introductions from China, Taiwan, and Nepal, respectively (Kawasaki, 1991) (Fig. 3).

Table 1 - Wild species of the subgenus *Cerasus* in Japan

Yamazakura group	Yamazakura	*P. jamazakura*
	Ooyamazakura	*P. sargentii*
	Kasumizakura	*P. verecunda*
	Oshimazakura	*P. Iannesiana*
Mamezakura group	Mamezakura	*P. incisa*
	Takanezakura	*P. nipponica*
Chojizakura group	Chojizakura	*P. apetala*
Edohigan group	Edohigan	*P. pendula*
Miyamazakura group	Miyamazakura	*P. mazimowiczii*
Karamizakura group	Karamizakura	*P. pseudo-cerasus (China)*
Kanhizakura group	Kanhizakura	*P. campanulata (Taiwan)*
Himarayazakura group	Himarayazakura	*P. cerasoides (Nepal)*

Fig. 3 - *Cerasus* species. Flowering of different cultivars. Top left 'Edohigan', Top middle 'Yamazakura', Top right 'Oshimazakura', bottom left 'Chojizakura', bottom right 'Kanhizakura'. Courtesy of Mr. Makoto Tsuruta.

Distribution of wild Cerasus *species in Japan*

Two wild species, Yamazakura and its close relative Oyamazakura, are broadly distributed throughout Japan (Fig. 4). While Edohigan is distributed from Kyushu Island to the Tohoku region (northern area of mainland Japan), this species is absent from some large peninsulas, such as Kii, Noto and Boso. As Edohigan blooms faster than Yamazakura and other Japanese *Cerasus* species, it was possibly spread by farmers along with rice cultivation from the south to the north of Japan. Mamezakura and its variant Kinki-Mamezakura are restricted to the mountainous area in the middle of the mainland. The wild population of Oshimazakura is endemic to the Izu and Boso Peninsulas and to Oshima Islands. As the flowers of Os-

Fig. 4 - Distribution of wild *Cerasus* species in Japan (Kawasaki, 1991).

himazakura have large white petals with a pleasant fragrance, it has been used as a parent to develop various cultivars of flowering cherry. Its green leaves are also used to decorate cakes. The flowers of Edohigan bloom faster than its leaves extrude. In contrast, flowers and leaves appear at the same time in Yamazakura and other species. Wild Edohigan and Yamazakura trees reach a height of 20-25 m, while Mamezakura and Oshimazakura have a relatively short stature (*ca.* 10 m).

Cultivars of flowering cherry

The history of the breeding of flowering cherry in Japan can be classified into three phases: Selection phase (Ancient to Azuchi-Momoyama era), Mutant phase (first half of Edo era), and Cross-hybridization phase (last half of Edo era to present). Two important incidents, the establishment of the Tokugawa Government (1603) and the practice of the Kyoho Reforms (1716), are related in the division of the three phases (Fig. 5). Before the Azuchi-Momoyama era, about 20 cultivars were selected from the native population of flowering cherry. 'Ukon,' with yellowish petals, and 'Shidare zakura,' with droopy branches, were representative cultivars in the Selection phase. During the first half of the Edo era, because Shoguns, especially the 3rd Shogun Tokugawa Iemitsu, asked Daimyos to donate fantastic or unusual flowers and trees, many ornamental cultivars of more than 100 species were brought together at Edo Castle. About 30 cultivars of flowering cherry with mutant phenotypes, such as many petals 'Ito kukuri' and dwarf stature 'Asahiyama' were collected in the Mutant phase. During the last half of the Edo era, flowering cherry cultivars were developed using natural or artificial cross-hybridization. In the Cross-hybridization phase of the last half of the Edo era, more than 80 cultivars, such as 'Ichiyo' with a leafy stigma and 'Surugadai-nioi' with a fragrance, were created by hybridization with Oshimazakura as parent (Fig. 6).

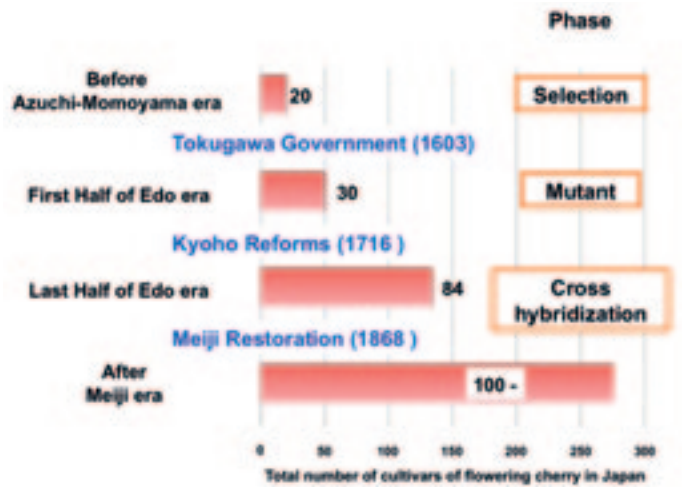

Fig. 5 - Three phases in the breeding of flowering cherry.

Fig. 6 - Cultivars (Cross hybridization phase) during last half of Edo era. Courtesy of Mr. Makoto Tsuruta..

3. Political and horticultural backgrounds

260-year peace during Edo period

Ieyasu Tokugawa (1542-1616), who was the 1st Shogun, struggled to establish the Tokugawa Government (1603-1868). It is thought to be the longest period of peace in the world at that time. The 260 years of peace under the Tokugawa Government was an important background to the development of various cultures, including horticulture. Takatora Todo (1556-1630) was probably a key person for the establishment of the Tokugawa Government and for the basis of the origin of 'Somei-yoshino.' He constructed more than 20 castles, including Edo Castle. And he once owned Kisyu Kokawa-Han and visited Mt. Yoshino, which was famous for its large Yamazakura population. Later, Takatora moved to Ise-Han where both Iga and Koga Ninja lived. Thus, Takatora organized the Ninja into a CIA (Central Intelligence Agency, USA)-like 'Onmitsu' system in order to keep an eye on the behavior of Daimyos for the Tokugawa Government. His house was located in 'Somei' village of Edo city and the name of his gardener was Ito Ihei, who was probably a secret manager of 'Onmitsu' under the control of the Todo clan. After Ieyasu died, Takatora constructed 'Kan-eiji' temple for Ieyasu's grave in 1625. Iemitsu Tokugawa (3rd Shogun) and Takatora directed the transplantation of wild Yamazakura trees from Mt. Yoshino to 'Kan-eiji' temple. As a result, 'Kan-eiji' temple became a famous 'Hanami' place in Edo city. Note: 'Hanami' means looking at blossoms whilst eating and drinking

Collection of cultivars and mutants

During the lifetime of Iemitsu Tokugawa (1623-1651) of the 3rd Shogun of the Tokugawa Government, most social systems, including transport and the economy, were established. Iemitsu, who was rather eccentric and an enthusiast of ornamental flowers and trees, asked Daimyos to donate fantastic or unusual plants, and as a result many mutant cultivars of more than 100 plant species were brought to Edo Castle. These collected plants were later

given to the Ito Ihei clan through the Todo clan. Hybridizations among the collected genetic resources led to the development of many horticultural cultivars, such as morning glory, Japanese azalea, camellia, orchid, and fern, by the Ito Ihei clan (gardeners) and people in Edo city. They frequently held competitive exhibitions of the cultivars that they had developed. For example, an unusual phenotype 'Shiro saizaki botan' of morning glory appeared at a frequency (one out of 20,000 seeds), corresponding to recombination among 6-7 recessive alleles. Thus, Japanese gardeners and others might have recognized a principle of inheritance before Mendel (1865) (Fig. 7).

