Application of the protoplast co-culture method for evaluation of allelopathic activities of volatile compounds, safranal and tulipalin A

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The effects of two plant volatile compounds (VOCs), safranal and tulipalin A were investigated using the protoplast method with digital image analysis, which was developed to investigate allelopathic activities of plants at the cellular level in a 50 μL liquid medium. Both VOCs showed inhibition on lettuce protoplasts growth at all three stages, i.e., the cell wall formation stage, the cell division stage, and the yellow colour accumulation stage. Among the three stages of protoplast growth, differences of inhibition patterns were observed. Inhibitions at the cell wall formation stage were stronger than those at the latter two stages in both VOCs. Tulipalin A showed higher inhibitory activity (total inhibition at 100 μM) than that of safranal (44% inhibition at 1 mM) at cell division stage. They were also compared with the direct exposure method in an enclosed vial using germinated lettuce seedlings, which was developed to investigate allelopathic activities of VOCs at plant level. Tulipalin A showed less inhibitory activity on radicle growth of lettuce, than that of safranal reported. Inhibition on hypocotyl growth was stronger than that on radicle in both VOCs. The causes of the differences between two VOCs and two bioassay methods, and the application of the protoplast method, were discussed for clarifying the contribution of VOCs as allelochemicals at cellular level.© 2020 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Allelopathy is a survival strategy of plants by releasing allelochemicals into the surrounding environment to inhibit the growth of neighbouring plants that share the same habitat. Nevertheless, a broad definition of allelopathy includes also stimulation effects on insects, microorganisms and animals [1,2].

Protoplast co-culture method was first developed to investigate the allelopathic activities of test plant protoplasts of an herbaceous leguminous plant on the growth of recipient plant protoplasts, using a 50 μL liquid medium in a well of 96-well culture plate. Similar results with the in vitro seedlings growth test and field tests have been obtained [3]. Understanding the mechanism(s) of action of allelochemicals at the cellular level is important when introducing as a new bioactive compound identified from allelopathic plants to reduce future environmental risk [4].

The protoplast method was applied to know the effects of pyridine metabolites on cell wall formation and cell divisions of lettuce protoplasts. Also, the role of trigonelline synthesis was found as detoxification of nicotinic acid [5]. Using the same protoplast method among several tested metabolites of purine alkaloids, the inhibitory activity by caffeine was the strongest [6].

There are increasing use of the protoplast method to explore the main allelochemicals of plant species and their underlying cellular mechanism(s) [7]. However, volatile allelochemicals and related metabolites have not been investigated using the protoplast method.

The stigma of saffron (Crocus sativus) could inhibit the growth of lettuce seedling in the dish pack bioassay method using 6-well dish, a method which is developed for volatile allelochemicals [8]. The volatile monoterpene aldehyde, safranal (Fig. 1), was identified from the stigma of saffron and its strong allelopathic activity was tested with the cotton swab method [9]. The cotton swab method (a direct exposure method with GC–MS), was developed to measures the strong inhibition of allelochemicals on radicle growth of lettuce seedling in enclosed vials. However, the protoplast method have not been applied on the VOC.

Using the dish pack method, strong allelopathic activities of leaves of Spiraea thunbergii were tested and the VOC, tulipalin A (α-Methylene-γ-butyrolactone) (Fig. 1), was identified from the leaves of the plant using GC–MS [10]. However, such a VOC has neither been evaluated as an allelochemical of S. thunbergii nor its effects at cellular level has been clarified. Although, tulipalin A has been known as a volatile bioactive insecticidal compound [11], its derivatives have been known for their strong potential of fungicidal activity [12].

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In this report, the two volatile compounds (VOCs), safranal and tulipalin A, were investigated for their allelopathic activities at cellular level using the protoplast method. Their effects on cell wall formation and cell division stages of lettuce protoplast growth were examined. Also, digital image analysis (DIA-PP method) was applied to know the effects on the yellow carotenoid accumulation stage during growth of lettuce protoplasts [7,13–15].

