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Brief Report

In Vitro Hypoxic Environment Enhances Volatile Compound Production in Persian Violet Flowers

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Abstract: Flowers of Persian violet (*Exacum affine* Balf. f. ex Regel) that are grown in nature typically produce a scent. However, whether Persian violet flowers developed inside sterile containers produce odors has yet to be studied. Therefore, this research aimed to study and compare the effects of ex vitro and in vitro environments on the volatile composition of Persian violet flowers. Persian violet flowers obtained from an in vitro culture and potted plants were analyzed for volatile constituents using gas chromatography–mass spectrometry (GC-MS). The main constituent of the volatile compounds in the Persian violet flowers grown in both conditions was alcohol, with 3-hexen-1-ol, which produces a grassy-green odor, being the dominant substance. In addition, the in vitro Persian violet flowers contained the highest amount of ethanol, which produces a wine aroma—followed by the terpene alcohol β-citronellol, which produces a rose scent. However, 3-carene (citrus odor), caryophyllene (floral odor), humulene (woody odor), and β-ionone (floral odor) were detected only in Persian violet flowers grown in natural conditions. Therefore, these results indicate that hypoxia possibly occurred during plantlet growth in the in vitro environment and caused some different volatile compound production from that in natural conditions.

Keywords: *Exacum affine*; in vitro flower; in vivo flower; odor-active compound; plant tissue culture

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1. Introduction

Persian violet (*Exacum affine* Balf. f. ex Regel) is a plant member of Gentianaceae, subfamily Exaceae, and section Africana. About 70 species of the *Exacum*, Persian violet, have only been cultured as indoor or outdoor small potted plants [1–3]. This species is an annual plant with shiny green ovate foliage and small, purple flowers having bright yellow pollen masses in the center [4,5].

Mainly, Persian violet is traded to consumers as a pot plant through florists and greenhouses. Denmark produces approximately 65% of the world's supply, while The Netherlands adds 20%. The common name of Persian violet may lead to the misconception that this ornamental plant only produces purple flowers; however, its cultivars come in various flower colors, but the commercially available ones are primarily purple/blue and white. Traditionally, seed propagation is used for this plant, but the reduction in fertility this causes has become a primary problem. Therefore, vegetative propagation, especially micropropagation, is gaining popularity over conventional methods [2,3,6].

In vitro flowering is an exclusive phenomenon in plant tissue culture technology. This artificial incident shows that the transition from vegetative to reproductive growth can occur in sterile conditions and complete the plant life cycle [7,8]. Many cultured plants can produce attractive in vitro flowers. Therefore, this has resulted in the introduction of plants that bloom in closed culture vessels, for example, Persian violet, which is currently sold on many platforms in Thailand [9–11]. Residences in various urban areas have been transformed into condominiums, often using potted plants to decorate. Persian violet flowers from culture and induced in a sterile container can be used to decorate offices and replace potted plants to decorate houses without requiring watering and fertilizing.

In addition to the shape and colors of Persian violet flowers, their unique floral aroma characteristics are also attractive to humans and, more significantly, influence consumers. The flower fragrance, principally in the petals, arises from a mixture of small volatile molecules (100–250 Da), classified into three major classes, including terpenoids (the largest class), phenylpropanoids/benzenoids (the second-largest class), and fatty acid derivatives [12–15]. Many reports show that floral scents are essential to plant survival and reproductive success. This odor signal predominantly functions to attract insects and animal pollinators. The emission of floral volatile compounds also functions as a repellentand protects against abiotic or biotic stresses and allelopathy [16–18].

The maturity of flowers is often related to fragrance production. For instance, newly opened young snapdragon flowers produce fewer odors than mature flowers, and mature jackfruit inflorescences emit more volatiles than immature blossoms. Moreover, the format of volatile organic compound emission during flowering can be continuous at a constant level or rhythmic with a maximum at daytime or nighttime. Though Persian violet flowers are always open, their fragrance is emitted in a rhythm. Persian violet plants in flower shops or markets have been reported to produce the highest odor at approximately 1 p.m., while the fragrance intensity is lower in the morning and evening [19–21]. The fragrance emission rhythm may involve the pollinator type or pollination time.

So far, the study and comparison of volatile constituent emission from in vitro and in vivo flowers is still rare, especially in Persian violet. Moreover, Persian violets bloom beautifully under sterile conditions; the scent of the flower that arises within the container cannot leak to the outside. It is, therefore, interesting to determine if the volatile components of in vitro Persian violet flowers are the same as that in ex vitro. Thus, the present research aimed to investigate and compare the effects of ex vitro and in vitro environments on the volatile organic compounds emitted from Persian violet flowers obtained from cultured plantlets under aseptic conditions and potted plants.

