Allelopathic activities of three carotenoids, neoxanthin, crocin and β-carotene, assayed using protoplast co-culture method with digital image analysis

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Abstract Allelopathic activities of three carotenoids of a natural pigment group, neoxanthin, crocin and β-carotene, were assayed by the protoplast co-culture method with digital image analysis (DIA-PP method). Effects on three different growth stages of lettuce protoplasts, i.e., cell wall formation, cell division, and yellow pigment accumulation, were investigated using 96-well culture plates. Cell division was inhibited 65–95% by all three carotenoids at 33–100 µM. Inhibition of cell division stage was stronger than at the cell wall formation stage in neoxanthin, and the water-soluble carotenoid, crocin, whose yellow pigment was incorporated into the vacuole of lettuce protoplasts. Neoxanthin at 33 µM and crocin at higher than 100 µM inhibited more than 100% of the yellow pigment accumulation. By contrast, at low concentrations (0.01–1 µM) β-carotene stimulated growth at the cell division stage. At high concentrations of β-carotene (100–500 µM), inhibition was prominent at all three stages, and also in neighboring wells of zero control, which suggested emission of a volatile compound by β-carotene. They were compared with the report of the volatile compound, tulipalin A. Differences in patterns of inhibition of carotenoids on lettuce protoplast growth were compared with reports of another natural pigment, anthocyanin, and anthocyanin-containing red callus cultured in the light, and with that of neoxanthin-containing yellow callus cultured in the dark.

Key words: allelopathy, carotenoid, in vitro bioassay, protoplast co-culture.

Introduction

Allelopathy is a strategy of plants to survive by production of allelochemicals to inhibit the growth of neighboring plants that share the same habitat. A broad definition of allelopathy includes stimulation effects on insects, microorganisms and animals (Fujii 2000; Rice 1984).

In vitro bioassay methods for allelopathy were used for surveying many plant species using lettuce seedlings as a recipient, such as the sandwich method (Fujii et al. 2004), which measure the activity of dried leaves; the plant box method, which measure the activity of intact root of small seedlings of test plant (Fujii et al. 2007).

A new bioassay method for allelopathy, protoplast co-culture method, was developed to elucidate the underlying cellular mechanisms of allelopathy and to predict the future environmental risks in the field (Sasamoto et al. 2013). Using the same protoplast co-culture method, activities of allelopathic plants and their putative allelochemicals were investigated, e.g., L-DOPA in Mucuna pruriens and M. gigantea (Mori et al. 2015; Sasamoto et al. 2013); trigonelline and nucleotide metabolites (Sasamoto and Ashihara 2014); three Sonneratia mangrove species of different salt tolerance (Hasegawa et al. 2014); mimosine in Leucaena leucocephala (Mori et al. 2015); rotenone in a mangrove Derris indica (Inoue et al. 2015); caffeine and metabolites (Sasamoto et al. 2015); coumarin and abscisic acid in Prunus species (Fujise et al. 2018). Cotyledon protoplasts of lettuce were mainly used as a recipient, though differences were found with the recipient plant species, e.g., lettuce, rice (Sasamoto et al. 2013) and Prunus (Fujise et al. 2018).

The protoplast co-culture method with digital image analysis (DIA-PP method) was further developed using lettuce protoplasts as a recipient (Sasamoto et al. 2017a, b). Different effects of protoplasts of test plants and
putative allelochemicals in co-culture were investigated on the three stages of lettuce protoplasts growth, i.e., cell wall formation, cell division, and yellow pigment accumulation stages. Different patterns of inhibition were observed among the three stages, e.g., *Arabidopsis thaliana* (Sasamoto et al. 2017a, b); four bamboo species (Ogita and Sasamoto 2017); canavanine and cyanamide in *Vicia villosa* (Sasamoto et al. 2019). And a natural pigment, anthocyanin, cyanidin-3,5-di-O-diglucoside (cyanin), was found as the allelochemical from its content in red callus of a mangrove *Sonneratia ovata* (Sasamoto et al. 2019). Water-soluble carotenoids, crocins, in stigma and corolla, to saffron synthesis, localization and transfer in the *Crocus sativus* plant (Rubio-Moraga et al. 2010) was discussed. Another VOC, water-soluble tulipalin A, found in *Spiraea thunbergii* leaves was also tested using the DIA-PP method (Mardani-Korrani et al. 2020).

