いちご水耕栽培を改善する揮発性香気成分の同定と性質決定

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Characterization and identification of volatile compounds improving the hydroponic technique for strawberry

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Hydroponic systems for vegetable production are nowadays essential to maximize productions and increase yields. However, the technical issues concerning control microbial diseases and pests, and the low level of flavor have not yet been improved. Here we report volatile organic compounds (VOCs) released from companion plants can be used as chemical signals providing better quality for strawberries during the hydroponic culture. First, we identified the chemical stimuli emanating from companion plants with strawberries via spectrometry analyses and found VOCs being responsible for the strawberry growth. The strawberries grown with tomato and lavender as companion plants were significantly higher in growth than non-companion strawberry. Especially, tomato VOCs stimulated strawberry growth. Therefore, the VOC was identified the chemical structure and biological properties.

KEYWORDS: glioblastoma, Aromas, Autophagy.

1. Introduction

Plant VOCs can serve as semiochemicals to protect plants from insect and pathogen attack, attract beneficial animals, and as communication signals within and among plants [1]. Terpenoids and other VOCs emitted from plants in response to stimuli disrupt parasitoids and predators to distinguish between infested and non-infested plants and thus aid in locating hosts or prey [2]. In addition, the companion-planting induced plant volatiles vary quantitatively and qualitatively related to various biotic and abiotic factors and the VOCs are specific to each plant–plant association [3]. It is well-known that a variety of stimuli leads to biochemical changes in plants. Plants respond to a variety of stimuli by activating their defense system [4], but they can also trigger indirect defenses, such as the emission of VOCs [5]. Plants use VOCs to perform a variety of tasks, as different as: indirect plant defense against insects; pollinators attraction; plant-to-plant communication; thermo-tolerance and environmental stress adaptation; defense from predators [3),4)]. In this paper we apply plant-to-plant communication analyses, and in particular hydroponic culture technics to identify beneficial companion plants in plants species network, on the basis of their similarities in terms of VOCs emissions.

The strawberry industry is considered one of the largest fresh fruit industries in Tochigi prefecture with an annual average production of 25,000 tones and a value of 27 billion yen (ca. 27 million USD) in 2018. Strawberry cultivation is considered a high-risk activity, mainly due to the great susceptibility to the attack of diseases and pests, oscillation in the market prices and great requirement of inputs and services. The high costs for implementing and maintaining

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the crop and the demand of the market for better quality products stimulate the search for new cultivation and management alternatives.

Hydroponic is the cultivation of crop without soil environment. It reduces the disease of crops those which are disseminated by the soil and also reduce the rotting of strawberry fruits. Moreover, this system shows the good result in quality of strawberry and other growth parameter. However, not every plant does equally well in hydroponic growing conditions, at least with current technology. As with nutrient content, the flavor of plants depends on the growing conditions. Hydroponic cultivation grows plants in a closed system using water. In the case of plant infections or pests, they can escalate fast to plants on the same nutrient reservoir. Pesticides for the control of pests and diseases are still needed [6].

Plant VOCs can serve as semiochemicals to protect plants from insect and pathogen attack, attract beneficial animals, and as communication signals within and among plants. It is well-known that VOCs lead to biochemical changes in plants. Plants use VOCs to perform a variety of tasks, as different as: indirect plant defense against insects; pollinators attraction; plant-to-plant communication; thermo-tolerance and environmental stress adaptation; defense from predators [7].

The aim of this study was firstly to develop a simple and inexpensive screening method of identifying VOCs that could emit from companion plantings, and secondly to validate this method by investigating the VOC variability of a series of diverse companion plants growing with strawberry. Subsequently, the chemical structure of the VOC involved in the strawberry growth was identified by using FT-IR, ^{13}C NMR, and ^{1}H NMR. In this study, we also identified the VOCs from companion plants stimulating growth of strawberries and enhancing accumulation of plant pigments. Four different companion plants, tomato, sweet pepper, leek, and lavender were tested to determine being responsible for the strawberry growth and VOC emissions. First, the VOCs emitted from the companion plants with and without strawberries were extracted with hexane and analyzed by spectrometer to determine whether that VOCs are attributable to strawberry growth and determine possible VOCs. Subsequently, we investigated the influence of strawberry exposure to VOCs from companion plants upon the growth and disease resistance.

