Running head: cell division and enlargement in persimmon

Article type: General Paper

Strapping and a Synthetic cytokinin Promote Cell Enlargement in 'Hiratanenashi' Japanese Persimmon

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Key words: cell number, *Diospyros kaki*, fruit size, parenchyma cell, synthetic cytokinin

Abstract

The effects of *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea (CPPU) with and without strapping on fruit growth of 'Hiratanenashi' Japanese persimmon were evaluated by measuring parenchyma cell size and the number of cell layers in mesocarp throughout fruit development. Three-year-old branches were strapped with a wire two weeks before full bloom, and 10 mg L^{-1} of CPPU was applied to the fruitlet at 10 days after full bloom. CPPU alone prolonged the growth period, resulting in a larger fruit diameter at harvest than the control. Strapping promoted the fruit coloration and increased final fruit diameter. CPPU plus strapping shortened the growth period compared to CPPU alone, and the mature fruit diameter was similar to CPPU alone. The increases in fruit diameter caused by CPPU and strapping were involved with the increases in length of parenchyma cell. The number of cell layers was almost similar among the treatments. These results showed that strapping promotes the rate of parenchyma cell expansion, and CPPU with and without strapping prolong the duration of cell enlargement and promote the rate of the cell expansion.

Introduction

High quality, large fruit size, high sugar content, and a fine appearance are important characteristics for the commercial production of Japanese persimmon fruit. Fruit size in many temperate fruits and nut crops is particularly important in determining market price. Studies have been conducted to control fruit size using the application of plant growth regulators (Zhang *et al*., 2007); fruit size is also controlled in numerous temperate crops by thinning, reducing the potential for bloom, and girdling, as well.

A synthetic cytokinin *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea (CPPU) has been demonstrated to promote fruit growth in grape (Zabadal and Bukovac, 2006), kiwifruit (Patterson *et al*., 1993), apple (Stern *et al*., 2006), Japanese persimmon (Hasegawa *et al*., 1991), and pear (Shargal *et al*., 2006) as well as other crops. CPPU is commercially applied in some crops, including persimmon in Japan, to improve fruit set and growth. However, CPPU also delays color development in some applications, which can indicate delayed physiological maturity (Hasegawa *et al*., 1991; Zabadal and Bukovac, 2006). On the other hand, strapping with wire, a treatment similar to girdling, has positive effects in increasing fruit growth and promoting maturation in the Japanese persimmon (Hasegawa *et al*., 2002). Thus, combining CPPU and strapping to overcome undesirable maturity delay may be a solution and this experiment tested that premise.

Final fruit size is affected by cell number at anthesis, the length of the cell division period after anthesis, and the extent of cell enlargement (Coombe, 1976). Generally, fruit

development is dependent on the interactions of five major classes of plant hormones (auxin, GAs, cytokinin, ABA, and ethylene) (Zhang *et al*., 2007) and endogenous cytokinins regulate cell division (D'Agostino and Kieber, 1999; Haberer and Kieber, 2002). Indeed, Riou-Khamlichi *et al*. (1999) have demonstrated that cytokinins activate cell division in *Arabidopsis* through the induction of *CycD3*. CPPU, a synthetic cytokinin, has been shown to affect cell division (Shargal *et al.*, 2006), cell enlargement (Patterson *et al*., 1993) or both (Yu *et al*., 2001; Antognozzi *et al*., 1996). Although CPPU application significantly increases the fruit size in Japanese persimmon (Hasegawa *et al*., 1991; Itai *et al*., 1995; Sugiyama and Yamaki, 1995), it is unknown whether CPPU effects are direct on division and/or enlargement, or indirect through interactions with other plant growth regulators or their metabolism.

The aim of this study was to evaluate the effects of CPPU application, with and without strapping, on the cell division and enlargement of fruit in 'Hiratanenashi' Japanese persimmon.

Materials and Methods

Plant materials and fruit growth

In 2005, experiments were conducted on two 45-year-old 'Hiratanenashi' Japanese persimmon (*Diospyros kaki* Thunb.) trees growing at the farm of the Faculty of Agriculture, Kochi University (lat. 33°55′N, long. 133°68′E). For each treatment, 20 three-year-old

branches were randomly labeled and flower buds were thinned to 1 per shoot prior to anthesis in both the cultivars. All flowers were under conditions of open pollination. After post-bloom small-fruit drop ceased, hand-thinning was performed at the same level for all-treatments.