Functions of carotenoids in plants have usually been discussed with chlorophyll pigment in the photosynthetic apparatus (Takaichi et al. 2006). However, environmental effects of these compounds in a dark condition has not been studied well. Allelopathic activities of carotenoids have not been directly determined. The reported content of β-carotene was the highest in carotenoid-storing carrot callus cultured in the dark (Oleszkiewicz et al. 2018), though no information on the allelopathic activities of β-carotene was available.

In this study, effects of the three carotenoids, neoxanthin, crocin and β-carotene, were investigated using the in vitro bioassay method of allelopathy, DIA-PP method.

**Materials and methods**

**Materials**

Protoplasts were prepared from cotyledons of *Lactuca sativa* (lettuce) seedlings (Sasamoto et al. 2013, 2019). In brief, lettuce seeds ‘Great Lakes 366’ were wrapped in a Miracloth bag (CALBIOCHEM, Cat; 475855), washed with a neutral detergent and tap water, and sterilized with 1.5% NaClO solution for 15 min and then washed with autoclaved water three times (1 min each time). Seeds were cultured on 0.8% agar medium and allowed to grow in the continuous light condition (60 µmol m⁻² s⁻¹) at 25°C for 6–8 days.

Lettuce cotyledons were cut and treated for 20–24h with 1% each of Cellulase RS and Macerozyme R10 in 0.4 M or 0.6 M mannitol solution at 27°C in the dark. After filtration through 63 or 80 µm pore size nylon mesh, they were washed three times with osmotic solution by centrifugation at 100 g (800 rpm) for 5 min.

**Protoplast culture of lettuce**

As described previously (Sasamoto et al. 2019), 5 µl of each protoplast suspensions in mannitol solution in ten times concentration of 6 to 100×10⁵ ml⁻¹ was put into 50 µl of liquid medium (inside 60 wells of a 96-well plastic culture plate, Falcon No. 3072). The medium was MS (Murashige and Skoog 1962) basal medium containing 1 µM of 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 µM of bezyladene (BA), 3% sucrose and 0.4 or 0.6 M mannitol. The pH was adjusted to 5.8 before autoclaving at 121°C for 20 min. Crocin (crocetin digentiobiosyl ester, No. 17304 Sigma-Aldrich) was diluted in pure water (Milipore Direct Q UV), filter sterilized (Milipore PVDF membrane GV®), and diluted with autoclaved medium. As β-carotene (No. 031-05533 Fujifilm-Wako) and neoxanthin (No. 512-23951 Fujifilm-Wako) are almost insoluble in water, they were dissolved in DMSO (Wako, Japan 99.9%), which was filter sterilized with PTFE membrane (Milipore LG). One microliter of neoxanthin or β-carotene solution of different concentrations in DMSO was applied to make 2% concentration of DMSO in the medium containing 0.4 M mannitol as described for rotenone (Inoue et al. 2015). β-carotene at a high concentration was also dissolved in filter-sterilized ethanol before it was added to the medium; the final ethanol concentration in the medium was 5%. Between each β-carotene-containing well was placed a zero control well prepared as in the test of volatile compounds (Mardani-Korrani et al. 2020). Chemical structures of three carotenoids are shown in the Supplementary Figure S1.

Finally, 100 µl of autoclaved pure water was added in between the wells and the plate was tightly sealed with two layers of Parafilm®. Cultures were kept in the dark at 28°C in a humid incubator (CO₂-incubator without the supply of CO₂ gas, APC-30DR, ASTEC Co., Ltd.). As described previously (Sasamoto and Ashihara 2014), under an inverted microscope (Olympus CK40), numbers of non-spherically enlarged protoplasts, and divided protoplasts were counted after 4 and 5 days of culture, and numbers of divided protoplasts including colonies composed of more than four cells were counted after 8 and 12 days of culture. The percentage of control without carotenoids was calculated at each lettuce protoplast density, and the percentages of control were averaged with standard error (SE) at different cell densities of lettuce (6×10¹ to 10⁵ ml⁻¹).