Fig. 7 - 'Shiro saizaki botan', Morning glory (6-7 recessive allele). Courtesy of Dr Yoshiaki Yoneda http://protist.i.hosci.ac.jp/Asago/Yoneda_DB/J/menu.html.

Accumulation of horticultural knowledge

During the period of peace, not only gardeners but also enthusiasts in Edo city enjoyed breeding ornamental flowers and trees. Much knowledge and many horticultural techniques were accumulated during the process of developing the new cultivars of ornamental plants. Many books and illustrations describing the knowledge and developed cultivars were published during the last half of the Edo era. For example, Kan-En Iwasaki published a book 'Somoku sodate-gusa' (Plant Breeding) in 1818. In this book, he illustrated six different grafting, four cutting and three layering techniques, which are comparable to the present horticultural techniques. In those days, as more than 80 cultivars of flowering cherry had been developed by using natural or artificial cross-hybridization, this horticultural knowledge and these techniques must have contributed to the development of new cultivars of flowering cherry (Fig. 8, 9).

'Ippon-zakura' to 'Gun-zakura'

Yoshimune Tokugawa (1684-1751) used to be a Daimyo in Wakayama, close to Mt. Yoshino, which was famous for its beautiful scenery covered with Yamazakura trees. After he became the 8th Shogun, he enacted the 'Kyoho Reforms' to rebuild the finances of the Tokugawa Government. Also he opposed the powers of the Todo clan and Kan-eiji temple. Yoshimune abolished 'Onmitsu' under the control of the Todo clan and then organized his subordinates into the 'Oniwaban,' which was also a CIA-like system. This change meant that Ito Ihei might lose his salary as a secret manager of the 'Onmitsu.' Against the Kan-eiji temple, Yoshimune tried to develop new 'Hanami' places because 'Kan-eiji' was the famous 'Hanami' place in Edo city. He wanted to reconstruct the scenes of flowering cherries on Mt. Yoshino in Edo city. Therefore, he directed transplantation of a group of wild Yamazakura trees, propagated by grafting in Edo Castle, to several new 'Hanami' places, such as Shinagawa, Sumida-gawa and Asuka-yama. Then, he encouraged people to eat and drink under blossoms because he knew the starting time of rice cultivation was very important for farmers to achieve the best rice yield.

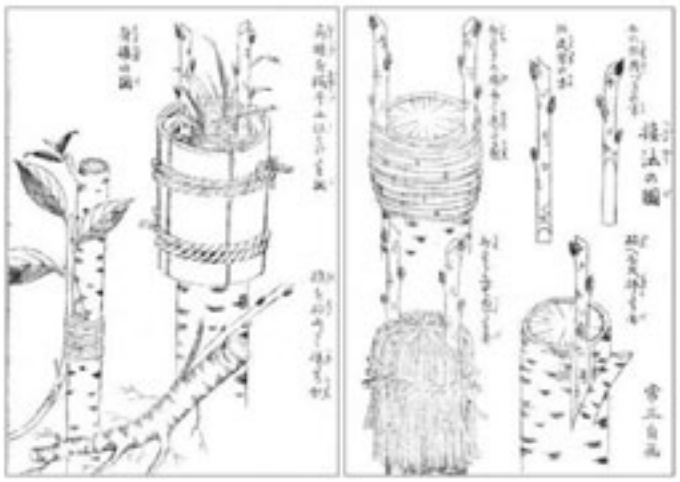

Fig. 8 - Illustrations of six different grafting techniques. Image credit: http://dl.ndl.go.jp/info:ndljp/pid/2569455.

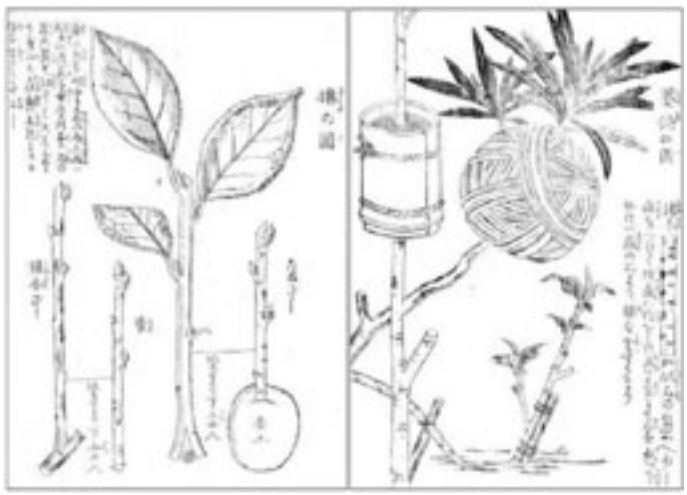

Fig. 9 - Illustrations of 4 cutting (left) and 3 layering (right).

During the first five to ten years, people probably enjoyed 'Hanami' under blossoms but after 15 years, the wild Yamazakura trees grew to be over 15 m high. Yoshimune did not realize that the tree height of flowering cherry was so important because people were able to look at flowers closely in spite of the height of the trees on the slopes on Mt. Yoshino (Fig. 10). In flat places of Edo city, the wild trees of Yamazakura grew too high to look at the blossoms

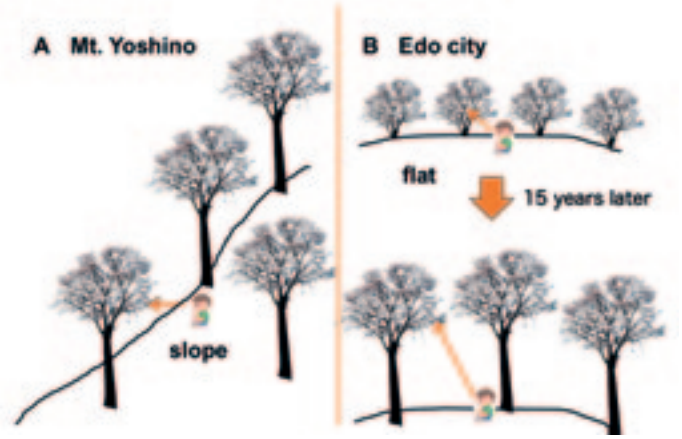

Fig. 10 - Sitting person's view of flowering cherry on the slopes of Mt. Yoshino (A) and on flat places in Edo city (B).

closely from under the trees but no one complained for fear that Yoshimune would punish them. Nowadays, all Yamazakura trees, planted by Yoshimune, have been replaced by 'Somei-yoshino.'

