カキ'西条'における耐凍性の系統間評価

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Evaluation of Cold Tolerance among Japanese Persimmon 'Saijo' Strains

Keisuke MOCHIDA*[†], and Hiroyuki ITAMURA**

(**Faculty of Life and Environmental Science, Shimane University, Matsue, Shimane 690–0823)

Abstract

Cold tolerance was compared among the three 'Saijo' persimmon strains "Abe", "Endo", and "Izumo". We also determined the carbohydrate and non-tannin flavans content (mainly anthocyanins) in the stems and buds of 1-year-old twigs of the three strains. In "Abe", the total sugar content in stems in early spring, just before sprouting, was significantly lower than those of "Endo" and "Izumo". This was because the amounts of sucrose and sorbitol contained in "Abe" were significantly lower than those in the other two strains. The glucose content of "Abe" buds in early spring was likewise significantly lower than that of the other two strains. The non-tannin flavan contents of the buds and bark tissues of "Abe" stems were lower than those of "Endo", while in mid-March the electrolyte leakage of stems and buds of "Abe" during treatments at -3 and -6° C was found to be significantly higher than in the other two strains. Browning was observed in "Abe" is the least tolerant to cold among the three strains. The sprouting disorders frequently observed in "Abe" may result from slight freezing injuries to buds.

Key words: Anthocyanin, Flavan, Freezing injury, Sugar.

1. Introduction

The Japanese persimmon (*Diospyros kaki*) cultivar 'Saijo' consists of many different strains, which differ in terms of their fruit shapes and ripening periods. These strains are distributed throughout the Chugoku districts, Hiroshima (the origin of 'Saijo') and its surroundings, Okayama, Tottori, Shimane, and Yamaguchi. The genetic variations have arisen due to bud mutations.

The Shimane Prefecture Agricultural Experiment Station selected 6 desirable early-ripening 'Saijo' strains from more than 10 strains cultivated in Shimane: "Abe", "Endo", "B-dwarf" (a dwarf strain of "Endo"), "Yamasaka", "Koto", and "Wada". These strains were classified as "B-type" persimmons based on their fruit shapes (obovate with four deep grooves) and fruit ripening time (harvestable from early October) (Kono *et al.*, 1980). However, apparent differences were observed in the tree growth and fruit productivity among the six strains, and "Endo" was selected as an elite line (Mochida *et al.*, 2007). "Abe" in particular had relatively poor fruit productivity and frequent physiological disorders, such as a delayed sprouting period, decreased sprouting rate, and poor shoot elongation. Together, these physiological attributes resulted in a decreased leaf count and leaf area index in "Abe".

Late frost damage in persimmons causes tree decline, while browning disorders on sprouts can be caused by late frosts. These can occur in orchards located in hollows or valleys, where frost protection instruments such as wind machines are not used. Frost injury appears at -1.5 to -2.0° C at sprouting time in almost all persimmon cultivars (Kikuchi, 1991). The 'Saijo' strain sprouts in early- to late-March in Shimane. The minimum temperature at the persimmon orchards in Shimane prefecture occasionally drops below zero at this time of year, hence the cold tolerance of buds and stems during this period is important for initial tree growth and productivity.

In this study, we investigated the carbohydrate

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[†]Corresponding author: mochida-keisuke@pref.shimane. lg.jp

contents and cold tolerance of buds and stems of three 'Saijo' strains. Twig cold tolerance was reportedly improved by increased non-tannin flavan content (mainly anthocyanins) during cold acclimation in persimmon, apple, and peach (Leng *et al.*, 1997; Leng *et al.*, 2000). Therefore, we investigated the non-tannin flavan contents in these 'Saijo' strains.

2. Materials and Methods

2.1 Plant Material

The experiment was conducted at the Masuda Branch of the Shimane Agricultural Experiment Station (131°51' E, 34°40' N, altitude 150 m). Three trees each of the early-ripening strains "Abe" and "Endo", and the mid-ripening strain "Izumo" were used for the experiment. The tree growth of "Abe" was inferior to that of "Endo" and "Izumo". All trees were 14 years old in the first year of investigation (2003). Trees were cultivated as described previously (Mochida et al., 2007), and all trees were cultivated in the same field. The sprouting dates in Masuda city in 2003 were 23 March for "Endo" and "Izumo" and 26 March for "Abe". In 2006 sprouting occurred from 17 March for "Endo" and "Izumo", and 19 March for "Abe". The sprouting date for "Abe" was slightly delayed in both years as a result of sprouting disorders.