2. Materials and method

2.1. Lettuce protoplast isolation and purification

Protoplasts were prepared from cotyledons of *Lactuca sativa* (lettuce) seedlings [3,7]. In brief, lettuce seeds ‘Great Lakes 366’ were wrapped in a Miracloth bag (CABIOCHEM, Cat:475855), washed with a neutral detergent and tap water, and sterilized with 1.5% NaClO solution at room temperature for 20 min. Then, seeds were cultured in 0.75% agar medium (inside 60 wells of a 96-well plastic culture plate, Falcon 2.0001, Becton Dickinson and Co., USA). As described previously [5], under an inverted microscope (Olympus CK40), numbers of non-spherically enlarged protoplasts (4 days in 0.4 M mannitol- and 5 days in 0.6 M mannitol-containing media) and divided protoplasts including colonies composed of more than four cells of protoplasts (8 days in 0.4 M mannitol- and 12 days in 0.6 M mannitol-containing medium), were counted. The percentage of control without VOCs 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 × 10³/mL to 10⁵/mL). Experiments were repeated once, independently.

2.2. Lettuce protoplast culture

As described previously [7], 5 μL of each protoplast suspension in ten times concentrations of 6 to 100 × 10³/mL was put into 50 mL of liquid medium (inside 60 wells of a 96-well plastic culture plate, Falcon No. 3072). The medium was MS [16] basal medium containing 1 μM of 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 μM of benzyladenine (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. Tulipalin A solution was prepared in pure water (MiliPore Direct Q UV), filter sterilized (Milipore PVDF membrane GVⅢ), and diluted with medium. The final concentrations were 0, 0.01, 0.1, 1, 10, 100, and 1000 μM. One μL of safranal solution of different concentrations in DMSO (Wako, Japan 99.9%) was sterilized with PTFE membrane (Milipore LG) and added to each representative well. Final concentrations were 0, 0.1, 1, 10, 100, 125, 250, 500 and 1000 μM. The final concentration of DMSO in each well was 2%. 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, APS-30DR, ASTEC Co. Ltd.). As described previously [5], under an inverted microscope (Olympus CK40), numbers of non-spherically enlarged protoplasts (4 days in 0.4 M mannitol- and 5 days in 0.6 M mannitol-containing media) and divided protoplasts including colonies composed of more than four cells of protoplasts (8 days in 0.4 M mannitol- and 12 days in 0.6 M mannitol-containing medium), were counted. The percentage of control without VOCs 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 × 10³/mL to 10⁵/mL).

2.3. Digital image analysis of yellow colour accumulation (DIA-PP method)

Image analysis of yellow colour accumulation of lettuce protoplasts (DIA-PP method) was performed as described previously [7,13–15,17]. Digital image of a 96-well culture plate was captured using a scanner (Epson GTX-970) after 27–42 days of culture. The image analysis was performed by Image J software (NIH, [18]). 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). The yellow values were deduced at each concentration of VOCs. The percentage of yellow value of control without VOCs was calculated at each lettuce protoplast density. Finally, the percentages of control were averaged with SE at different cell densities of lettuce (6 × 10³/mL to 10⁵/mL).

2.4. Direct exposure method on lettuce seedling growth

In order to investigate the effect of VOCs on the growth of lettuce seedling, a direct exposure method, which was modified from the cotton swab method [9], was used. Briefly, 4 pre-germinated lettuce seeds (Great Lakes 366, Takii Co., Japan) were placed into 20 mL head-space vial containing 10 mL of solidified agar (0.75%). Then a 7.6 cm double-tipped cotton swab was cut in half and inserted into the center of agar. Standard tulipalin A was dissolved in DMSO and 30 μL of 0.001, 0.01, 1, 10, 20 mM solution was each added to the tip of cotton swab using micro-capillary syringe (Hamilton, 80300). Similar procedure was also repeated for safranal (0.001 to 5 mM). The vials were then immediately sealed by a Teflon pressure cap and placed in an incubator under dark conditions at 25 °C. The length of the radicle and hypocotyl were measured after 3 days of incubation. The experiment was done in three replications. The percentage of extension was calculated in comparison to the radicle and hypocotyl elongation of the control seedlings.