2. Materials and Methods

Plant Material:

Plants used for the study were strawberry (*Fragaria x ananassa cv*. 'Sky Berry') and as companion plants; tomato (*Lycopersicum esculentum Mill. var. cerasiforme*), leek (*Allium schoenoprasum L. var. foliosum* Regel), sweet pepper (*Capsicum annuum L. var. grossum* Sendtn), and lavender (*Lavandula angustifolia*).

Plant Culture:

The plants were cultivated in a hydroponic system, using expanded vermiculite as substrate and were fertirigated with modified Hoagland's solution. The dimensions of the container were 10.0 cm × 10.0 cm × 30.0 cm (L × W × H). Containers were placed inside growth chambers with or without companion plants. Chambers were then placed on growth shelves, and plants were subjected to a 12/12 h (day/night) photoperiod and maintained at 25°C. Each shelf was equipped with 2-LED light tube, generating a photosynthetic flux of 360 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at plant canopy level. The experimental design was a randomized complete block, with three replications.

Plant assessments:

Plants were then divided into roots, crowns, leaves (including petioles), and fruiting structures (flowers and fruit). The number of leaves was counted, and the length of leaves and the height of plants were measured.

Extraction and Isolation of VOCs:

The plant leaves were extracted with methanol and hexane and the spectroscopic properties were measured. For the isolation of VOCs from tomato leaves, hexane extracts were concentrated under reduced pressure at 45°C to obtain the crude extract, which was fractionated over silica gel eluted with hexane to give two subfractions (D1 and D2).

Subfraction D1 was concentrated and rinsed with sodium hydroxide. The hexane layer was subjected to silica gel to give three subfractions (1D1–1D3). Fraction 1D1 was further purified by reversed-phase HPLC, to obtain compound 1.

Structural analysis of VOCs:

All NMR spectra were recorded on JEOL RESONANCE DELTA2 NMR spectrometers (500 and 100MHz for ^{1}H- and ^{13}C-NMR, respectively) in $CDCl_3$. Chemical shifts were reported in ppm referenced to $CHCl_3$ as internal standard (δ 7.26 for proton and δ 77.0 for carbon). IR spectra were recorded on a Thermo Nicolet NEXUS 470 FTIR. Column chromatography (CC) was performed on silica gel 60; the fractions were monitored by Vis-UV spectrometer.

Extraction of VOCs and plant pigments:

The plant leaves were extracted with methanol and hexane and the spectroscopic properties were measured.

Antifungal activity of the extracts from the strawberries and companion plants:

The methanol and hexane extracts were individually tested for inhibition of mycelial growth and conidial formation of *B. cinerea* on PDA. The bioassay was done in closed petri dishes (9cm in diameter) in the presence of *B. cinerea* and the extracts. In the test for inhibition of mycelial growth, a mycelial agar plug removed from the colony margin of a 2-day-old PDA culture of *B. cinerea* was inoculated in the center of petri dish containing 20 ml of PDA. The extracts tested were individually applied onto the plates.

Microscopic observation:

The strawberry leaves grown with or without companion plants were observed under microscopes. The blade surfaces, blade and petiole cross-sections of each culture were observed under light microscope. The scanning electron microscopy (SEM) method was used to examine morphological changes in strawberry surface. All the samples were not conductive; they were coated with 10 mm of gold (sputtering technique) and were examined with SEM (JEOL JSM-6510, Japan).

Preparation of crude enzyme solution:

The strawberry leaves were ground in a mortar with a suitable amount (equal to wet cell wt.) of sea sand in 0. 1 M acetate buffer (pH 5.0), then centrifuged at 12,000 rpm and 0 °C for 30 min. The supernatant was dialyzed overnight against 0.02 M acetate buffer (pH 5.0). The dialyzed solution was designated the crude intracellular enzyme.