2.2 Meteorological observations

We measured temperature at the experimental site from January to April in 2003 and 2006 using a thermocouple (T-type) located 1.5 m above the ground. Data was recorded using a data logger (R7326B type, ADVANTEST Co., Ltd., Japan). Data on the precipitation and sunshine duration from August to November in 2002 and 2005 were extracted from AMEDAS data from the Masuda station, which is located 3 km away from the experimental site.

2.3 Measurement of cold tolerance in February and March (2006)

Cold tolerance in early spring was evaluated by electrolyte leakage. Five 20-cm twigs (branches that bore fruits in the previous year) each of "Abe", "Endo" and "Izumo" were collected on 21 February and 19 March, 2006 (bud sprouting period), and divided into five series (one of which served as the control). They were placed in plastic bags in a programmed freezer at a temperature below freezing. On 21 February, the temperature in the four series was reduced from +5 to -5, -10, -15, or -20°C respectively. On 19 March the temperature was reduced from +5 to -3, -6, -9, or

 -12° C. The temperature was decreased within a period of 5 h for each series. The final temperature of each series was maintained for 5 h, and then returned to $+5^{\circ}$ C over 5 h (Leng *et al.*, 1993). Each cold treatment had five replicates.

After the following 0°C treatments, 1-year-old twigs were cut into 5 cm stem portions, and the finely sliced 5 g samples (buds; 0.5 g) were soaked in 40 mL of water at 20°C for 10 h. The initial electric conductance (C_1) was measured with a conductivity gauge (Horiba, C-173, Japan), while the second electric conductance (C_2) was measured after the sample was killed in boiling water for 30 min and soaked in 40 mL water at room temperature for 12 h. The electrolyte leakage (E) was calculated as follows; $E=C_1/C_2\times 100$. Three buds and 1-year-old stems were chosen from every tree after each sub-zero temperature treatment on 19 March. Each sample was dissected longitudinally through the centerline of the bud and stem, and evaluated for injury on the cut surface on the bud, cambium, xylem, and pith tissues of the stem. The degree of browning was classified as visual evidence of injury based on the following scale: 0, no browning; 1, slight browning; and 2, severe browning.

2.4 Determination of the carbohydrate content of stems and buds during the bud sprouting period (2003)

The carbohydrate contents were determined in early spring. Five mature 1-year-old twigs (branches that bore fruit in the previous year) were collected from the central canopy of three trees, each of "Abe", "Endo" and "Izumo" strains. Twigs were collected on 26 March, 2003 and separated into buds and stem portions of approximately 5 cm. The stems were cut into lengths of approximately 5 mm. Stem pieces and buds were dried at 90°C for 10 days, and then ground using a masticator (WB-1, Osaka Chemical Co., Ltd., Japan). Dried samples (500 mg stems or 200 mg buds) were placed into test tubes, and 30 mL 80% ethanol was added. The samples were then boiled at 80°C in a water bath for 30 min, and then centrifuged. Each sample was extracted twice with 80% ethanol, and the combined ethanol extracts were evaporated with a rotary evaporator at 40°C to the water phase under reduced pressure. The water phases were diluted to an adequate concentration for HPLC analysis with the addition of distilled water, and filtered through a 0.45 µm membrane filter. Sucrose, fructose, glucose, and sorbitol were measured by HPLC (Waters; RI detector; Sugar-Pak column; H_2O elution solvent; column temperature 80°C; flow rate 1 mL/min). The residues were then dried for starch determinations. To determine the starch content, 200 mg dried samples of stems and 100 mg dried samples of buds were placed into test tubes, and extracted three times with 8.14 N HClO₄. The extracts were then diluted to 100 mL with distilled water and the starch contents determined using by the Phenol-H₂SO₄ test (Sakano, 1989). The total carbohydrate contents were expressed in the form of the total of soluble sugars and starch. All analyses were replicated twice.