Before the mid-Edo era, people enjoyed seeing a distant scene of a single big flowering cherry 'Ippon-zakura.' On the contrary, Yoshimune introduced the concept of a group of flowering cherry 'Gun-zakura' to people in Edo city.

Involvement of Masatake Ito Ihei

Masatake (1676-1757) of 4th Ito Ihei and his father Sannojo (?-1719) were excellent plant breeders who developed many ornamental cultivars, Japanese azalea, camellia and sweet flag. As the Ito Ihei clan probably earned a salary from the Todo clan as managers of the 'Onmitsu,' they enjoyed plant breeding and gardening as a hobby. Sannojo and Masatake wrote many horticultural books, such as 'Kadan-chikin-syo' (1695) and 'Koeki -chikin-syo' (1719). Sannojo illustrated 120 plant species of ornamental flowers and trees including 46 cultivars of flowering cherry. Masatake also illustrated 197 species of ornamental plants, however, there was no description of flowering cherry. It is very unlikely that Masatake was uninterested in the breeding of flowering cherry. He probably excluded descriptions of flowering cherry from his books in order to avoid conflict with Yoshimune who initiated the 'Kyoho Reforms' in 1716. Masatake might have found that wild Yamazakura trees were not suitable to 'Gun-zakura' but he could not blame Yoshimune because Yoshimune might not just punish Masatake, he might wipe out the entire Todo clan. Thus, Masatake may have tried to develop a new cultivar for 'Gun-zakura' in secret within Kan-eiji temple.

4. DNA analysis to reveal the origin of 'Somei-yoshino'

'Somei-yoshino' was first identified and named by Yorinaga Fujino in 1900 and Dr. Jinzo Matsumura of Tokyo University registered it as *Prunus* x *yedoensis* at 1901. Dr. Ernest H. Wilson (1916) proposed, through morphological observations, the hypothesis that 'Somei-yoshino' is a hybrid of Oshimazakura and Edohigan. Dr. Kaname Takenaka (1962, 1965) confirmed Wilson's hypothesis by observing morphologies of the hybrids, Izu-yoshino and Amagi-yoshino, produced through artificial hybridization between the two species. However, these hybrids show higher stature and whiter petals compared with 'Somei-yoshino.'

Analyses of restriction fragment length polymorphism of chloroplast DNA (Kaneko *et al*., 1986) and plastid subtype identity sequence (Ohta *et al*., 2006) clearly indicate that the maternal lineage of 'Somei-yoshino' is Edohigan. In contrast, nuclear DNA analysis of flowering cherry is difficult because *Cerasus* species have complete self-incompatibility. We found that sequence variations of *PolA1* gene are useful for analyzing phylogenetic relationships in rice and wheat (Takahashi *et al*., 2009; Rai *et al*., 2012). *PolA1* is a single copy nuclear gene encoding the largest subunit of RNA polymerase I complex. Thus, DNA fragments containing intron 19 and exon 20 sequences of *PolA1* gene were amplified by PCR using template DNAs from wild strains of *Cerasus* species in Japan (Fig. 11). PCR products were purified and analyzed by direct sequencing technique. Sequence analysis of exon 20 indicates that

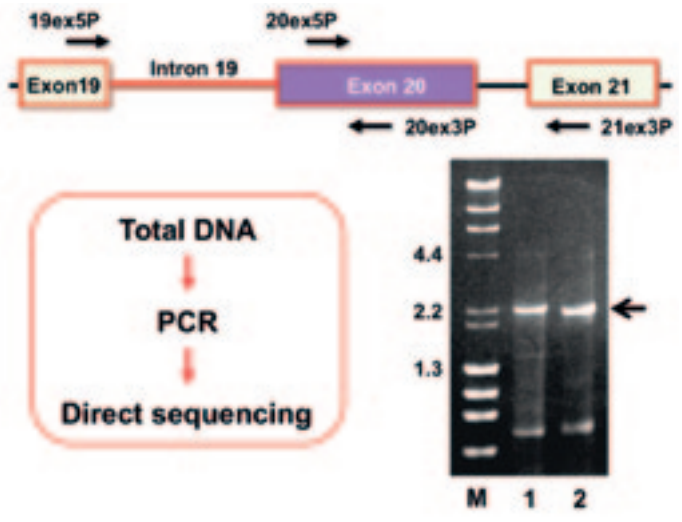

Fig. 11 - Sequence analyses of intron 19 and exon 20 of *PolA1* gene in *Cerasus* species.

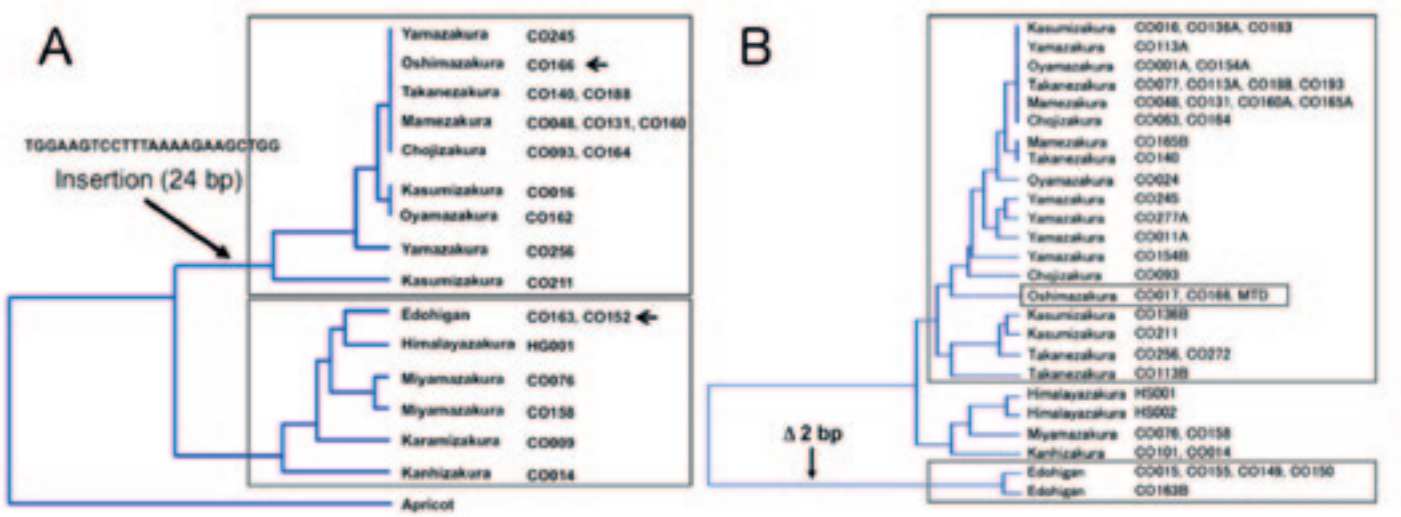

Fig. 12 - Relationships of exon 20 (A) and intron 19 (B) sequences of *PolA1* gene among *Cerasus* species, in Japan.

