INTRODUCTION

During fruit ripening, a various chemical and physical changes occur such as sugar accumulation, fruit softening, decline of organic acid and pigment accumulation. Peach fruits accumulate sugars during fruit maturation, predominantly sucrose while glucose and fructose decrease as fruit ripen (Falchi et al., 2013). Peach also accumulates anthocyanin, a red pigment in fruit skin during ripening (Jiao, Ma, Shen, Yan, & Yu, 2014; Rumainum, Worarad, Yamaki, & Yamane, 2016). It is suggested that sugar accumulation is involved in anthocyanin synthesis.

As well as sugars, plant hormones also play important roles in fruit ripening. Auxin has been reported to regulate fruit ripening by declining in non-climacteric fruit (Given, Venis, & Gierson, 1988) and increasing in climacteric fruit (Ohmiya, 2000) during ripening. It has been suggested that increasing endogenous auxin level prior to maturity regulates ethylene production, and ethylene triggers ripening-associated events (Tadiello et al., 2016). There are several studies concerning anthocyanin accumulation on the peach skin (Jiao, Ma, Shen, Yan, & Yu, 2014; Tuan et al., 2015), however, only few studied about the effect of sugars and plant hormones particularly in flesh of peach.

Recently, polyphenols have gained popularity as functional components of food (Celik, Ozgen, Sere, & Kay, 2008; Manganaris, Goulas, Vicente, & Terry, 2014). Peach contains various nutritional...
compounds such as phenolic compounds and vitamin C that have been considered as antioxidants (Cantín, Moreno, & Gogorcena, 2009). Flesh color could be used as antioxidant parameter by which dark color relates to high polyphenolic content (Fikrinda, Susanto, Efendi, & Melati, 2015). Therefore, it is necessary to understand the mechanism of the accumulation of polyphenolic compounds in the fruit flesh. ‘Tenshin Suimitsuto’ (‘Tianjinshuimi’ in Chinese) is a peach cultivar that has distinctive flesh color, reddish purple, and contains remarkable amount of anthocyanin compared to other cultivars (Yu et al., 2013; Zhang et al., 2008). This cultivar has a potential as an antioxidant source (Rumainum, Worarad, Yamaki, & Yamane, 2016).

2,3,5-triiodobenzoic acid (TIBA) has been reported to prevent auxin efflux inhibit so thus inhibit auxin transport (Christie & Leopold, 1965). In cucumber, TIBA increased endogenous IAA level when it was treated with peduncle of parthenocarpic fruit by inhibition of polar auxin transport (Hikosaka & Sugiyama, 2015). In zucchini, TIBA induced fruit set and early fruit development by inhibition of ethylene production (Martínez et al., 2013). Carvalho, Quecini, & Peres (2010) found out that a promotion effect on anthocyanin accumulation by TIBA in tomato hypocotyls possibly via suppression of endogenous auxin level. In peach, TIBA enhanced flavonoid accumulation on the skin of bagged fruit (Rumainum, Worarad, Yamaki, & Yamane, 2016). Experiment with detached fruit is more complex since there are many environmental and developmental factors involved. Therefore, in this experiment mesocarp discs were used and incubated under controlled light and temperature condition to identify the effects of sucrose, auxin and TIBA on anthocyanin, flavonoid and phenol accumulation.

The objective of this study was to identify the effects of sucrose and auxin on anthocyanin, flavonoid and phenolic accumulation in the mesocarp of peach fruits.

MATERIALS AND METHODS

The experiments were conducted in Laboratory of Horticulture, Utsunomiya University during 2013, 2014 and 2015 growth seasons.

Experiment 1) Effects of Sucrose and NAA on Anthocyanin Accumulation

To examine the effect of sucrose and auxin on anthocyanin accumulation, mesocarp disc were applied to sucrose and 1-naphtalene acetic acid (NAA).

Preparation and Incubation of Mesocarp Discs

Fruits of white-fleshed cultivar ‘Ikeda’ and red-fleshed ‘Tenshin Suimitsuto’ that have entered stage III (maturation stage) were used in this experiment in 2014 and 2015 growing season, respectively. Fruits were sterilized in 5% NaOCl solution containing 50 mL^-1 of Tween20 and washed with distilled water. Tissue cylinders were excised using a cork-borer of 13 mm in diameter. The peel was removed and each tissue cylinder was cut approximately 3 mm in thickness to make discs.