Bata-glucosidase activity:

Enzyme activities were measured by the activity against 0.25 % salicin. A 0.05-mL sample of an appropriately diluted enzyme solution was added to 0.05 mL of substrate in 0.1 M acetate buffer (pH 4.0), and the mixture was incubated at 30 °C. The reaction was stopped by the addition of a copper reagent, and the released reducing sugar was measured by the Somogyi- Nelson method.

RNA Extraction and RNA Amplification:

The RNAs from strawberry leaves were extracted using AGPC method. cDNA synthesis and RNA amplification were performed using the RT-PCR kit (Takara). The specific primers used for the RT-PCR. The genome was sequenced by using dideoxy method. Sequencing data was assembled and annotated by using BLAST at Bioinformation and DDBJ Center. Then the sequences were submitted to swiss modeling at SIB Swiss Institute of Bioinformatics to create their three-dimensional structures.

3. Results and Discussion

3. 1 Analysis of variance for strawberry traits under different companion plants

Analysis of variance showed that interaction between companion plants to the leaf length and height of strawberry was significant in 5% level of probability. The longest leaves were obtained from treatment (lavender) with an average of 4.87 cm and the least amount of treatment (leek) with an average of 2.13 cm was obtained (Figure 1). The greatest height of strawberry

was obtained from the tomato treatment with an average 14.94 cm and the shortest was gotten from the leek treatment with an average of 3.13 cm (Figure 1). The VOCs released from tomato and lavender might increase leaf length and plant height of strawberries.

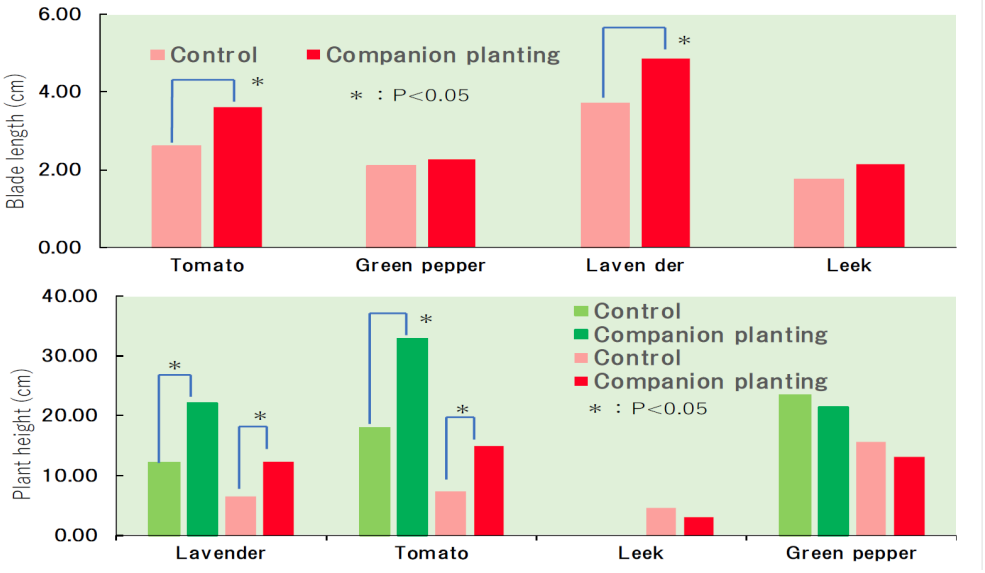

Figure 1 Effects of companion planting on the growth of strawberries and plants companied with. The growth of strawberries (red bars) and companion plants (green bars) was measured. The indexes of the growth are the blade length (top)and the plant height (bottom).

3. 2 Identification of VOCs induced by the companion planting

Hexane extracts from leek, lavender and tomato grown with strawberry absorb strongly around 200 nm. Hexane extracts from green pepper grown with strawberry have a high absorbance at 447 nm. However, the compound having an absorbance at 446 and 474 nm produced by the tomato in control culture were decreased, while the compounds having a absorbance at 328 were present in in the control plants was not detected in lavender grown with strawberry (Figure 2).