	25	36	38	43	101	128	131	146	171	174	178	199	298	305	309	349 - 350	392	416	457	477	492	500
'Somei-yoshino'	A	T	T	T	A	A	C	A	C	T	G	A	T	C	T	del (2)	A	A	C	A	C	G
	T	C	C	A	T	G	T	G	A	C	A	C	C	T	G	AA	C	T	A	G	T	A
Edohigan	A	T	T	T	A	A	C	A	C	T	G	A	T	C	T	del (2)	C	A	C	A	C	G
	A	T	T	T	A	A	C	A	C	T	G	A	T	C	T	del (2)	C	A	C	A	C	G
'Komatsuotome'	A	T	T	T	A	A	C	A	C	T	G	A	T	C	T	del (2)	C	A	C	A	C	G
	A	T	T	T	A	A	C	A	C	T	G	A	T	C	T	del (2)	A	A	C	A	C	G
Oshimazakura	T	C	C	A	T	G	T	G	A	C	A	C	C	T	G	AA	C	T	A	G	T	A
	T	C	C	A	T	G	T	G	A	C	A	C	C	T	G	AA	C	T	A	G	T	A
Yamazakura	A	C	C	A	A	G	T	G	C	C	A	C	C	T	G	AA	C	T	A	G	T	A
	A	C	C	A	A	G	T	G	C	C	A	C	C	T	G	AA	C	T	A	G	T	A

Fig. 13 - Comparison of two allelic intron 19 sequences within *PolA1* gene among 'Somei-yoshino,' Edohigan, 'Komatsuotome,' Oshimazakura, and Yamazakura.

Yamazakura and Edohigan can be clearly distinguished (Fig. 12). A particular insertion of 24 bp was found in the Yamazakura group including Oshimazakura as well as Mamezakura and Chojizakura groups (Table 1). This data indicates that these three groups share the same ancestor. The sequence analysis of intron 19 shows a similar result to that of exon 20 (Fig. 12), however Oshimazakura is clearly distinguished by three single nucleotide polymorphisms (SNPs) from Yamazakura and other species.

Sequence analysis of intron 19 shows that 'Somei-yoshino' contains a haplotype containing the three SNPs at the positions of 25 101, and 171, which are specific to Oshimazakura (Fig. 13). This result is very important for considering the origin of 'Somei-yoshino' because Oshimazakura is an endemic species to the Izu and Boso Peninsulas (Fig. 4). Another haplotype of 'Somei-yoshino' is identical to that of the wild Edohigan except for one SNP at the position of 392. The same SNP was found in a haplotype of 'Komatsu-otome.' 'Komatsu-otome' is a cultivar of Edohigan with a short stature (*ca*. 6 m) and is found as an original tree in Ueno Park, which was in the precincts of Kan-eiji temple in the Edo era. These results suggest that 'Somei-yoshino' originates from hybridization between a maternal parent, a semi-dwarf cultivar closely related to 'Komatsu-otome,' and a paternal parent, a cultivar of Oshimazakura (Fig. 14). Also 'Somei-yoshino' could originate from hybridization between hybrids containing each haplotype.

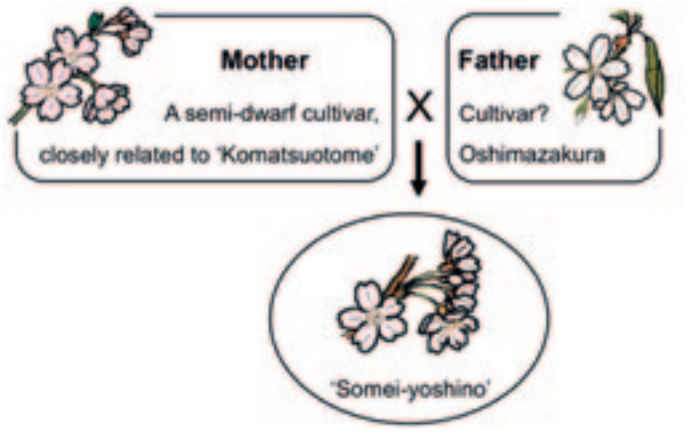

Fig. 14 - Schematic representation of the origin of 'Somei-yoshino' (Nakamura *et al*., 2007).

'Komatsu-otome' (tree No. 135, assigned by Ueno Park) was grown around the ruins of the Bell Tower in Kan-eiji temple (Fig. 15). Now, a total of ten trees related to Edohigan grow around the Bell Tower. Four trees (133, 134, 136, 138) have been identified as 'Somei-yoshino.' Sequence analysis of chloroplast DNA shows that the maternal lineage of all ten trees is Edohigan. And sequence analysis of the *PolA1* gene indicates that three trees (141, 142, 144) and 'Somei-yoshino' contain a haplotype (O) specific to Oshimazakura. One tree (145) is homozygous of the haplotype (K), which is shared by 'Komatsu-otome' and 'Somei-yoshino.' In addition, one haplotype (T) found to be specific to the chloroplast DNA of 'Some-yoshino' (Ando *et al*., unpublished) is shared with another tree (142). These results suggest that flowering cherry trees around the Bell Tower were developed by artificial hybridizations between Edohigan and Oshimazakura, and that there were sufficient genetic resources to develop 'Somei-yoshino' and 'Komatsu-otome.' DNA analysis of offshoots of four 'Somei-yoshino' clones revealed that these clones were propagated by using layering or grafting with a weak stock. This result suggests the action of a professional gardener. The purpose of planting the four clones together might be related to the evaluations of the 'Gun-zakura' by Buddhists of the Kan-eiji temple.

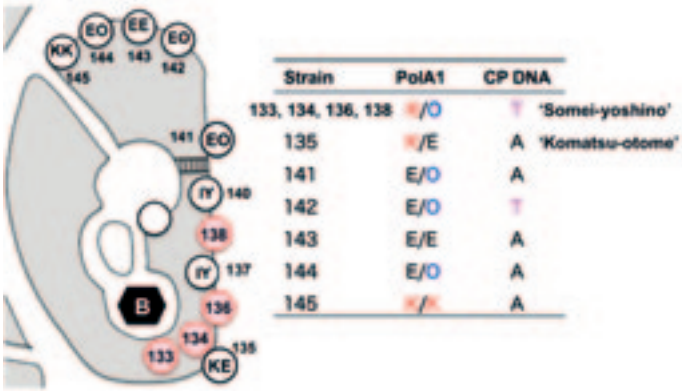

Fig. 15 - Haplotypes of intron 19 in *PolA1* gene and of chloroplast (CP) DNA analyzed for ten trees around the ruins of the Bell Tower (B) in Kan-eiji temple.