At full bloom, 10 flowers were sampled and the diameter of the ovaries was measured. Fruit growth on the trees was monitored from 2 weeks after full bloom (WAFB) until harvest, by measuring the diameter of the fruits on all the labeled branches.

Application of CPPU and strapping treatment

The treatments were as follows 1) no treatment (untreated control, open-pollinated), 2) CPPU（Fulmet; Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan）application alone, 3) strapping alone, and 4) CPPU plus strapping. The fruitlets (ovary and sepals) were sprayed with 10 mg L^{-1} CPPU (approximately 1 mL) using a hand-held sprayer at 10 DAFB. Full bloom occurred on 10 May in ‘Hiratanenashi’. The timing and rate of CPPU applications were based on a previous report by Hasegawa *et al*. (1991). Using pliers, each experimental branch was bound with a vinyl chloride-coated steel wire, 2 mm in diameter. The wire was then twisted at 5 cm above the knot of the binding such that the diameter of the binding wire was halved (approximately 1 mm). Strapping was performed two weeks before the anticipated full bloom. The wire was removed two months after the treatment, and germicide (0.07% methylthiofanate; Topsin M water-dispersible powder, Hokko Chemical

Industry Co., Ltd., Japan) was sprayed on the wound.

Measurement of the cell size and cell number

Every fortnight, five fruits from each cultivar from full bloom to harvest were sampled. After sampling, fruit size was determined by measuring fresh mass, fruit diameter, and fruit length. Slices of mesocarp and epidermis 1.5 cm in width were excised laterally along the equatorial plane and longitudinally on each sampling date. Tissues samples were vacuum-infiltrated with formalin-acetic acid (FAA; formalin: 70% ethanol: acetic acid = 5: 90: 5 (v/v/v)) preservative. Small fruitlets sampled before 4 WAFB were dehydrated in a graded alcohol series, embedded in paraffin, and transversely sectioned into 10-μm sections on a rotary microtome. The sections were stained with 0.05% (w/v) toluidine blue, and permanent mounts were prepared. The fruits sampled after 6 WAFB were removed from FAA, rinsed in distilled water, and sectioned by hand.

All the sections were observed under a light microscope (Olympus DX-50; Tokyo, Japan), and digital images were continuously captured from the pith to the epidermis using a digital microscope camera (Olympus DP-12; Tokyo, Japan). The images were recorded on a personal computer, and size of 100 cells each for parenchyma and tannin cells of the outer and inner walls of the pericarp per sampled fruit determined by measuring the lengths of the long and short axes using the computer software Scion Image (Scion Corporation, Fredrick, USA). The size of each cell was estimated using the mean value obtained for 100 cells per

fruit. From these measurements a mean cell size was determined and cell number was estimated as the number of cell layers that was calculated by dividing the fruit long diameter by the mean length of the long axis of the cells, according to the modified method of Yamaguchi *et al*. (2002).

The period of cell division was estimated based on the change in cell layer number and allometric growth of parenchyma cell length and fruit diameter as an exponential function: $y = bx^{\alpha}$, where y is the independent variable; x, the dependent variable; b, the intercept; and α, the growth coefficient. Linear regressions were performed on log-transformed data. The inflexion point of the growth curve was determined from the point at which the two lines intersected. The period of cell division was estimated to be the time from full bloom to the inflexion point.

Results and Discussion

Effects of CPPU application with and without strapping on the fruit size

Strapping did not affect the sizes of flower and fruitlet at full bloom (Table 1). All treatments increased mature fruit diameter compared with that observed in the control (Table 2). There was no significant difference between control and CPPU for fruit diameter at 22 WAFB (Fig. 1A) and the CPPU treated-fruit remained immature. Application of CPPU alone prolonged the growth period, which was the mean value from full bloom until harvest, by approximately 10 days as compared to the control (Table 3). The matured fruit was

greater in CPPU than control. Although strapping shortened the fruit growth period by approximately 5 days compared to the control, the mature fruit diameter following strapping was larger than that of the control. CPPU application plus strapping prolonged the fruit growth period by approximately 5 days compared to the control, and the mature fruit diameter was significantly larger than both the control and strapping alone (Table 3).