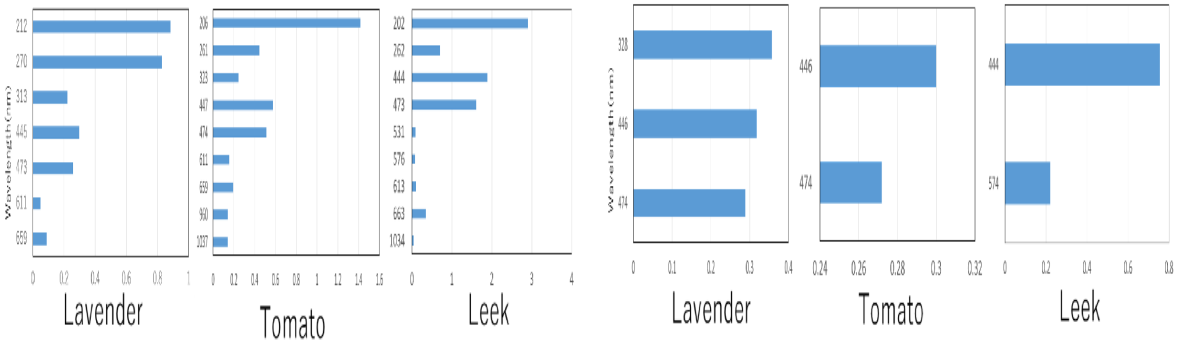

Figure 2 Spectroscopic analysis of plant extracts. Strawberries were grown with (left; companion planting) or without (right; control) lavender, tomato, leek and green pepper. The plants were extracted with hexane and the absorption spectra of each extract were measured.

3. 3 Molecular Structure of the VOC from tomato leaves grown with strawberry

The tomato leaves were extracted with hexane and subjected to silica gel column chromatography. The structure of the

compound was identified on the basis of spectroscopic techniques. The compound was obtained as a colorless oil; UV (CH_3OH), λ max (logε): 209 (3.38) nm; FT-IR cm^{-1}: 2965, 2915, 2854, 1716, 1669, 1439, 1409, 1375, 1356, 1266, 1229, 1156, 1106, 1047, 982, 833, 738. The IR spectrum of compound 1 showed the presence of carbonyl groups (1710 cm^{-1}).

^1H NMR (500 MHz, $CDCl_3$) : 1.59 (s, 6 H, $2CH_3$), 1.61 (s, 6 H, $2CH_3$), 1.39-2. 26(m, 10 H, $5CH_2$), 5.06-5.07 (2, 2H, J = 6.5 Hz, vinyl H), 5.14 (t, J = 6.5 Hz, 1 H, vinyl H); ^{13}C NMR (100 MHz, $CDCl_3$) : 16.0, 16.0, 17.6, 25.7, 26.7, 26.7, 29.9, 39.7, 39.7, 122.5, 124.0, 124.0, 131.2, 135.0, 136.3, 208.9 (Figure 3A).

Therefore, the compound 1 was determined as (5E,9E)-6,10,14-trimethylpentadeca-5,9,13-trien-2-one (Figure 3B)[8].

A

position	$\delta^{13}C$	mult	δ^1H
1	29.9	CH_3	2.13 (s)
2	208.9	C	
3	43.7	CH_2	2.46 (t 7.5)
4	22.4	CH_2	2.26 (m)
5	122.5	CH	5.07 (t 6.5)
6	136.3	C	
7	39.7	CH_2	1.98 (m)
8	26.7	CH_2	2.07(m)
9	124.0	CH	5.14 (t 6.5)
10	135.0	C	
11	39.7	CH_2	2.27,2.03 (m)
12	26.7	CH_2	1.50,1.39 (m)
13	124.0	CH	5.06 (m)
14	131.2	C	
15	25.7	CH_3	16.1 (s)
16	17.6	CH_3	16.1 (s)
17	16.0	CH_3	1.59 (s)
18	16.0	CH_3	1.59 (s)