**Digital image analysis**

Procedures were the same as previously described for co-culture with protoplasts of *Arabidopsis thaliana* (Sasamoto et al. 2017a, b). Image analysis of yellow pigment accumulation
of lettuce protoplasts (DIA-PP method) was performed as described previously (Ogita and Sasamoto 2017, Sasamoto et al. 2017a, b, 2018, 2019). Digital image of a 96-well culture plate was captured using a scanner (Epson GTX-970) after 21 or 28 days of culture. The image analysis was performed by Image J software (NIH, Rasband, 1997–2016). An image of the blue channel (jpg file) was selected. A horizontal straight line was drawn at the center of wells. The plot profile of the line was then analyzed. The data of the blue plot values were saved as an excel file. In excel software, the average of “blue plot values” were determined for each well. The yellow value was converted by deduction of each averaged blue value from the highest blue value (control well without lettuce nor carotenoid). The yellow values at each concentration of carotenoids were subtracted from yellow values of both lettuce- and carotenoid-containing wells. The percentage of yellow value of the control without carotenoids, was calculated at each lettuce protoplast density. Finally, the percentages of control without carotenoids were averaged with SE at different cell densities of lettuce ($6 \times 10^3$ to $10^5$ ml$^{-1}$).

### Results and discussion

**Neoxanthin**

The effects of neoxanthin on the three stages of lettuce protoplast growth were shown in Figure 1 and Supplementary Table S1. The inhibitory effect of neoxanthin was more distinct at the cell division stage and less at the cell wall formation stage. At 1–10 $\mu$M, inhibition on yellow pigment accumulation was in between of inhibition on cell wall formation and on cell division. While it was strongly inhibited (142%) at the highest concentration tested (33 $\mu$M), at which 70% inhibition of cell division was observed.

These values of neoxanthin were different and inhibition was stronger than those of another natural pigment, anthocyanin, cyanin, which was identified as an allelochemical in the red callus of *Sonneratia ovata* cultured in the light condition. 60% inhibition of cell division at 100 $\mu$M, and no inhibition on the yellow pigment accumulation were observed at up to 500 $\mu$M of cyanin. Inhibition patterns of three growth stages were the same in both cyanin and the protoplasts of red *S. ovata* callus. In which, 60% inhibition of cell division at $2.5 \times 10^5$ ml$^{-1}$ and 87% inhibition at $10^5$ ml$^{-1}$ was observed. The content of cyanin in the red callus was ca. 1 mM of fresh weight (Sasamoto et al. 2018).

In the neoxanthin-containing yellow callus of *Avicennia alba* cultured in the dark (Sasamoto et al. 2020), 72% inhibition of cell division was observed at $2.5 \times 10^4$ ml$^{-1}$ and 98% inhibition of cell division was obtained at $10^4$ ml$^{-1}$. These values were stronger than above cyanin-accumulating callus of *S. ovata*. Effect of neoxanthin on cell wall formation stage was less inhibitory than on cell division stage, and inhibition on yellow pigment accumulation was in between of the two stages up to $10^4$ ml$^{-1}$ (Figure 1). Similar tendency was observed up to $10^4$ ml$^{-1}$ in the yellow callus of *A. alba* (Sasamoto et al. 2020). In the electron microscope images of yellow callus, specific electron dense particles and crystalloids were found in plastids and some electron dense aggregates were observed in vacuole of yellow *A. alba* callus (Sasamoto et al. 2020), which were similar to the carotenoid-accumulating calluses of carrot cultured in the dark (Oleszkiewicz et al. 2018) and engineered citrus (Cao et al. 2012). Calculated content of neoxanthin in the yellow *A. alba* callus was ca. 4 $\mu$M (4 nmol g$^{-1}$ fresh weight) (Sasamoto et al. 2020). Taken together, neoxanthin was proven to be the allelochemical of the yellow *A. alba* callus cultured in the dark.