3. Results and discussion

3.1. Effects of safranal and tulipalin A on the three stages of lettuce protoplast growth

The effects of safranal on the three stages of lettuce protoplast growth are shown in Fig. 2A and B. The inhibitory effect of safranal was more distinct at the cell wall formation stage and less at the cell division stage. The yellow colour accumulation was inhibited after 27 days (88%) only at the highest concentration (1 mM) tested. Due to the very low solubility of safranal in water, DMSO was used to prepare different concentrations. Since DMSO inhibits lettuce protoplast growth, the final concentration was adjusted to 2% in the medium containing 0.4 M mannitol as described for rotenone [19]. In the safranal experiment, after 8 day of culture, numbers of divided lettuce protoplasts were counted, but almost no colony formation was observed. No inhibition of safranal on the neighbouring wells was seen at the highest concentration tested. Tulipalin A showed almost complete inhibition at 100 μM on the cell division stage of lettuce protoplasts (Fig. 2C). About 50% inhibition of the cell wall formation was observed at 10 μM. In the control wells without tulipalin A, 92% of protoplasts were non-spherically enlarged after 5 days of culture (cell wall formation stage), and 20% developed colonies after 12 days of culture (cell division stage). As shown in Fig. 2C, inhibition by 0.1–10 μM was stronger at the cell wall formation stage than at the cell division stage. In current literature, this is the first report that shows the effects of tulipalin A on plant cells.
Yellow colour accumulation after 28 days of culture (Fig. 2C and D) was not inhibited up to 10 μM of tulipalin A, but the highest inhibition was observed at 100 μM. Although the yellow colour can be distinguished under an inverted microscope in the both non-spherically enlarged and divided cells and colonies [13], the yellow colour accumulation can be quantified only after three weeks of culture [17]. Data of scanned image did not change after 6 weeks of culture (data not shown). Previously, a carotenoid was spectroscopically identified from the hexane extract of yellow lettuce protoplasts culture [14]. Yellow accumulation might reflect the synthesis and accumulation of carotenoid in lettuce protoplasts. Therefore, inhibitory allelopathic activities can be described as the decrease of yellow values in a well of 96-well culture plate using digital image analysis.

In our initial experiment of tulipalin A, up to 10 mM (data not shown) caused stronger inhibition compared with Fig. 2C data. The zero control (0 μM) which were the neighbouring wells of 10 mM wells were totally inhibited and total inhibition of cell divisions and yellow accumulation were observed in 100 μM wells. The 50% inhibition was observed at 1 μM. By using two separate plates of low and high concentrations and two columns of zero control (Fig. 2C), we confirmed no inhibition on the neighbouring wells up to 10 μM of tulipalin A. However, inhibition by 100 μM of tulipalin A on the neighbouring wells of zero control was 24% (5 day), 49% (12 day), and 9% (28 day), respectively. Such inhibition at high concentrations on the neighbouring wells can be caused by the high volatility of tulipalin A.

Overall, both applied VOCs, safranal and tulipalin A, exerted inhibitory activities at the three growth stages of lettuce protoplasts. However, the inhibition by tulipalin A (Fig. 2C) on protoplasts of lettuce was significantly higher than that of safranal (Fig. 2A).

Less inhibitory activity on the yellow colour accumulation stage than cell wall formation or cell division stages was observed in tulipalin A (Fig. 2C). Similar results have been observed in several non-VOCs allelochemicals such as canavanine [7], and anthocyanin, cyanin [15] and in allelopathic plant protoplasts containing these allelochemicals. However, this phenomenon was not observed in the case of safranal experiment (Fig. 2A). Inhibition on early cell wall formation stage was stronger than on cell division stage. Such a tendency was observed by both safranal and tulipalin A and in the previous study for canavanine, but not for cyanin. These phenomena might reflect in the uptake efficiency of each allelochemical into cells, and/or different cellular mechanism of different allelochemicals.