B

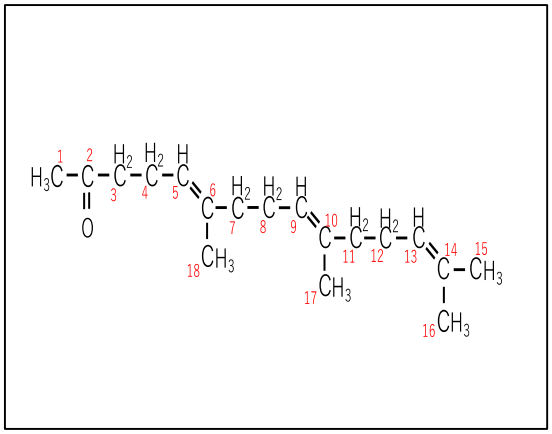

Figure 3 Molecular Structure of the VOC from tomato leaves grown with strawberry. A, ^1H and ^{13}C NMR data for compound 1. B, Structure of Compound 1.

3.4 Identification of pigments induced by the companion planting

Methanol extracts from strawberry grown with lavender and tomato absorb strongly at 436 nm. However, the compound having an absorbance at 1036 nm was decreased There were no significant composition differences between with and without companion plants, but the compounds having absorbances at 800-1060 nm were significantly decreased in strawberries grown with tomato and lavender (Figure 4).

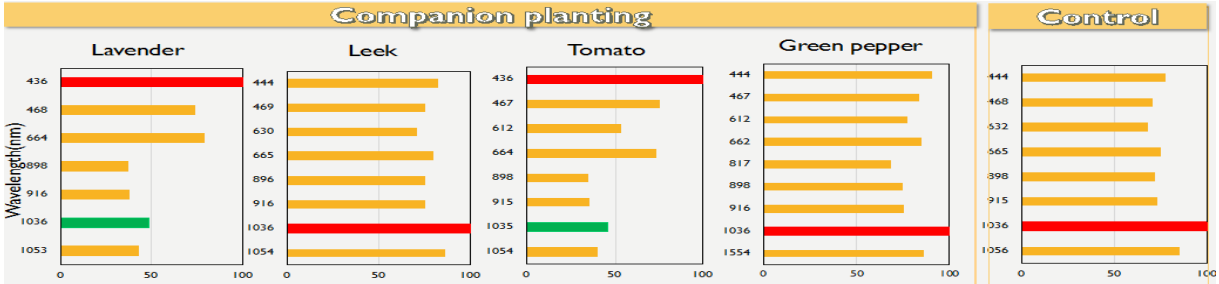

Figure 4 Spectroscopic analysis of plant extracts. Strawberries were grown with (companion planting) or without (control) lavender, tomato, leek and green pepper. The plants were extracted with methanol and the absorption spectra of each extract were measured.

3.5 Antifungal activity of the extracts from the strawberries and companion plants

Bioassay was used to determine the presence of antifungal compounds in different plant extracts (Figure 1). The active compounds against *B. cinerea* were found in the methanol extracts of strawberry leaf grown with tomato and lavender. While no active compound was observed in hexane extract (Table 1).

Table 1 Antifungal activity of the extracts

Hexane extract	Lavender	Tomato	Leek	Strawberry	Green paper
Starting plant	-	-	+-	-	-
Plants (control)	+-	-	+-	+-	+-
Strawberry (companion planting)	-	-	-		-

Methanol extract	Lavender	Tomato	Leek	Strawberry	Green paper
Starting plant	+	+	-	-	+-
Plants (control)	-	+	+	-	-
Strawberry (companion planting)	+	++	-		+-