Masatake Ito Ihei (1676–1757) lived at the same time as Yoshimune Tokugawa (1684–1751). Masatake had the ability to develop 'Somei-yohsino' because he had developed many ornamental cultivars of various plant species (Iwasaki, 1989, 1991). Masatake might be the person who planted hybrids of flowering cherry around the Bell Tower because Kan-eiji temple had a close relationship with the Todo clan and because the precinct of Kan-eiji was an extraterritorial place from the Tokugawa Government. Except for Masatake, there was no reason why anyone would record the origin of 'Somei-yoshino.'

After the Meiji Restoration

Ken-eiji temple was destroyed during the 'Boshin' war (1868) and the temple grounds were established as Ueno Park (1873). The Meiji Government held Japanese industrial exhibitions several times in Ueno Park in order to promote Japanese industry. 'Somei-yoshino' shoots were massively propagated using grafting with a strong stock by gardeners in 'Somei' village, and then the shoots were probably sold in the exhibitions. The oldest known 'Somei-yoshino' tree is one of the 1,000 shoots that were planted within Hirosaki Castle in 1882, the year after the 2nd Japan Industry Exhibition (1881). As no one could buy the 1,000 shoots without seeing the real 'Somei-yoshino,' it was probably planted within Ueno Park at that time. At present, many 'Somei-yoshino' trees are beginning to show gaps in their stems, which seems to support the idea that 'Somei-yoshino' has a 60-year life span. As these gaps are caused by imbalanced growth between the scion and stock after grafting, if clones of 'Somei-yoshino' are propagated using grafting with a weak stock, they would be able to survive for hundreds of years.

5. Conclusions

There is no record of the origin of 'Somei-yoshino' but the results presented in this paper suggest that Ito Ihei Masatake, or someone else, developed 'Somei-yoshino' in secret as a suitable cultivar for 'Gun-zakura' within the grounds of Kan-eiji temple. 'Somei-yoshino' was created by horticultural techniques developed during the long period of peace and economic stability of the Edo era. 'Somei-yoshino' has been a parent of more than 100 cultivars since the Meiji Restoration. Dr. Yozaburo Shirahata (2000) suggested that 'Hanami' consisting of 'Gun-zakura,' ('Eating and drinking', and 'Mass gathering') is a popular culture unique to Japan, and the Japanese love of 'Hanami' is showing no sign of diminishing.

Acknowledgements

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Attempt for postharvest ripening of immature fruits of Haskap (*Lonicera caerulea* L. var. *emphyllocalyx* Nakai), an emerging fruit in Northern Japan

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Key words: agricultural mechanization, fruit softening, postharvest ripening.

Abstract: Haskap or Japanese blue honeysuckle (*Lonicera caerulea* L. var. *emphyllocalyx* Nakai) is a deciduous shrub berry crop, which is recently listed as one of promisingly emerging berry crops. In the present short survey, an attempt for postharvest ripening of immature fruits of haskap was testified by examining the changes in fruit hardness, peel color, pigment synthesis and sugar-acid balance during storage at 5, 10, 15 or 20°C. Softening and coloring were shown to be induced during postharvest storage, especially at 20°C. The extent of maturation was largely enhanced by longer storage period. It is conclusive that haskap berries can be harvested at premature stage if postharvest maturation was allowed during storage and/or transportation to the markets.

1. Introduction

Haskap (Japanese blue honeysuckle; *Lonicera caerulea* L. var. *emphyllocalyx* Nakai), a deciduous berry shrub, growing to 1.5-2.0 m tall, bearing a type of blue-berried honeysuckle about 1 cm in diameter, has recently been listed as one of four promising, emerging berry crops with commercial potential despite its current status of low economic importance (Hummer *et al*., 2012). Therefore, these crops have attracted the attention of world agriculturalists and breeders even if the history of their agricultural handling is still brief (Hummer *et al*., 2012). Blue-berried honeysuckles, including haskap, are native throughout the cool temperate Northern Hemisphere; haskap is cultivated in Hokkaido, the coldest local region in Japan. Haskap berry is now known as the earliest fresh fruit harvested in Hokkaido prefecture, Japan, starting in late June (Fu *et al*., 2011).

In the last two decades, an American research team has conducted a survey on the adaptability of various blue honeysuckle plants to the northwestern United States. They have considered qualities from several geographic sources including botanical varieties *edulis*, *kamtchatica*, *altaica*, and *boczkarnikovae* from Russia, *edulis* and *boczkamikovae* from northeast China, and *emphyllocalyx* from Hokkaido, Japan, and concluded that the Japanese variety *emphyllocalyx* (Haskap) has superior adaptability in Oregon (Thompson, 2006; Thompson and Barney, 2007). Therefore, a breeding program utilizing this variety was initiated in 2003 in the states of Oregon and also Idaho, aiming for outstanding selections in hopes of identifying superior individuals to release as cultivars as the basis for a new berry industry in the United States.

To date, a research team at Hokkaido University has surveyed the ploidy level and geographical distribution of wild haskap, based on the flow cytometric analysis revealing the presence of DNA diploid and DNA tetraploid plants sampled in Japan (Miyashita *et al*., 2011). Accordingly, chromosomal analysis confirmed that diploid and tetraploid plants showed 2n=2x = 18 and 2n = 4x = 36, respectively. The DNA diploid populations were found only in lowland mires, Betsukai, Bekanbeushi, Kushiro and Kiritappu located in eastern Hokkaido prefecture. On the other hand, DNA tetraploid populations were widely distributed in most areas in Hokkaido prefecture, and also in mainland Japan.

In fact, commercial cultivars of haskap have been selected only from wild plants and thus, fruit traits and other agricultural characteristics have been largely limited until recently (Miyashita and Hoshino, 2010). Breeding and plant biotechnological attempts to obtain novel haskap cultivars have followed, resulting in enhanced yield and quality. Around a decade ago, selection of haskap wild lines showing notable edible qualities and some horticultural char-

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acteristics were performed for a further breeding program (Takada *et al*., 2003). Accordingly, interspecies crosses between *L. caerulea* var. *emphyllocalyx* and *Lonicera gracilipes* var. *glabra* Miquel were examined to increase genetic variability of *L. caerulea* var. *emphyllocalyx* (Miyashita and Hoshino, 2010). Furthermore, Miyashita *et al*. (2009) reported the regeneration of haskap plantlets from endosperm culture of selected lines to enhance the breeding strategy.