Mesocarp discs were put over Murashige-Skoog medium (pH 5.8) solidified with 1% agar containing various concentrations of sucrose (100 and 200 mM) and NAA (1 and 10 µM), and their combination (suc 100 mM + NAA 1 µM, suc 100 mM + NAA 10 µM, suc 200 mM + NAA 1 µM, suc 200 mM + NAA 10 µM). In the control and the auxin treatments, sucrose was not supplemented. Discs were then incubated at 25°C, under 16/8 h (light/dark cycle; light intensity 121 µmol·s^-1) for 4 (‘Ikeda’) or 6 (‘Tenshin Suimitsuto’) days. After incubation discs were collected for anthocyanin assay.

Measurement of Anthocyanin

The extraction of anthocyanin was conducted following the method described by Ohmiya (2000) with slight modifications. One gram of fruit skin or flesh was ground with a mortar and pestle, immersed in methanol containing 1% (v/v) HCl at 4 °C for 12 h, and then centrifuged at 6,000 x g before being filtered through filter paper (No. 2, Whatman). The total anthocyanin content in the supernatant was determined by measuring the absorbance at 528 nm using a spectrophotometer (U-2001, Hitachi, Tokyo, Japan), and was expressed as µg of cyanidin 3-glucoside per g FW.

Experiment 2) Effects of TIBA on Anthocyanin, Flavonoid and Phenolic Accumulation

Preparation and Incubation of Mesocarp Discs

Fruits that had entered stage III (maturation stage) of white-fleshed cultivar ‘Akatsuki’ and red-fleshed ‘Tenshin Suimitsuto’ were used in this experiment in 2014 and 2015 growing seasons, respectively. The mesocarp discs were prepared as described in the first experiment. Mesocarp discs were put in MS medium (pH 5.8) containing auxin (10 µM) only or auxin (10 µM) and TIBA (50, 100 and 200 µM), and TIBA (200 µM) only (in ‘Tenshin Suimitsuto’). Sucrose was not supplemented in all
medium. Discs were incubated at 25°C, under 16/8 h light/dark cycle for 4 days. Discs were then collected for anthocyanin, flavonoid and phenolic assay.

**Measurement of Total Anthocyanin, Flavonoid and Phenolic**

Tissue discs (approximately one gram) were extracted as described previously in methanol containing 1% HCl for 12 hours at 4°C. Methanolic solution was used to determine the total of anthocyanin, flavonoid and phenolic. The total anthocyanin was measured as described above and expressed as µg of cyanidin 3-glucoside per g FW.

The total flavonoid content was measured following the method described by Cantín, Moreno, & Gogorcena (2009). Briefly, 0.3 mL of 5 % sodium nitrite was added to 1 mL of methanolic extract diluted with water (1:2), and vortexed. After 5 minutes, 0.3 mL of 10 % aluminum chloride was added, and mixed for 1 minute. Then, 2 mL of 1 N sodium hydroxide was added and mixed by vortexing for 30 seconds before measurement. The absorbance at 510 nm was recorded, and the total flavonoid concentration was expressed as mg of catechin equivalents per g FW.

The measurement of total phenolic concentration was conducted using the Folin–Ciocalteu method as described by Cantín, Moreno, & Gogorcena (2009). A drop of 0.5 mL Folin–Ciocalteu reagent was added to 0.5 mL of methanol extract diluted in distilled water. After 3 minutes, 1 mL of 1 N sodium carbonate was added. The solution was mixed for 15 seconds, and allowed to stand for 1 h at 25°C. The absorbance was measured at 725 nm. The total phenolic concentration was expressed as mg of gallic acid equivalents per g FW.

**Statistical Analysis**

All data were subjected to the Tukey–Kramer’s multiple comparison tests or the Student’s t-test to evaluate the significance of the data.

Remarks: S100 = Sucrose 100 mM; S200 = 200 mM; N1 = NAA 1 µM; N10 = 10 µM

**Fig. 1.** Pigmentation of mesocarp discs in ‘Ikeda’ (A) and ‘Tenshin Suimitsuto’ (B) after 4 and 6 days of incubation, respectively.
RESULTS AND DISCUSSION

Experiment 1) Effects of Sucrose and Auxin on Anthocyanin Accumulation

Mesocarp discs were incubated in MS medium containing various concentrations of sucrose and NAA and their combination. Fig. 1 shows mesocarp discs of ‘Ikeda’ (A) and ‘Tenshin Suimitsuto’ (B) after incubation for 4 and 6 days, respectively. In Ikeda, red coloration was observed in treatments of 100 mM sucrose, both NAA treatments and the combination of 100 mM sucrose and 10 µM NAA. Anthocyanin concentration was 57.8 µg g⁻¹ FW on the media with 10 µM NAA, significantly ($P< 0.1$) higher than the control which was 18.1 µg g⁻¹ FW (Fig. 2A).