3.2. Inhibitory activity of safranal and tulipalin A on the growth of lettuce seedlings (direct exposure method)

Inhibitory effect of safranal on the radicle of germinated lettuce have already been reported using the cotton swab method calibrated by GC–MS [9]. The 50% inhibition by safranal was reported at 1.2 μg/L (ppb). In this present paper, similar direct exposure bioassay was applied without GC–MS calibration. Stronger inhibitory effect of safranal was found on the growth of hypocotyl than on radicle. The growth was completely inhibited at 5 mM concentration (Fig. 3). Tulipalin A also showed inhibitory activity on the growth of the germinated seeds of lettuce in direct exposure method (Fig. 3). The effect of tulipalin A was also more

![Fig. 2.](image-url)

*Fig. 2.* Effects of safranal (A and B) and tulipalin A (C and D) on the three stages of lettuce protoplast growth, at cell wall formation (4 or 5 days), cell divisions (8 or 12 days) and yellow colour accumulation (27 or 28 days). The used medium was MS basal medium containing 1 μM 2,4-D and 0.1 μM BA, 3% sucrose, and 0.4 M mannitol (A, B) or 0.6 M mannitol (C, D). B and D are the scanned images of 96-well plates.
noticeable on the hypocotyl in comparison to the radicle of lettuce seedlings. The seedling growth was completely inhibited at 20 mM concentration (Fig. 3). As the inhibition on radicle growth is usually stronger than on hypocotyl in other lettuce seedlings growth test for bioassay of allelopathy [8], the above data of VOCs using the direct exposure method are unique.

3.3. Differences between the inhibitory activity of safranal and tulipalin A

The inhibition by safranal was 4–10 times stronger than that of tulipalin A in direct exposure method. The inverse relationship in the strength of inhibitory activities between direct exposure method and the protoplast method (Figs. 2 and 3) could be due to the differences in the volatility of both compounds. It is possible that lower boiling point of safranal makes it more volatile in the ambient temperature and therefore easier to affect the growth of the seedlings. On the other hand, in the protoplast method, the greater hydrophilic tendency of tulipalin A increases its exposure to the lettuce protoplast in a liquid medium and therefore may increase its inhibitory activity.

Despite these differences, stronger inhibition by tulipalin A was observed (100%) at a concentration much lower than 5 mM in the protoplast method. The possible mode of action of safranal in plants as an enzyme inhibitor in high concentration [20] on plant cells may differ from that of tulipalin A which is due to forming highly reactive bonds with free sulphydryl groups of proteins or glutathione [21,22]. These differences in the mode of action might be another reason behind the results observed in this experiment.

3.4. Application of the protoplast method

Investigations on the effects of the metabolites related to the synthesis and degradation of the VOCs in plant cells will clarify the different aspects of cellular mechanism(s) of allelopathy. The possible metabolic pathways of safranal and carotenoids, crocin(s), synthesis, localization and transfer in the saffron plant have been proposed [23–25]. Currently, a carotenoid “crocin” is under investigation using the protoplast method with digital image analysis [26].

In S. thunbergii leaves, cis-cinnamoyl glucoside has been found as one of the allelochemicals in the plant [27]. Quantitative comparison of the effects of such non-VOCs-allelochemicals and related metabolites with the inhibitory effect of volatile tulipalin A and protoplasts of S. thunbergii leaves [28] will contribute to finding key allelochemical metabolite(s) in S. thunbergii plant.

In conclusion, this is the first report on in vitro bioassay of allelopathic activities of volatile compounds, safranal and tulipalin A using lettuce protoplast method with digital image analysis. Their allelopathic activities were compared with those assayed using direct exposure method.

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