Although haskap is one of most promising berry crops in Hokkaido, the amount of cultivation and supply to the market is largely limited due to the attention required to handle soft ripe berries. In Japan's small-scale orchards, farmers carefully harvest the haskap berries by hand so as to avoid losing any juice through damage of the delicate peel (Fu *et al*., 2011). To date, conventional agricultural machinery has failed to be introduced for the harvest. There could be two distinct approaches applicable to improve harvesting efficiency: (1) development of novel machinery or devices for automated or semi-automated harvesting with maximal care from aids such as sensors and actuators; and (2) development of novel working algorithms to simplify the overall processes so that conventional agricultural machines or equipment can be readily introduced, as recently proposed for other horticultural crops (Kawano *et al*., 2012).

Some examples of recent approaches can be found in a series of studies conducted by Fu *et al*. (2011). After testing various combinations of separating, collecting and cleaning methods, they concluded that the harvesting rate for haskap went from 1.45 kg/h (conventional hand picking) to a maximum of 10.36 kg/h.

The present study aims to contribute to the documentation, designing a novel harvesting strategy based on the ripening physiology of haskap berries, which could be readily automated with minimal efforts. Mature haskap berries are easily damaged by mechanical operations during harvest, selection and transportation. Therefore, mature haskap berries are hard to handle by conventional harvesting machines and those harvested in the Hokkaido region bear transportation with difficulty over great distances to markets in large cities in mainland Japan. To avoid these problems or difficulties which could be attributed to fruit maturity, it is tempting to propose harvesting immature green berries which are less sensitive to mechanical stresses, to then be followed by postharvest forced maturation during storage or transportation. In this brief survey, we report an attempt at postharvest ripening of immature fruits of haskap by examining the changes in fruit hardness, peel color, pigment synthesis and sugar-acid balance during storage at different temperatures.

2. Materials and Methods

Plant materials

Mature and immature haskap berries were picked from two-year-old shrubs cultivated at the Experiment Farm at Hokkaido University. The berries, harvested by hand, were immediately packed for transportation under temperature-controlled conditions (exposure to heat or cold was avoided), and used for the experiments within two days after harvest.

Color measurements

As described elsewhere (Kawano and Shimokawa, 2003), changes in color of the fruit peel were monitored using a handy CIELAB color reader (CR-13, Konica Minolta Sensing Inc., Osaka, Japan) by measuring the *a** and *b** values of CIE (Commission International de l'Eclairge) 1976 *L*a*b** color space units (CIELAB system), at 0, 1, 3, and 7 days of storage. As the *a** value corresponds to a red-green scale (red, positive; green, negative) and the *b** value corresponds to a yellow-blue scale (yellow, positive; blue, negative) (Kawano, 2013), we assumed that the *a** and *b** values represent degreening due to chlorophyll loss and blue color development due to the synthesis of anthocyanin, respectively.

Hardness

Changes in fruit hardness during storage were determined using a universal fruit hardness meter (max., 1 kg; model KM, Fujiwara Scientific Co. Ltd, Tokyo).

Storage

As the studied species are native to a cold region, we tested the effect of a low to moderate range of temperature, namely, 5, 10, 15 and 20°C, by keeping the samples in temperature-controllable cool incubators (model CN-25C, Mitsubishi Electronic Engineering Co. Ltd., Tokyo, Japan).

Sugar and acid contents

Freshly squeezed berry sap was used for determination of sugar content using a pocket sugar meter (model PAL-1, ATAGO Co. Ltd., Tokyo, Japan) and organic acid content using a portable amperometric acid sensor (model FS-101N, ATAGO Co. Ltd., Tokyo, Japan). For determination of sugar and organic acid, 0.5 mL and 0.1 mL of berry sap were used, respectively. Quantification of organic acids is based on the voltammetric reduction of 3,5-di-tert butyl 1,2-benzoquinone (in quinone reagent mixture provided by ATAGO Co. Ltd.) in the presence of acids (Kotani *et al*., 2008).

Pigment analysis

Anthocyanins were extracted from 0.1 g fresh weight of homogenates in 20 ml of 50% (w/v) acetic acid kept at 23°C for 24 h. Then, 1 ml of crude extract was subjected to centrifugation at 10,000 rpm (8217 x *g*) at room temperature for 10 min and the resultant supernatant was used for optical reading at 550 nm using a spectrophotometer (U-3310, Hitachi, Tokyo). Total anthocyanin content (expressed as of cyanidine-3-glucoside) was estimated according to Matsuzoe *et al*. (2006).

3. Results and Discussion

Appearance of berries before and after the storage

Prior to and after storage, the color of berry peels was compared with that of mature samples with the naked eye

(Fig. 1). While the peel of mature berries were apparently highly pigmented with a deep blue color, the immature berry samples lacked the blue pigmentation but were green due to the remaining chlorophylls. After a week of storage at 20°C, the peel of immature berry samples was slightly colored blue (Fig. 1 bottom). These observations suggest that postharvest ripening of haskap berries could likely be achieved under controlled conditions.

Softening and coloring

The progress in postharvest maturation in haskap berries kept under various temperature was scored by the changes in mechanical hardness (Fig. 2) and peel color (Fig. 3). The results suggest that the softening of berries is affected by the storage temperature. At time 0, the difference in hardness between mature and immature samples was significantly large. The hardness of the immature samples kept at 20°C was gradually lowered and finally attained a level comparable to naturally-matured berry samples (Fig. 2).

Similarly, coloring was enhanced in the samples stored at higher temperature. Peel color was non-destructively determined by a colorimeter and the changes in peel color were expressed as values in CIELAB system (Fig. 3). At 5°C, neither lightness (L^* values), greenness (negative a^* values), nor blueness/yellowness (b^* values) showed significant change during seven days of storage. In contrast, under higher temperatures, all coloring values were altered with time, suggesting that chlorophyll degradation and anthocyanin biosynthesis were induced. Loss of chlorophylls (de-greening) is one key feature of fruit ripening found in various fruits with ethylene-producing climacteric (Kawano