2.5 Determination of non-tannin flavans (mainly anthocyanins) in stems and buds in February (2006)

To clarify the non-tannin flavans (non-tannin flavonoids, mainly anthocyanins) in buds and stems, five 1-year-old twigs were collected from the central canopy of three trees each of "Abe", "Endo" and "Izumo" on 3 February, 2006. Twigs were separated into buds and stems, while the stem tissues were separated into bark and wood. The fresh tissues (bark and wood, 1.0 g; bud, 0.5 g) were ground using a masticator, mixed with 25 mL 80% MeOH, and kept at 4°C for 24 h. The macerated mixture was then filtered and the residue re-extracted with 25 mL 80% MeOH. The combined methanol extracts were subsequently evaporated on a rotary evaporator at 40°C to the water phase under reduced pressure, and the water phase was fractionated with an equal volume of petroleum ether to remove lipids. The water phase was brought up to 50 mL for wood and bud tissues and 100 mL for bark tissues with distilled water, while non-tannin flavans were assayed by the partitioning of these crude extracts according to the modified method of Peri and Pompei (1971)

(Ito *et al.*, 1987). Extractions were replicated three times. The supernatants (A) of the reaction mixtures of cinchonine sulfate and these crude extracts were insolubilized by adding formaldehyde under acid conditions. These precipitations were the non-tannin flavan fractions, while the supernatants (B) were simple phenols. Phenolics were determined in supernatants (A) and (B) using the modified Folin-Denis method (Swain and Hillis, 1959), with D-catechin used as the standard. The concentration of non-tannin flavans were calculated by subtracting B from A.

3. Results

3.1 Meteorological observations

Total precipitation was 348 mm from August to November in 2002, and 382 mm during the same period in 2005. Precipitation during this period in a normal year is 545 mm. There were 697 h hours of sunshine in 2002, and 622 h in 2005 (620 h in a normal year) in Masuda. The minimum temperature every 5 days (extreme value) is shown in Fig. 1. Sub-zero temperatures occurred several times in the field during January to March in 2003 and 2006.

3.2 Cold tolerance of stems and buds in February and March (2006)

The electrolyte leakages (*E*) of 1-year-old stems and buds of "Abe", "Endo" and "Izumo" subjected to sub-zero temperature treatments in winter and at the sprouting period are shown in Fig. 2. On 21 February, following the -10° C treatment, the electrolyte leakage in stems of the three strains ranged from 13.0 to 14.4%, and 17.2 to 24.3% in buds. On 19 March, following the -9° C treatment, *E* values ranged from 19.2 to 26.2%, and 37.2 to 45.4% in stems and buds, respectively. Therefore, the electrolyte leakage was higher in March



Fig. 1. Changes in minimum temperature every 5 days (extreme value) from Jan. to Apr. in 2003 and 2006.



Fig. 2. Freezing resistance in stems and buds of 1-year-old twigs of three 'Saijo' strains in early, spring (21 Feb. and 19 Mar., 2006)

Freezing tolerance expressed as percentage electrolyte leakage from stems and buds respectively, after freezing tests at -5, -10, -15, and -20° C on 21 Feb 2006, and at -3, -6, -9, -12° C on 19 March, 2006.

^z No significant differences among three strains (ANOVA).

^y Strains with different letters in the same freezing test differ significantly (5% level; Tukey test).

* Percentage electrolyte leakage from stems and buds at room temperature.



Fig. 3. Relative electrolyte leakage in stems and buds of 1-year-old twigs of three 'Saijo' strains after −3°C treatment, compared to that at room temperature (19 March, 2006).

² Vertical bars indicate standard errors (n=3).

than in February, and higher in buds than in stems. On 19 March, the electrolyte leakages of stems and buds in "Abe" at -3° C were 17.5 and 17.8%, respectively. These values were significantly higher than those of "Endo" and "Izumo".

The relative electrolyte leakage of stems and buds subjected to the -3° C treatment compared with those at room temperature (control) on 19 March is shown in Fig. 3. The relative electrolyte leakage of stems in "Abe" was 9.4%, which was relatively higher than that of other strains, although the difference was not significant. On the other hand, the relative electrolyte leakage of buds in "Abe" was 22.0%, which was significantly higher than the relative E values of "Endo" and "Izumo".