Biosynthetic pathway of a growth retardant plant hormone, abscisic acid (ABA) is related to 9′-cis-neoxanthin in chloroplasts (Takaichi et al. 2006). However, the content of ABA was too low to explain the inhibition of protoplast growth of *Prunus* suspension cells themselves by ABA (Fujise et al. 2018), and to explain the inhibition by *A. alba* cells cultured in the dark (Hasegawa et al. 2011; Sasamoto et al. 2020). The stimulation of growth at up to $10\mu$M of ABA was observed in the recipient lettuce protoplast culture (Sasamoto et al. 2013). The inhibitory effect of neoxanthin might not be directly related to cis-ABA synthesis.

**Crocin**

The effects of crocin (crocetin digentiobiosyl ester) on the three stages of lettuce protoplast growth are shown in Figure 2 and Supplementary Table S2. The inhibitory effect of crocin was more distinct at the cell division stage and less at the cell wall formation stage as with...
neoxanthin (Figure 1). At 100 µM, 74% inhibition, and at 1 mM, 100% inhibitions of cell division were observed. Inhibition on the yellow pigment accumulation was similar to the inhibition on other two stages up to 10 µM. However, at higher concentrations than 100 µM, very strong inhibition on the yellow pigment accumulation stage was observed (226% inhibition at 1 mM). Such values of more than 100% inhibition were shown with the 33 µM of neoxanthin (Figure 1, Supplementary Table S1).

In the digital image analysis of co-culture using a 96-well culture plate, high concentrations of chemicals or high protoplast densities of test plants affected the yellow values. The yellow values of each concentration of each chemical or test plant protoplasts without lettuce protoplasts are deduced as a control. Although the yellow colour can be distinguished under an inverted microscope in both non-spherically enlarged and divided cells and colonies of lettuce protoplasts (Sasamoto et al. 2017a), the yellow pigment accumulation can be quantified only after three weeks of culture (Ogita and Sasamoto 2017). A carotenoid was spectroscopically identified from the hexane extract of yellow lettuce protoplasts culture (Sasamoto et al. 2017b). As shown in Figure 3b, yellow pigment of crocin was incorporated into the vacuole of lettuce protoplasts in co-culture, which might reduce the yellow values of co-cultured wells, while yellow color of crocin without lettuce protoplasts remained after 28 days at higher than 100 µM. Dim yellow color was also observed at 33 µM of neoxanthin without lettuce after 28 days. Inhibition of carotenoid synthesis, and degradation of exogenously supplied carotenoids by co-cultured lettuce protoplasts might have occurred. Therefore, more than 100% inhibition on the yellow pigment accumulation was obtained by high concentrations of neoxanthin and crocin, and was also reported in a bamboo, Sasa kurilensis protoplasts, but not in other three bamboo protoplasts, using DIA-PP method (Ogita and Sasamoto 2017).

Inhibitory effects of water-soluble carotenoid, crocin (Figure 2) were stronger than of a VOC, water-insoluble safranal, which showed 50–70% inhibition at the three stages at the concentration of 1 mM. Safranal is biosynthesized in C. sativus plant, as is crocin. Another VOC, water-soluble tulipalin A showed more than 90% inhibition at 100 µM. In these two VOCs, inhibition of cell wall formation was prominent than of cell division (Mardani-Korrani et al. 2020). This might reflect the difference of cellular action site of each chemical. Effects of the two VOCs on the neighboring wells of zero control were described in the next β-carotene paragraphs.

**β-carotene**

Figure 4 shows the effects of β-carotene dissolved in DMSO on three stages of lettuce protoplast growth. β-Carotene at low concentrations (0.01–1 µM) stimulated...
the colony formation after 12 day of culture. At 8 days of culture, at which 2% was colonies at zero control, similar stimulation of cell division was observed (data not shown). Cell wall formation was not inhibited at these concentrations. Inhibition of cell division, 65% (12 day) and 77% (8 day) were observed at 37 μM, while after 4 day of culture, 84% of cell wall formation was inhibited. The patterns of β-carotene at three growth stages of lettuce were very different from those of neoxanthin (Figure 1) and crocin (Figure 2), which showed a gradual decrease of cell division and cell wall formation depending concentrations. Stimulation patterns at each growth stage at low concentrations of chemicals or low protoplast densities of allelopathic plants have been reported in several materials, e.g., trigonelline (Sasamoto and Ashihara 2014); caffeine metabolites and coffee callus (Ogita and Sasamoto 2018; Sasamoto et al. 2015); bamboo species (Ogita and Sasamoto 2017); cinnamic acid and Spiraea species (Suzuki et al. 2018). Physiological and ecological roles of trigonelline and caffeine metabolism in plants were summarized (Ashihara et al. 2020).