Antifungal activity: strong ++, mild +, slightly +-, none –

3.6 Microscopic examination of cross sections of plant leaves

Since we observed that mycelial growth was restricted by the extracts from strawberry grown with companion plants, we microscopically examined the strawberry leaves. Light microscopy showed that the cellular content was organized and organelles such as chloroplasts were clearly observed in the strawberry leaf treated by tomato. In the tomato interaction, we also noted pronounced thickness of strawberry leaf tissue at the cuticle layer, and accumulation of purple pigment in the petiole. Since anthocyanin is also one of the major pigment which is responsible for the purple coloration of strawberries, the strawberry treated by tomato might accumulate anthocyanin in vascular tissue (Figure 5A). The scanning electron microscopic (SEM) images revealed that there were two forms of wax crystals on the strawberry leaf surface; regular (rougher) like a spider web structure and irregular (smoother) crystals. In the control, leaf surfaces were covered with regular-shaped wax crystals and formed a less dense network. The size of the wax crystal (irregular-shaped) was thicker and a dense network was observed in plants treated with tomato in comparison to controls. The crystal was deposited in the epicuticle layer when plants were treated with tomato, and this appeared to result in an increase in thickness in the wax crystal under companion planting. Overall, the results clearly showed that the treatment by tomato increased epicuticular wax crystals, displayed morphology changes at the strawberry leaf surface (Figures 5A and 5B).

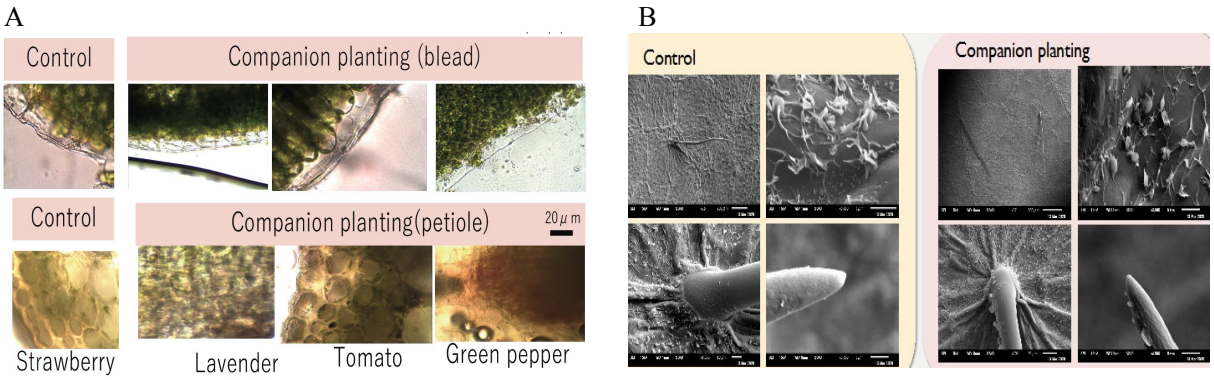

Figure 5 Representative images of strawberry leaves grown with companion plants. A, The blade (top) and petiole (bottom) cross-sections of each culture were observed under microscope. Scale bar, 20 μm. B, The scanning electron microscopic (SEM) images.

3.7 Beta-Glucosidases of strawberry grown with tomato

Beta-Glucosidase (β-d-glucoside glucohydrolase, EC 3.2.1.21) catalyzes the hydrolytic cleavage of β-glycosidic bonds between two glycone residues or between glucose and an alkyl or aryl aglycone. This enzyme is of great importance in physiological and biotechnological processes and it is used for the enzymatic saccharification of cellulosic materials, and the liberation of flavor compounds. This enzyme also helps to release phenolic compounds, pigments, phytoalexins and plant hormones from fruit and vegetable residues. Thus, the activity of β-Glucosidases in the strawberry leaf was measured. The highest activity of β-glucosidase was found in the strawberry grown with tomato, while β-glucosidase activity was low in the strawberry grown with sweet pepper. These results suggest that β-glucosidase induced by tomato could play an important role to release phenolic compounds, pigments, phytoalexins and plant hormones in strawberry (Figure 6A).