The browning of 1-year-old stem and bud tissues after sub-zero temperature treatments is shown in Fig. 4. Slight browning was observed on the buds of "Abe" following the -3°C treatment, while the browning level at -6° C was 1, and that at -9 and -12° C in all samples was 2 respectively. The total browning of all samples at the lower temperatures meant that no difference emerged among the three strains. No browning was observed on the cambium in "Endo" and "Izumo" at -6°C treatment. However, the browning level in "Abe" was approximately 0.6, with slight visible injury. The browning levels at −9°C in "Abe" and "Izumo" were approximately 1, as opposed to approximately 0.6 in "Endo", which was relatively lower than the other two strains. Slight injury was evident in the woody tissues of "Abe" at -9°C treatment, while only slight browning was observed on the wood in "Endo" and "Izumo"



Fig. 4. Browning on stems and buds of 1-year-old twigs of three 'Saijo' strains after freezing tests at −3, −6, −9, and −12°C (19 March, 2006).

^z Browning was used as visual evidence of injury (0=no browning, 1=slight browning, 2=severe browning).

^y Vertical bars indicate standard errors (n=3).

following the -12° C treatment. Severe freezing injury occurred in "Abe" wood, with a browning level of approximately 1.3. The browning level on the pith at -12° C in "Abe" was approximately 0.6, which was more severe than that shown by the other two strains.

3.3 Carbohydrate content of stems and buds during the bud sprouting period (2003)

Table 1 shows the starch and the sugar components in 1-year-old stems and buds of each strain at the bud sprouting period in 2003. Total sugar contents in 1-year-old stems of "Abe" were significantly lower than those of other strains, while no significant difference emerged in the starch contents of 1-year-old stems among the strains. In all strains, sucrose was the most abundant sugar in 1-year-old stems, followed by fructose, glucose, and sorbitol, while the sucrose and sorbitol contents in 1-year-old stems of "Abe" were significantly lower than the other two strains. Although no significant difference emerged among the strains, sugar contents in the bud of "Abe" tended to be lower than those of the other two strains, while the glucose content in the buds of "Abe" was significantly lower

Table. 1	. Reserve	carbohydrate	contents	in	stems	and	buds	of	1-year-old	twigs	of	three	strains	of
'Saijo'	persimm	ons (measured	1 on 26 1	Mar	ch 20	03)								

	Strain	Starch	Total muses	composition of soluble sugar					
Portion			Total sugar -	Sucrose	Glucose	Fructose	Sorbitol		
		(%. DW)	$\begin{array}{c ccccc} \hline composition of soluble sugar \\ \hline Sucrose & Glucose & Fructose & S \\ \hline (\%. DW) & (\%. DW) & (\%. DW) & (\%. DW) & (\% \\ \hline 1.44 b^{z} & 0.91 b & 0.20 & 0.29 & (0) \\ \hline 1.84 a & 1.24 a & 0.25 & 0.29 & (0) \\ \hline 1.68 a & 1.12 a & 0.18 & 0.29 & (0) \\ \hline \ast & \ast & n.s. & n.s. \\ \hline 2.74 & 1.97 & 0.41 b & 0.36 \\ \hline 3.10 & 2.18 & 0.64 a & 0.27 \\ \hline 2.02 & 0.67 & 0.22 \\ \hline \end{array}$	(%. DW)					
	"Abe"	3.81	1.44 b ^z	0.91 b	0.20	0.29	0.03 b		
stem	"Endo"	3.68	1.84 a	1.24 a	0.25	0.29	0.06 a		
	"Izumo"	3.61	1.68 a	1.12 a	0.18	0.29	0.08 a		
Significancey		n.s.	*	*	n.s.	n.s.	*		
	"Abe"	3.04	2.74	1.97	0.41 b	0.36	0.01		
bud	"Endo"	2.94	3.10	2.18	0.64 a	0.27	0.00		
	"Izumo"	3.20	2.93	2.02	0.67 a	0.23	0.01		
Significance		n.s.	n.s.	n.s.	*	n.s.	n.s.		