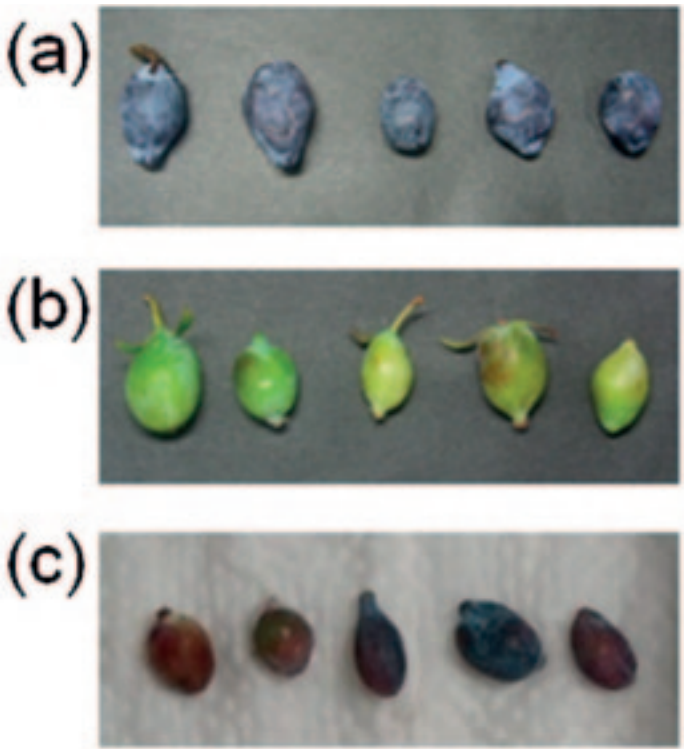

Fig. 1 - Images of typical haskap berries at three different stages of maturation. (a) Mature berries. (b) Immature berries prior to storage. (c) Semi-colored berries obtained after storing the immature samples for 1 week at 20°C.

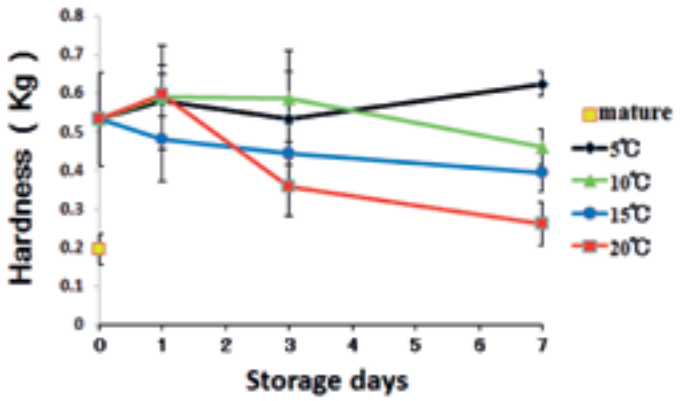

Fig. 2 - Changes in mechanical hardness of haskap berries during storage under various temperatures. Vertical bars on the graph indicate SE (n=4).

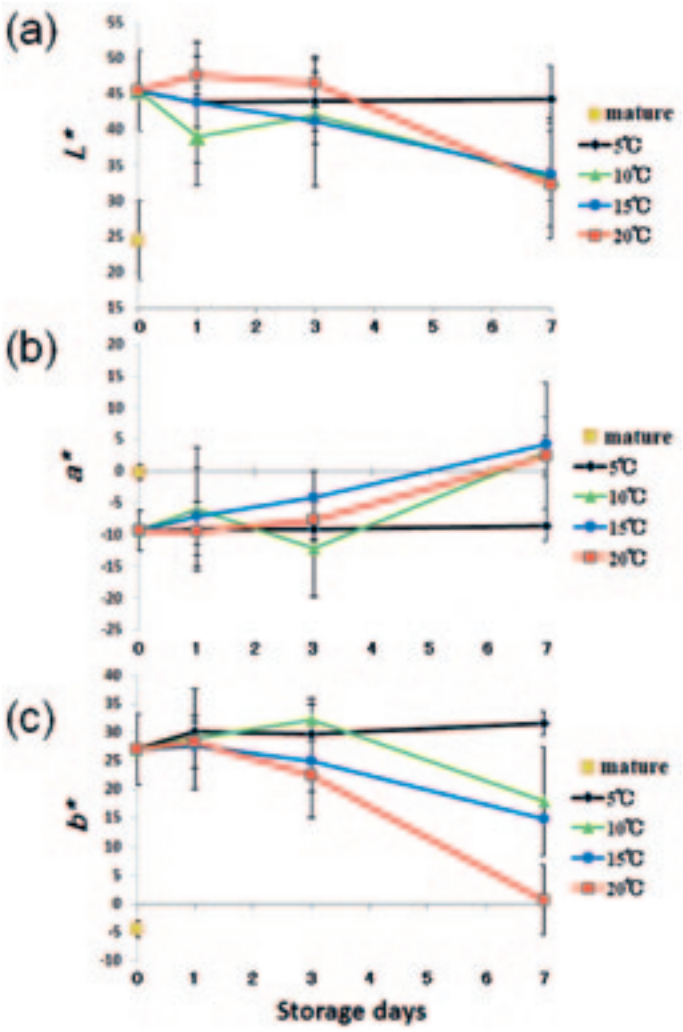

Fig. 3 - Colorimetric changes in peel color in haskap berries during storage under various temperatures. Peel color was determined by colorimeter and expressed as values in CIELAB system, namely L^* (a), a^* (b), and b^* (c). Vertical bars on the graph indicate SE (n=4).

and Shimokawa, 1994; Kawano *et al*., 1999; Kawano and Shimokawa, 2004, 2005 a) and non-climacteric natures (Kawano and Shimokawa, 2003, 2005 b). Thus, further investigation of the de-greening mechanism is encouraged.

Anthocyanin content

As the decrease in *b** value in the peel color (Fig. 3 c) reflects the increase in blue pigments, the changes in anthocyanin content during postharvest storage were monitored (Fig. 4). The mature samples were rich in anthocyanin (Fig. 4 a-i) and immature samples contained no detectable anthocyanin (Fig. 4 a-ii). Among the samples which were subjected to postharvest maturation, only the samples kept at 20°C showed signs of induced pigmentation (Fig. 4 a-vi).

Similarly to the colorimetrically determined blueness (Fig. 3), the blue pigmentation in the samples stored at 20°C was shown to be linearly increased with time (Fig. 4). Based on the observed tendency, we can further expect that enhanced pigmentation can be manifested by longer storage.

Sugar/acid contents

It is well known that both the absolute values and balance in Brix index and acidity in the berry sap largely determine the consumption quality of fresh haskap berries (Takada *et al*., 2003). During postharvest maturation under various temperatures, no significant increase in sugar content in the immature berries was observed (Fig. 5 a). Instead, compared to green immature samples examined prior to storage, there was significant decrease in acidity over the seven days of storage in most samples kept at different temperatures (Fig. 5 b), thus contributing, although slightly, to the increase in sugar/acid ratio (Fig. 5 c). In the end, significance between the sugar acid ratio in mature samples and that in stored samples was lost, suggesting that sugar/acid ratio was amended by the postharvest maturation process employed.