CPPU significantly prolonged the growth period by delaying fruit coloration, resulting in larger fruit size at harvest than the control, as reported in Japanese persimmon (Hasegawa *et al*., 1991; Itai *et al*., 1995; Sugiyama and Yamaki, 1995). In contrast, strapping promoted the fruit coloration and increased fruit diameter in 'Hiratanenashi', which was concordant with the results obtained by Hasegawa *et al*. (1991). CPPU application plus strapping exhibited synergic effects on the fruit growth; in addition, the delay in maturation by CPPU was reduced by strapping.

It has been suggested that CPPU affects the rate and/or the duration of cell division because cytokinin is closely involved with cell division. However, it is unknown whether CPPU affects cell division and cell enlargement in Japanese persimmon. In this experiment, we evaluated the histological factors underlying the increase in the mature fruit diameter caused by CPPU with and without strapping.

Effects of CPPU application with and without strapping on the enlargement of parenchyma and tannin cells

The rate of increase in the length of the parenchyma cells reflected the changes in fruit diameter (Fig. 1B). The final parenchyma cell size in CPPU application plus strapping was the largest, followed by that of CPPU alone, strapping alone, and the control in that order (Table 2). At full bloom, there was no difference in the parenchyma cell size among the treatments (Table 1), indicating that the differences in the final cell size were due to an increase in the rate and/or the duration of the parenchyma cell growth.

The tannin cell size was not affected by CPPU and strapping (Table 1 and 2).

These results show that strapping promoted the rate of parenchyma cell growth; CPPU with and without strapping not only extended the growth period of the parenchyma cells but also promoted the rate of the cell growth.

Effects of CPPU application with and without strapping on cell number and cell division

The seasonal changes in the number of cell layers were almost similar among all treatments (Fig. 1C). The number of cell layers increased until 6 WAFB in 'Hiratanenashi', followed by being almost constant until harvest. CPPU tended to increase the number of cell layers in the mature fruit compared to the control although not significantly (Table 2). The change in the number of cell layers (Fig. 1C) and allometric growth (Table 3) revealed that the duration of cell division was almost similar among the treatments, which was between 26 and 33 days. This result was supported by that of Hirata and Hayashi (1978), who reported that the period of cell division in 'Hiratanenashi' fruit was 14 and 28 DAFB in the inner and

outer walls of the mesocarp tissue, respectively.

Cytokinins were one of the most important key factors influencing the processes in plant development and linked to virtually all the stages of the cell cycle (D'Agostino and Kieber, 1999). Li *et al*. (2003) reported that CPPU promotes the expression of *CycD3* genes during the growth of parthenocarpic fruits, and the regulation of the expression of the *CycD3* gene appears to be a key mechanism by which the cytokinins influenced cell proliferation and fruit development in *Lagenaria leucantha*.

Shargal *et al*. (2006) demonstrated that CPPU and *N*-phenyl-*N*′-1,2,3-thiadiazol-5-ylurea (TDZ) affected the cell number by extending the phase of cell division in pears. Yu *et al*. (2001) reported that CPPU induced parthenocarpic fruit growth by directly reactivating cell division and cell expansion in *Lagenaria leucantha*. Patterson *et al*. (1993) reported that CPPU stimulated cell expansion in the pericarp sufficiently to explain the measured increases in total fruit volumes. In this study, the increases in fruit diameter caused by CPPU were closely involved with the increases in length of parenchyma cell, but not cell division in 'Hiratanenashi' Japanese persimmon.

The carbohydrates that accumulate in the canopy by girdling serve as a rich source of energy for all the stages of reproductive development, namely, flowering, fruit set, fruit enlargement, and ripening (Goren *et al*., 2005). Similarly, Hasegawa *et al*. (2002) reported that strapping, which is a form of girdling, increased the amount of carbohydrates in the shoots and fruits. Strapping would increase the available assimilates for fruits, resulting in

promotion of fruit growth and cell expansion.