² Different letters within column indicate significance at 5% level (Tukey test).

^y *Indicates significance at 5% level, n.s. indicates non-significance (ANOVA).





- ^z Vertical bars indicate standard errors (n=3).
- ^y Different letters within column indicate significance at 5% level (Tukey test).
- * No significant differences among three strains (ANOVA).

than those of the other two strains.

3.4 Non-tannin flavans (mainly anthocyanins) in stems and buds in February 2006

Non-tannin flavans (mainly anthocyanins) in the bark and wood tissues of 1-year-old stems and buds are shown in Fig. 5. The non-tannin flavan contents peaked in the bark, and were lower in the bud and wood. Although no significant difference emerged in the non-tannin flavan contents of wood tissue among the three strains, the non-tannin flavan contents in the bud and bark of "Endo" were higher than those of the other two strains. In particular, the non-tannin flavan contents in the buds of "Abe" were significantly lower than those of the other two strains.

4. Discussion

From autumn to winter in 2002 and 2005, the hours of sunshine fell within the normal range, but there was less precipitation than normal. Each strain produced a heavy crop (data not shown). Accordingly, there were presumably small amounts of reserve nutrients in buds and stems before sprouting in both 2003 and 2006. The low nutrient levels made these persimmon strains particularly susceptible to frost injury in 2003 and 2006, hence, these years were considered suitable times to clarify differences in cold tolerance among the strains.

The electrolyte leakage in the -3° C treatment in "Abe" during the bud sprouting period was significantly higher than the E values of "Endo" and "Izumo". In particular, the relative electrolyte leakage from "Abe" buds in the -3° C treatment compared to unfrozen controls was significantly higher than those from the other two strains (Figs. 2, 3), and slight browning was observed on the bud tissues (Fig. 4).

In the field, the intact buds and stems show frost injury at -3° C during the bud sprouting periods (Kikuchi, 1991). As the electrolyte leakage at -3° C exceeded that of the control, the cell membrane appeared to have been damaged. However, the -3°C treatment was non-lethal to the buds and stems during the laboratory experiment in this study. In our experiment, 1-year-old stems and buds were not humidified before the sub-zero treatment. The temperature at which cold injury occurred was considered to be lower than that under natural conditions. Moreover, experimental conditions in a laboratory cannot exactly replicate natural conditions, because the rate of temperature change and the duration of the target temperature in nature are highly variable. Despite these limitations, our results show clear differences in cold tolerance among the three persimmon strains.

According to Leng *et al.* (1995), the temperature at which 50% survival occurs (LT_{50}) corresponds to 25% electrolyte leakage. In late-February, LT_{50} values in the three strains were about -17° C to -18° C in 1-year-old stems, and -10° C to -13° C in buds. However, in mid-March, the LT_{50} values in stems of "Abe" were approximately -8° C and -12.5° C in both "Endo" and "Izumo", in contrast to LT_{50} values in buds of -6.0 to -6.5° C in "Abe", and -6.5 to -7.0° C in both "Endo" and "Izumo". The cold tolerance of buds was lower in comparison to stems in both stages.

In early to late March, the minimum temperature at persimmon orchards in Shimane prefecture occasionally drops below zero. Slight late frost damage occasionally occurs in orchards near the experimental site. For example, the buds of a 'Saijo' strain (exact strain unclear) showed browning on 1 April, 2001 in orchards located in valleys in Masuda city. In these valley areas, the cold conditions persist remain for longer than at other more open sites. In 2006, sub-zero temperatures occurred four times in March at our site, meaning these sub-zero temperatures may have caused slight injury to the least tolerant strain of "Abe", which may result in decreased yields due to inhibited tree growth.

Sakai and Yoshida (1968) reported that an increased concentration of sugars and sugar-alcohols in plant tissues correlated with increased cold tolerance. The sugar contents of 1-year-old "Abe" stems were significantly lower than those of other strains and a similar pattern was observed in buds. Glucose contents, which affect susceptibility to freezing injury (Sakai and Yoshida, 1968), were significantly lower in buds of "Abe" than in the other two strains (Table 1). This suggests that low glucose content in the buds and low total sugar content in the 1-year-old stems of "Abe" was one of the reasons for vulnerability to freezing injury at sprouting.