Texture, taste, flavor, and attractive components

Softening of the berries is one of the important factors determining the texture of haskap berries. As described above, the mechanical hardness of berries was significantly lowered during postharvest incubation at 20°C and attained a level comparable to naturally-ripened samples (Fig. 2). As shown in figure 5 a, the postharvest maturation approach was barely successful for enhancing the sweetness increase, although sweetness and sourness represented by the contents of sugar and organic acids are major factors determining the tastes of berries (Takada *et al*., 2003). However, due to the induced decrease in total acid

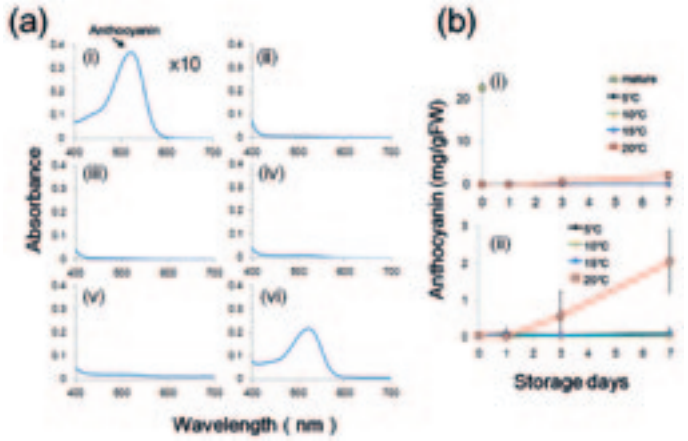

Fig. 4 - Changes in anthocyanin content in haskap berries during storage under various temperatures. (a) Typical spectral profiles of extracts from different samples. (i) Mature berries. (ii) Immature berries prior to storage. (iii, iv, v, vi) Immature berries subjected to one- week-long storage under 5, 10, 15, 20°C, respectively. (b) Increase in anthocyanin content in haskap berries during storage under various temperatures. (i) Comparison of anthocyanin content between mature samples and immature samples subjected to storage. (ii) Data in (i) were enlarged for ease of comparison among the samples stored under different temperatures. Vertical bars in (b) indicate SE (n=4).

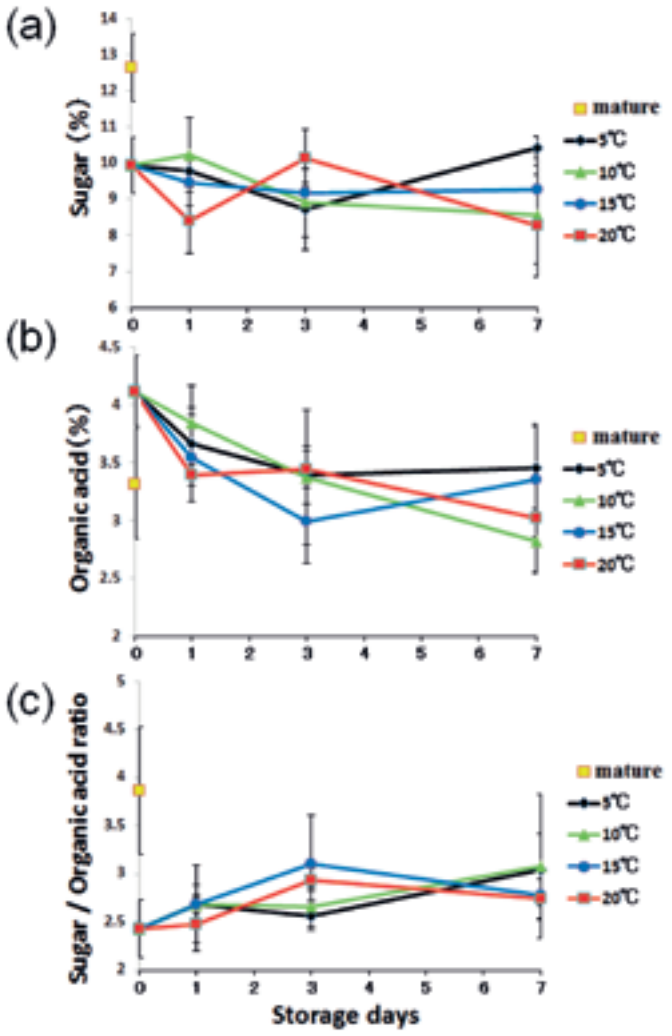

Fig. 5 - Changes in sugar and acid contents in haskap berries during storage under various temperatures. (a) Sugar content (percentage by weight). (b) Organic acid content (percentage by weight). (c) Sugar/acid ratio are compared. Vertical bars on the graph indicate SE (n=4).

level (with limited extent, Fig. 5 b), the sweetness/acidity balance might be enhanced reaching a level relatively close to the level of naturally-matured samples (sugar/acid ratio in Fig. 5 c). A preliminary organo-lip test performed by laboratory members is in support of the data on sugar/acid balance, but it is still early to provide any conclusion from such a limited survey.

One of major uses of haskap in Hokkaido is in bakery goods as sour taste accents and as color-attractive toppings. Therefore, rather than sweetness, production of pigments, chiefly anthocyanin, is of more importance from this point of view.

It is notable that haskap is considered a new berry crop with high antioxidant capacity due to its wealth in anthocyanins and related substances: anti-oxidative scores for haskap varieties were the highest among commercial fruits examined through multiple methods such as ferric reducing antioxidant power (FRAP) assay, oxygen radical absorbance capacity (ORAC) assay, the 1,1-diphenyl-2- picrylhydrazyl (DPPH) free radical scavenging assay, the aluminum chloride colorimetric method and the Folin-Ciocalteu method (Rupasinghe *et al*., 2012). More recently, Takahashi *et al*. (2014) examined the effects of dietary intake of anthocyanin-rich phenolic phytochemical (containing 13.2% anthocyanin) purified from haskap fruit on postprandial serum triglyceride and blood glucose levels in rats, concluding that a decrease in postprandial blood lipids and blood glucose by short or long-term haskap phytochemical ingestion is due to anthocyanin and other polyphenols contained in the haskap phytochemical. Reports on the antioxidant capacity of haskap and other health-related studies regarding haskap-derived pigments have been reviewed elsewhere (Celli *et al*., 2014).

In addition to pigments, the flavors or aromas of haskap berries are of commercial importance and have recently gained attention from food industries. For instance, haskap residues after juice extraction have been used preliminary as natural flavoring for teas (Sakamoto *et al.*, 2012). Thus, the impact of postharvest maturation of haskap berries on the production of flavors and aromas should be documented in future studies.

4. Conclusions

Two key parameters of fruit maturation, namely softening and coloring, were significantly amended by postharvest storage at moderate temperature and the extent of maturation is likely to be enhanced by a longer storage period. As enhanced coloring represents an increase in anthocyanin content, valued for its antioxidant action, the postharvest maturation approaches presented here may contribute to the market quality of this crop. However, the sugar content in the berry sap could not be altered during storage and the change in sap acidity induced during storage was limited. In conclusion, haskap can be harvested at premature stage if postharvest maturation is allowed during storage and/or transportation to the markets.

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