Likewise, Antognozzi *et al*. (1996) demonstrated that CPPU modified the carbohydrate metabolism of the fruit, increasing the soluble sugars and starch content throughout fruit growth. The increase in parenchyma cell size caused by CPPU is most likely due to the change of the carbohydrate metabolism of the fruit. We have expected that CPPU application within the period of cell division would affect the cell number of the mesocarp; however, this did not appear to be the situation.

In conclusion, CPPU application with and without strapping increased the fruit weight at maturation in 'Hiratanenashi' Japanese persimmon. Histological studies suggested that strapping would promote the rate of parenchyma cell expansion and CPPU application would prolong the duration of cell enlargement and promote the rate of the cell expansion.

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Figure legend:

Figure 1. The seasonal changes in the fruit diameter (A), parenchyma cell length (B), and the number of cell layers (C) in 'Hiratanenashi' fruit. Vertical bars represent S.E. (N = 5).

Figure 2. Cross sections of mesocarp tissue in 'Hiratanenashi' fruit at ripening. A: Control, B: CPPU, C: strapping, D: CPPU + strapping. Bars represent 200μm. TC, tannin cells.

Table 1 Effects of strapping on flower and fruitlet size, number of cell layers, and the area of parenchyma cell at full bloom in 'Hiratanenashi' Japanese persimmon.

Table 2 Effects of CPPU, strapping and CPPU + strapping on fruit size, number of cell layers, and the area of parenchyma and tannin cells of mature fruits mesocarp in 'Hiratanenashi' Japanese persimmon.

Table 3 Effects of CPPU, strapping and CPPU + strapping on the periods of fruit growth and

cell division in ‘Hiratanenashi’ Japanese persimmon.

Table 1 Effects of strapping on flower and fruitlet size, number of cell layers, and the area of parenchyma cell at full bloom in ‘Hiratanenashi’ Japanese persimmon.

Treatment	Fresh mass (mg)		Ovary diameter (mm)	Ovary length (mm)	Number of cell layers[z]	Parenchyma cell area (μm^2)	Tannin cell area (μm^2)
	Flower	Fruitlet					
Control	1,366	845	6.5	9.5	181	676	909
Strapping	1,339	759	6.3	9.3	183	641	801
Significant	NS[y]	NS	NS	NS	NS	NS	NS

Data are the means of five replicates.

[z] Number of cell layers = fruit diameter / parenchyma cell diameter.

[y] NS: not significant

Table 2 Effects of CPPU, strapping and CPPU + strapping on fruit size, number of cell layers, and the area of parenchyma and tannin cells of mature fruits mesocarp in ‘Hiratanenashi’ Japanese persimmon.

Treatment	Fresh mass (g)	Fruit diameter (mm)	Fruit length（mm)	Number of cell layers[z]	Parenchyma cell area (μm^2)	Tannin cell area (μm^2)
Control	160.5 a[y]	72.6 a	49.9 a	292 a	25,779 a	22,341 a
CPPU	180.7 b	76.6 b	51.5 ab	304 a	35,010 bc	26,691 a
Strapping	171.5 ab	75.4 b	50.4 ab	292 a	32,541 b	26,569 a
CPPU + strapping	184.3 b	76.2 b	51.6 b	302 a	37,349 c	27,456 a

Data are the means of five replicates.

[z] Number of cell layers = fruit diameter / parenchyma cell diameter.

[y] Means followed by the same letter in each column were not significantly different by Tukey-Kramer’s multiple range test ($P < 0.05$).

Table 3 Effects of CPPU, strapping and CPPU + strapping on the periods of fruit growth and cell division in 'Hiratanenashi' Japanese persimmon.

Treatment	Fruit growth period (day)[z]		Cell division period (day)[y]
Control	161.8	b	31.8
CPPU	171.5	d	32.7
Strapping	156.5	a	26.4
CPPU + strapping	166.8	c	29.1

[z] Average number of days from full bloom (10 May) until harvest.

[y] The period of cell division is estimated allometric growth of parenchyma cell length and fruit diameter as two exponential functions: contol ($y = 8.29x^{0.797}$, $y = 6.59x^{0.868}$,25.8 mm-fruit diameter), CPPU ($y = 8.86x^{0.778}$, $y = 7.09x^{0.846}$,26.7 mm), strapping ($y = 8.01x^{0.818}$, $y = 6.05x^{0.897}$, 34.3 mm), and CPPU + strapping ($y = 8.42x^{0.792}$, $y = 6.05x^{0.894}$, 25.4 mm).