The non-tannin flavan (mainly anthocyanins) content in buds of "Abe" was significantly lower than that in buds of "Endo" and "Izumo" (Fig. 5). Plant shoots accumulate anthocyanidin glycosides (anthocyanin) in vacuoles of epidermal and sub-epidermal cells, which turn red when they are subjected to low temperatures (Leng et al., 2000). An increased anthocyanin concentration in deciduous plants enhances cold tolerance. Anthocyanins accumulated in vacuoles may protect the vacuole membrane from low temperature injury (Leng et al., 2000). In fact, in Hedera helix L. leaves (Parker, 1962), and shoots of apple, peach (Leng et al., 2000) and persimmon (Leng et al., 1993), cold tolerance increased sharply in November and December, accompanied by a similarly sharp increase in anthocyanin concentration. As flavonoid compounds always exist as glycosides in plant tissues (Van Buren, 1970), the formation of non-tannin flavan glycosides (mainly anthocyanins) is thought to be largely influenced by sugar content. Miura and Iwata (1985) reported that the promotion of anthocyanin synthesis in Polygonum seedlings appeared to be closely correlated with the accumulation of carbohydrates, especially total sugars. The glucose content in "Abe" buds was markedly lower than that in the other two strains; therefore the formation of non-tannin flavan glycosides is assumed to be lower. This low concentration of sugars and flavan glycosides in "Abe" may enhance the freezing injury.

Matsumoto *et al.* (2006) suggested that sprouting disorders in 'Saijo' persimmon, such as suppressed or delayed sprouting, were exacerbated by sub-zero temperatures in winter, while Mochida *et al.* (2007) reported that among the early ripening "B-type" strains

of 'Saijo', sprouting disorders were most common in "Abe". This study strongly suggests that the sprouting disorder in "Abe" was caused by non-lethal freezing injury to buds at temperatures below 0°C during sprouting, which may lead to a decreased leaf count and shorter stems. Unfortunately, "Abe" is likely to be already planted widely throughout Shimane prefecture.

It is probable that "Abe" will not recover from its sprouting disorders. The carbohydrate contents in buds and stems, and flavonoid contents in buds will decrease due to the decline in tree growth, which will trigger another late frost injury. As Matsumoto et al. (2006) pointed out, abnormally accumulated condensed tannins in the xylem suppressed the rising of the breeding sap. In turn, this obstructed water and mineral movement, ultimately causing the entire system to deteriorate. We confirmed that there were higher concentrations of tannins and Ca, Mg, and Mn in browned parts of the xylem of older woody tissue than in other healthy parts of the xylem (data not shown). In future, the selection of superior strains with respect to cold tolerance could be effective in reducing late frost injury and sprouting disorders in early strains of the 'Saijo' persimmon.

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カキ '西条' における耐凍性の系統間評価

持田圭介*・板村裕之**

(*島根県農業技術センター
(**島根大学生物資源科学部)

約

要

"西条"における耐凍性の系統間差について、早生"B型"系統の"安部"系および"遠藤"系と中晩生の"出雲型" 系統で比較した。各供試系統の1年生枝の枝部分および芽について、炭水化物並びに非タンニンフラバン(主としてアントシアニン)含量を測定した。"安部"系は、萌芽前の枝中糖含量が"遠藤"系および"出雲型"系統と比較し有意に少なく、これはショ糖とソルビトール含量が 有意に少ないことが要因であった。また、同時期の"安部"系の芽中ブドウ糖含量が,他の2系統と比較し有意 に少なかった。芽および枝皮層部中非タンニンフラバン 含量は、"安部"系で少なく"遠藤"系で多かった。3月 中旬の枝と芽の-3℃および-6℃処理後の電解質浸出率 は、"安部"系が有意に高く、芽では-3℃で、1年生枝形 成層は-6℃で褐変障害が認められ、"安部"系は他系統 より耐凍性が劣った。"安部"系で多発している春季発芽 不良症状は、芽に軽度の凍害を被ったことが一因と推察 された。

キーワード: アントシアニン,フラバン,低温障害,糖