![Graph A](image1)

![Graph B](image2)

Remarks: S100 = Sucrose 100 mM. S200 = 200 mM, N1 = NAA 1 µM, N10 = 10 µM. * indicates a significant difference at $P< 0.1$ by Student’s $t$-test. ** indicates a significant difference at $P< 0.05$ by Student’s $t$-test. Vertical bars indicate SE (n=3)

**Fig. 2.** Effects of sucrose, NAA and combination of sucrose and NAA on anthocyanin accumulation in ‘Akatsuki’ (A) and ‘Tenshin Suimitsuto’ (B).
In 'Tenshin Suimitsuto' mesocarp discs, anthocyanin concentration was 84.9 µg g⁻¹ FW in treatment of sucrose 100 mM, significantly (P< 0.05) higher than the control (48.8 µg g⁻¹ FW). Treatment with NAA did not significantly increase anthocyanin accumulation. In contrast, NAA 1 µM significantly decreased anthocyanin content, i.e., 30.8 µg g⁻¹ FW. Combination treatments had no significant influence on anthocyanin accumulation.

Sugars have been reported to be involved in the synthesis of plant pigments including anthocyanin. Zheng et al. (2009) proved that sugars induced anthocyanin production directly and not via induction of osmotic pressure stress. In grape berries, treatment with sugars, i.e., fructose, glucose and sucrose increased anthocyanin accumulation (Dai et al., 2014). They also reported that the anthocyanin accumulation depends on sugars concentration. In this study, 'Tenshin Suimitsuto' showed a higher concentration of anthocyanin in the 100 mM sucrose treatment, suggesting that sucrose induced the synthesis of anthocyanin in red-fleshed peach fruit. However, a high concentration of sucrose (200 mM) did not significantly increase anthocyanin, it is probably due to the limited precursor of anthocyanin.

In this study, synthetic auxin (i.e., NAA) at high concentration (i.e., 10 µM) increased anthocyanin accumulation in red-fleshed 'Tenshin Suimitsuto'. To examine whether sucrose and auxin together may regulate anthocyanin production, mesocarp discs were treated with sucrose combined with auxin. However, the combination treatment of sucrose and auxin showed no marked influence in both white- and red-fleshed cultivars. Anthocyanin concentration was higher in 'Tenshin Suimitsuto', indicating that red-fleshed cultivar might be more responsive to induction by factors such as sugars and plant hormone. Or else, it may be possible that maturity of 'Tenshin Suimitsuto' flesh might have been sufficient for pigmentation since anthocyanin accumulation in fruit flesh occurs at mature stage (Rumainum, 2016), while 'Ikeda' did not accumulate anthocyanin in the flesh during ripening.

Experiment 2) Effects of TIBA on Anthocyanin, Flavonoid and Phenolic Accumulation

Pigmentation of mesocarp discs is shown in Fig. 3. Treatment with auxin showed clear red pigmentation in both cultivars. Anthocyanin accumulation was also seen in the control and the combination treatment of 10 µM NAA and 50 µM TIBA in both cultivars. However, combination treatment of NAA with high concentration of TIBA (100 µM and 200 µM) showed less coloration.

Remarks: S100 = Sucrose 100 mM; S200 = 200 mM; N1 = NAA 1 µM; N10 = 10 µM

Fig. 3. Pigmentation of mesocarp disc of 'Akatsuki' (A) and 'Tenshin Suimitsuto' (B) after 4 days of incubation.
Instead, mesocarp discs of NAA 10+TIBA 200 treatment had brown color. ‘Tenshin Suimitsuto’ discs treated with TIBA (200 µM) alone showed no pigmentation by anthocyanin (Fig. 3B).

In ‘Akatsuki’, anthocyanin concentration was 31.5 µg g⁻¹ FW in the control, 57.7 µg g⁻¹ FW in the single auxin treatment, and 34.6 µg g⁻¹ FW in the NAA 10+TIBA 50 treatment (Fig. 4A). An addition of TIBA in a high concentration reduced anthocyanin accumulation. Anthocyanin accumulation was negligibly detected in NAA 10+TIBA 100 and was not detected in NAA 10+TIBA 200 treatment. In ‘Tenshin Suimitsuto’, anthocyanin concentration was 37.5 µg g⁻¹ FW and 33.2 µg g⁻¹ FW in control and auxin only treatment, respectively (Fig. 4B). Treatment with TIBA at higher concentration significantly decreased anthocyanin level. Anthocyanin accumulation was not detected in the single TIBA treatment.