[x] Means followed by the same letter in each column were not significantly different by Tukey-Kramer's multiple range test ($P < 0.05$).

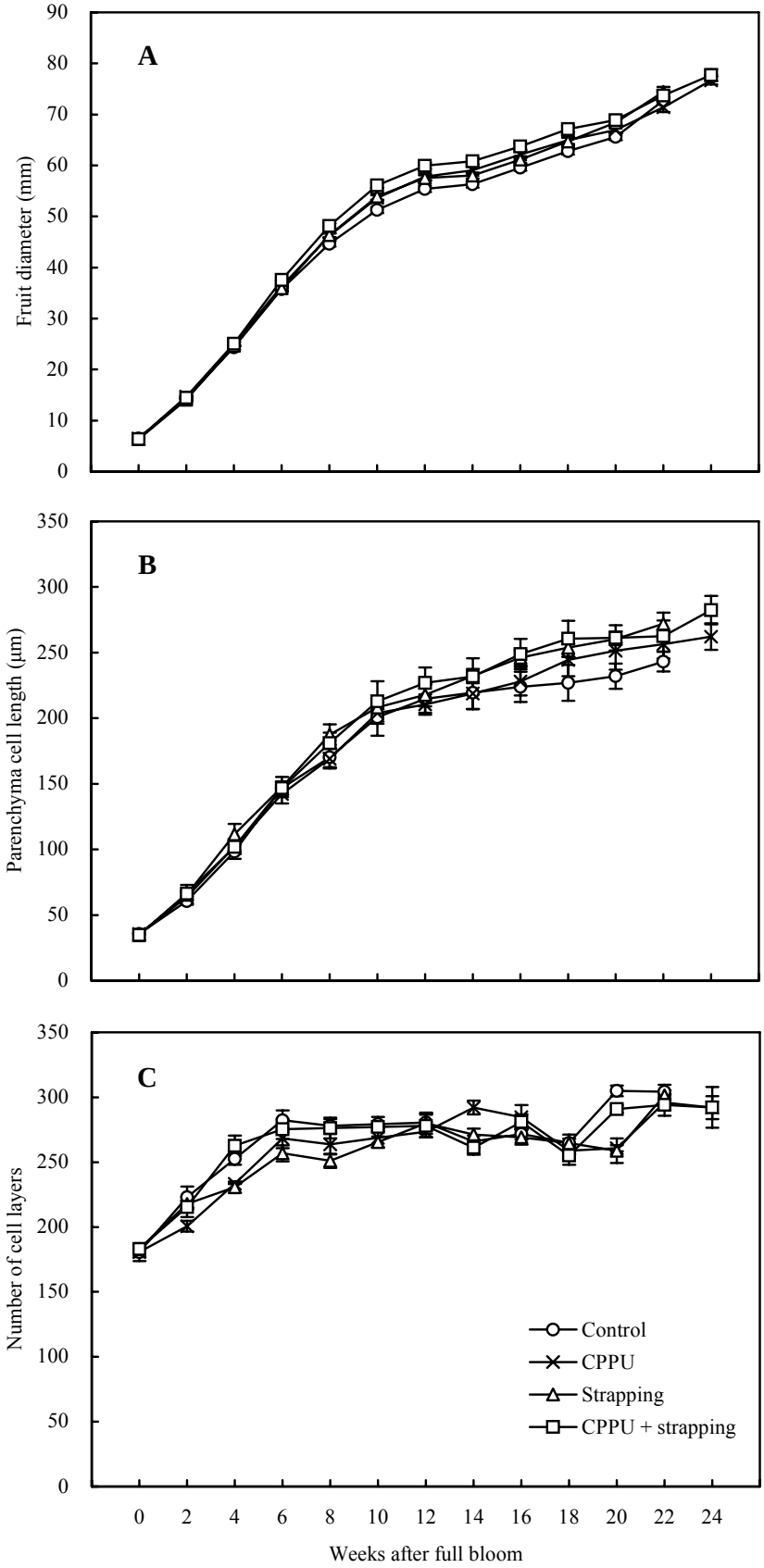

Figure 1. The seasonal changes in the fruit diameter (A), parenchyma cell length (B), and the number of cell layers (C) in 'Hiratanenashi' fruit. Vertical bars represent S.E. (N = 5).