Further experiments were conducted using high concentrations of β-carotene dissolved in ethanol. The final concentration of ethanol in the medium was 5%. As shown in Figure 5, strong 95% inhibition of cell wall formation and cell divisions at 100 μM, and complete inhibition at 250 and 500 μM, were observed. Inhibition of yellow pigment accumulation by β-carotene was 84% (100 μM), 89% (250 μM), 110% (500 μM), respectively.

Inhibition on the neighboring wells of zero control without β-carotene was also investigated. In the neighboring wells of 500 μM, inhibition of cell divisions up to 54% was observed (Figure 5). Inhibition on yellow pigment accumulation was less, but inhibition on lettuce protoplast growth was prominent at all three stages. Emission of a volatile compound by the high concentrations of β-carotene was strongly suggested. Such strong inhibition on the neighboring wells of zero control was not observed with 1 mM of crocin. As for the VOCs tested using the DIA-PP method (Mardani-Korrani et al. 2020), no inhibition by the 1 mM safranal on the neighboring wells was seen. While the inhibition by 100 μM of tulipalin A on the neighboring wells of zero control at the three stages was strong (24%, 49% and 9%, respectively).

In the agricultural field, a mutual beneficial relation is known between carrot and lettuce (Fujii 2000). Very recently, monoterpene lactone, (−)-loliolide, which is a metabolite of β-carotene, (Murata et al. 2019), was involved in barnyardgrass-induced rice allelopathy at rhizosphere environment (Li et al. 2020). Further study is needed on VOC(s) emitted by the lettuce protoplasts treated with high concentrations of β-carotene. Application of the DIA-PP method on the β-carotene-containing carrot callus cultured in the dark (Oleszkiewicz et al. 2018) might contribute to clarify the cellular mechanism(s) of allelopathy of β-carotene.

More than 750 carotenoids are known (Takaichi et al. 2006). The three carotenoids investigated in this paper, neoxanthin, crocin and β-carotene, whose chemical structures are shown in Supplementary Figure S1, belong to the three classes of carotenoids with different chemical structures, i.e., xanthophylls, apocarotenoids, and carotenes, respectively. Further comparative study using the DIA-PP method on other members of each class of carotenoids might also contribute to clarify a relationship between differences of patterns of allelopathic activities and the chemical structures of carotenoids.

Conclusion

This is the first report that showed the allelopathic activities of the three carotenoids, neoxanthin, crocin, β-carotene, using the protoplast co-culture method with digital image analysis (DIA-PP method).

All three carotenoids showed 65–95% inhibition of cell division at 33–100 μM. The three carotenoids all inhibited the yellow pigment accumulation of lettuce protoplasts at high concentrations.

Inhibition at the cell division stage was stronger than at the cell wall formation stage in neoxanthin and crocin, which yellow color was incorporated into the vacuole of lettuce protoplasts. The yellow pigment accumulation was inhibited more than 100% by neoxanthin at 33 μM and crocin at higher than 100 μM.

At low concentrations of β-carotene (0.01–1 μM), cell division of lettuce protoplasts was stimulated.
At high concentrations of β-carotene (100–500 µM), growth of lettuce protoplasts was strongly inhibited at all three stages. A VOC might be emitted and growth of lettuce protoplasts was inhibited in neighboring wells of zero control. They were compared with the report of the VOC, tulipalin A.

Different patterns of inhibition on the three growth stages of lettuce protoplasts were observed among the three carotenoids. They were compared with reports of another natural pigment, anthocyanin, and anthocyanin-containing red callus of S. ovata cultured in the light, and with that of neoxanthin-containing yellow callus of A. alba cultured in the dark.

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