![Graph A](image1.png)

![Graph B](image2.png)

Remarks: S100 = Sucrose 100 mM; S200 = 200 mM; N1 = NAA 1 µM; N10 = 10 µM. Different letters indicate a significant difference at P<0.05 by Tukey-Kramer's multiple comparison test. Vertical bars indicate SE (n=3)

**Fig. 4.** Effect of NAA and combination of NAA and TIBA on anthocyanin accumulation in ‘Akatsuki’ (A) and ‘Tenshin Suimitsuto’ (B).
There was no significant effect of NAA and TIBA treatments in regard to flavonoid accumulation of ‘Akatsuki’ mesocarp discs (Fig. 5A). Single NAA treatment showed similar amount of flavonoid with the control, 419.6 µg g⁻¹ FW and 413.9 µg g⁻¹ FW, respectively. On the other hand, in ‘Tenshin Suimitsuto’, it tended to be high in single NAA treatment which was 1926.8 µg g⁻¹ FW compared to the control (1587.0 µg g⁻¹ FW) (Fig. 5B). An addition of TIBA significantly reduced flavonoid concentration.

**Fig. 5.** Effects of NAA and combination of NAA and TIBA on flavonoid accumulation in ‘Akatsuki’ (A) and ‘Tenshin Suimitsuto’ (B).
Total phenolic is shown in Fig. 6. Similar to flavonoid, the single NAA treatment had no effect on phenolic accumulation. The concentration was 292.7 µg g⁻¹ FW, not significantly different from the control, i.e., 289.8 µg g⁻¹ FW (Fig. 6A). At high concentrations of TIBA (100 µM and 200µM), phenolic content significantly decreased to 94.4 µg g⁻¹ FW and 63.5 µg g⁻¹ FW, respectively. In mesocarp discs of ‘Tenshin Suimitsuto’, similarly, phenolic concentration of NAA only and NAA combined with 50 µM TIBA were not significantly different in comparison with control (Fig. 6B). It was 893.0 µg g⁻¹ FW in NAA only, 654.0µg g⁻¹ FW in NAA 10+TIBA 50, and 718.3 µg g⁻¹ FW in the control.

Remarks: S100 = Sucrose 100 mM; S200 = 200 mM; N1 = NAA 1 µM; N10 = 10 µM. Different letters indicate significant difference at $P<0.05$ by Tukey-Kramer’s multiple comparison test. Vertical bars indicate SE (n=3)

Fig. 6. Effects of NAA and combination of NAA and TIBA on phenol accumulation in ‘Akatsuki’ (A) and ‘Tenshin Suimitsuto’ (B).
The accumulation of anthocyanin was promoted by auxin and significantly reduced by TIBA (Fig. 5), indicating that auxin is a regulator in anthocyanin synthesis of mesocarp of both cultivars. TIBA significantly reduced anthocyanin production. TIBA was reported to inhibit auxin transport (Christie & Leopold, 1965; Hikosaka & Sugiyama, 2015), it might block the transport of auxin in the mesocarp discs. Treatment with TIBA only in ‘Tenshin Suimitsuto’ completely suppressed the accumulation of anthocyanin, supporting Ohmiya (2000) that auxin is required in anthocyanin synthesis. Anthocyanin also accumulated in the control mesocarp discs, it seemed injury by cutting the mesocarp and also light treatment during incubation induced the accumulation. Anthocyanin accumulation was also observed in ‘Akatsuki’ mesocarp discs (Ohmiya, 2000).

Flavonoid concentration was significantly decreased by TIBA treatment in ‘Tenshin Suimitsuto’ but not in ‘Akatsuki’. In contrast, phenolic concentration decreased significantly by high concentration of TIBA in both cultivars. This experiment clearly showed that the regulation of the accumulation of anthocyanin and other phenylpropanoid pathway products by auxin as shown by Lewis et al. (2011) in mesocarp of white- and red-fleshed peaches in vivo. These two different genotype fruits showed similar pattern regarding to auxin and TIBA treatment, although ‘Tenshin Suimitsuto’ contained higher content of flavonoids and phenolics. This suggests that auxin-induced anthocyanin synthesis, at least in vivo, is similar in white- and red-fleshed genotypes. Moreover, TIBA markedly inhibited not only anthocyanin but also flavonoid and phenolic accumulation, suggesting effect on upstream pathway and that TIBA might directly down regulate flavonoid synthesis.

**CONCLUSION AND SUGGESTION**

Treatment by sucrose induced anthocyanin accumulation in ‘Tenshin Suimitsuto’, suggesting that anthocyanin synthesis may be induced by sucrose in peach fruit. Application of TIBA significantly reduced anthocyanin accumulation, suggesting that auxin is required in the synthesis of anthocyanin. Furthermore, TIBA at high concentration inhibited flavonoid and phenolic accumulation, suggesting that it may directly downregulate flavonoid and phenolic synthesis. Fruit developmental stage may be important in further studies to identify roles of auxin.

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