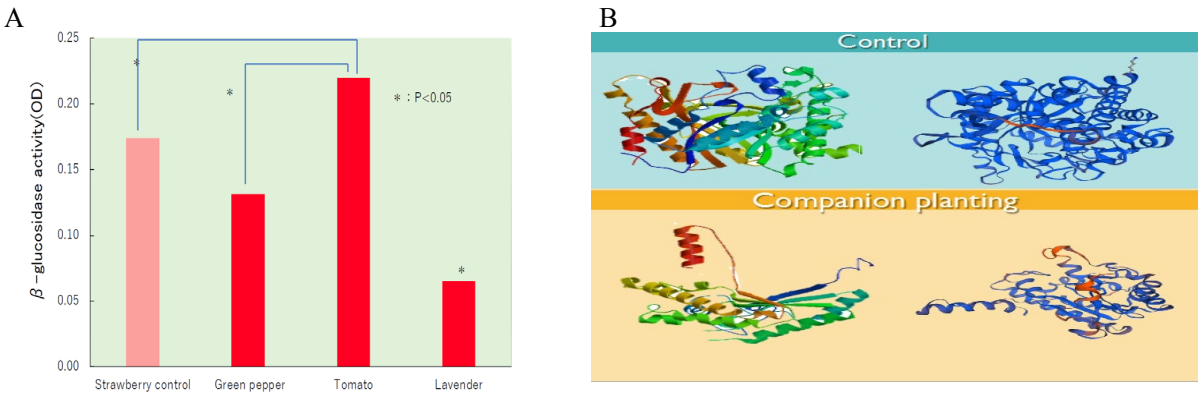

Figure 6 The β-glucosidases of strawberry leaves grown with companion plants. A, The β-glucosidase activities. The results are expressed as the mean. The differences (＊, $p<0.05$) were analyzed by t-test. B, The Structure of the β-glucosidases.

3. 8 Structural analysis of the β-glucosidases

The amino acid sequence deduced from the nucleotide sequence from the control strawberry was 500 residues with a molecular mass of 50 kDa. A data base search with the Blast program showed that it has 99%, 80%, and 80% sequence identity with *Fragaria x ananassa* β-glucosidase 1 (BG1), *Camellia sinensis* β-glucosidase 6 GH1 family (GH1BG6), and *Actinidia deliciosa* β-glucosidase 12 (BG12), respectively.

The amino acid sequence deduced from the nucleotide sequence from the strawberry grown with tomato was 340 residues with a molecular mass of 34 kDa. A data base search with the Blast program showed that it has 100%, 82%, and 80% sequence identity with *Fragaria x ananassa* β-glucosidase 2 (BG2), *Vitis vinifera* clone SS0AEB9YF02, *Hevea brasiliensis latex* cyanogenic β-glucosidase, respectively.

Domain organization, tertiary structure and active site arrangement for the two β-glucosidase were investigated and compared to related structures. The active sites of β-glucosidase from control strawberry (BG1) has a deep active-site pocket. On the other hand, β-glucosidase from strawberry grown with tomato (BG2) has a shallow active-site pocket (Figure 6B).

4. Conclusion

The VOCs from companion plants stimulate growth of strawberries. Hexane extracts having absorbance at 206 nm significantly increased in tomato leaf grown with strawberry. The tomato cultures produce more VOCs in the companion planting indicating that VOCs could play an important role in growing of strawberry. Therefore, the chemical structure of the VOC was identified by using FT-IR and NMR spectroscopy analysis. The VOC from tomato grown with strawberry

was determined as (5E,9E)-6,10,14-trimethylpentadeca-5,9,13-trien-2-one (farnesylacetone). Farnesylacetone provided by the cleavage activity of carotenoids (having absorbance at 350-500nm) in a biosynthetic route for most of the isoprenoids released by tomato [9] is induced by companion planting with strawberry.

The VOCs from companion plants stimulate growth of strawberries and enhance accumulation of plant pigments. Pigments having absorbance at 436 nm significantly increased and the active compounds against *B. cinerea* were accumulated in strawberry leaves grown with tomato and lavender. In the tomato interaction, strawberry leaves were thicker at the cuticle layer, accumulated purple pigment, increased epicuticular wax crystals, and induced the highest β-glucosidase activity. The β-glucosidases have amino acid sequence identity with BG1 (control) and GB2 (companion planting with tomato) from *Fragaria x ananassa*. Since the BG2 has wide open active site, the enzyme could be suitable for a rather large substrate. These results suggest that β-glucosidase induced by tomato could play an important role to release phenolic compounds, pigments, phytoalexins and plant hormones in strawberry fruits.

5. Acknowledgments

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