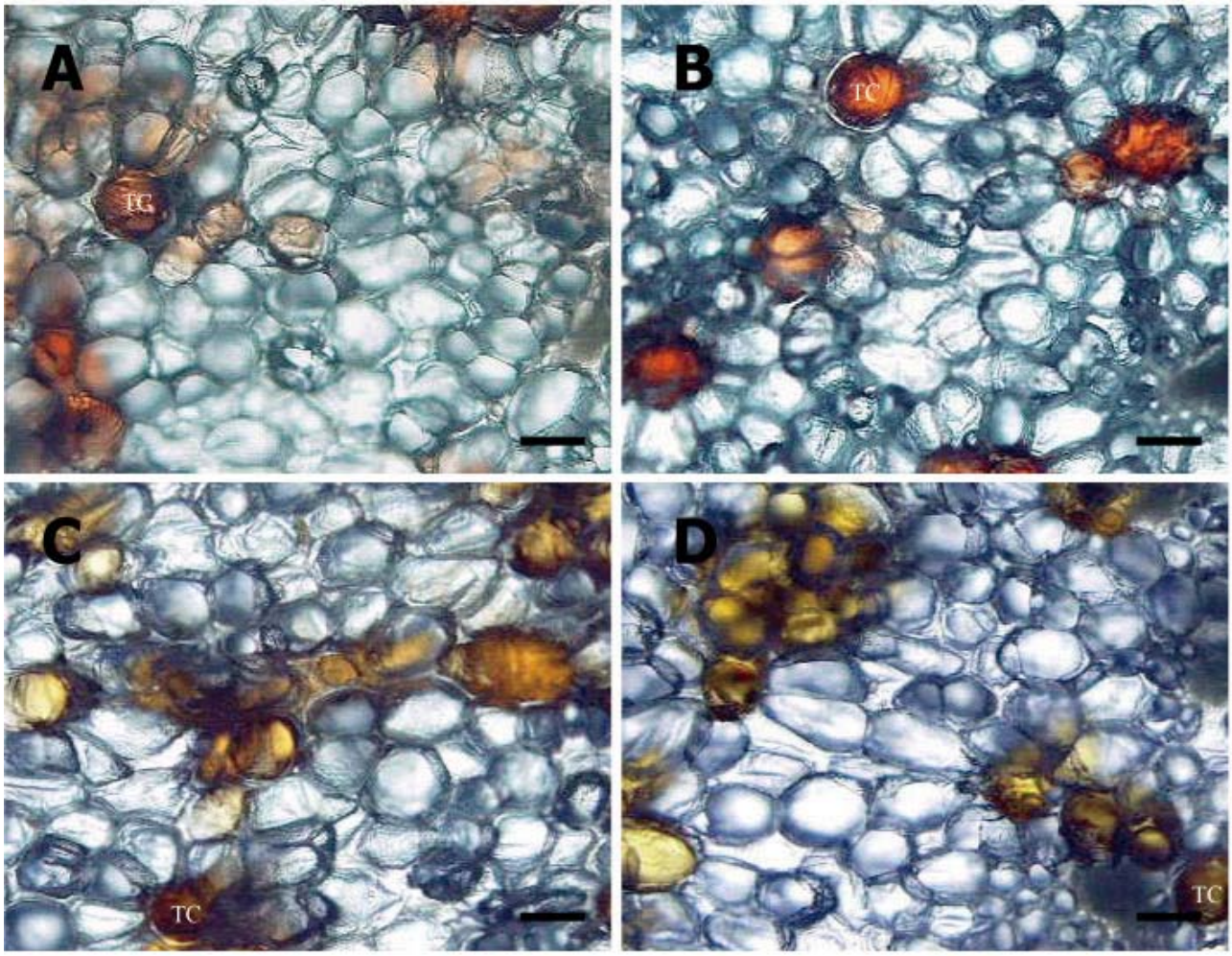

Figure 2. Cross sections of mesocarp tissue in 'Hiratanenashi' fruit at ripening. A: Control, B: CPPU, C: strapping, D: CPPU +strapping. Bars represent 200μm. TC, tannin cells.