2. Materials and Methods

2.1. Plant Materials

In vitro clean-cultured plantlets of Persian violet were obtained from the previous work [4]. A shoot tip explant (approximately 10 mm long) was excised and placed on a semi-solid MS basal medium [22]. This medium contained inorganic nutrients, organic nutrients, 3% (*w*/*v*) sucrose, and 0.9% (*w*/*v*) agar. The medium pH was adjusted to 5.7 before being sterilized by a high-pressure steam sterilizer (TOMY autoclave, model ES-315, Seiko, Tokyo, Japan). Then, the container with a shoot tip explant was transferred to a primary growth room and maintained at 25 ± 2 °C under 16 h light (with a PPF of 20.87 µmole m^{-2} s^{-1}) and 8 h dark periods for 4 weeks. After the full-bloom flowers had developed on the in vitro plantlets, they were collected and investigated for volatile components and compared with those from Persian violet potted plants purchased from a local shop (Thanhikorn Orchid Garden, Siam Orchid Center Building) near Chatuchak Market, Bangkok, Thailand. These potted plants grew in a pot (size 15 × 15 × 12 cm) with chopped coconut husks and garden soil (ratio 7:3) for 4 months (after transplant) before the full-bloom flowers were obtained.

2.2. Volatile Organic Compound Analysis

Persian violet flowers obtained from the in vitro culture or potted plants (Figures 1A, S1 and S2) were randomly cut and placed on a Petri dish (Figure 1B). Then, the floral parts were simultaneously separated (Figure 1C) using a scalpel. After that, they were immediately weighed to 2 g and placed into a 10 mL vial. The vial was later capped (Figure 1D) before being placed promptly into a static headspace sampler model 7697A (Agilent, Santa Clara, CA, USA). The headspace condition was 50 °C, and 100 µL of air (headspace) was introduced into the GC column. This headspace sampler was connected to a gas chromatograph–mass spectrometer (GC-MS) (model 7890, Agilent, Santa Clara, CA, USA) consisting of a mass selective detector (model 7000C, Agilent, Santa Clara, CA,

USA) and an HP-INNOWAX 19091N-133 (30 m × 0.25 mm; 0.25 µm film thickness) column. The GC-MS conditions for the analysis were as follows: a carrier gas flow rate of 1 mL/min, an injector temperature of 200 °C, oven temperature programming from 40 to 240 °C with increments of 5 °C/min, an EI source temperature of 230 °C, and an EI voltage of 70 eV. The analyzed data were compared with those in the NIST2011 library. The retention index of each substance was calculated by comparing the chromatogram with the *n*-alkanes (C7–C30) standard with the formula below [23]. In contrast, the relative content was calculated from the internal (thiophene) standard.

$$I^T = 100\left[\frac{t^T_{\mathrm{Ri}} - t^T_{\mathrm{Rz}}}{t^T_{\mathrm{R}(z+1)} - t^T_{\mathrm{Rz}}} + Z\right] \quad (1)$$

where:

I^T = retention index;
t^T_{Ri} = retention time of sample peak;
t^T_{Rz} = retention time of *n*-alkane peak eluting immediately before sample peak;
$t^T_{\mathrm{R}(z+1)}$ = retention time of *n*-alkane peak eluting immediately after sample peak;
Z = carbon number of *n*-alkane peak eluting immediately before sample peak.

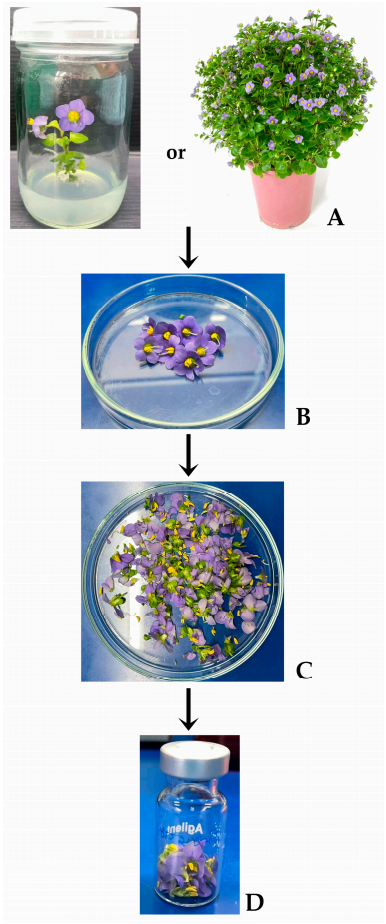

Figure 1. Plant material preparation for volatile organic compounds analysis of in vitro and in vivo Persian violet flowers. (**A**) Persian violet in vitro plantlet (left) or in vivo potted plant (right). (**B**) Persian violet flowers were cut and placed on a Petri dish. (**C**) The separated Persian violet floral parts on a Petri dish. (**D**) The separated Persian violet floral parts in a vial.

2.3. Odor-Active Compounds Determination

To identify the volatile constituents that are the primary odor-active compounds, the analysis of these compounds was performed by determining the odor activity values (OAVs) using the following formula [24]:

$$\text{OAV} = \frac{\text{Concentration of the volatile content}}{\text{Odor threshold value}}$$

Any volatile substance with an OAV of ≥1 indicated that it was a significantly volatile substance among the constituents of the odor. In addition, to examine the odor characteristics of those volatile compounds, data based on the information obtained from the available textbook were compared [25].

3. Results

After the Persian violet shoot tip was cultured in a primary growth room for approximately four weeks, blooming flowers in axenic culture conditions were obtained. The Persian violet in vitro and in vivo flowers were collected at 1 p.m. since it has been reported that this horticultural plant produces the most potent fragrance in the early afternoon [21].

When the flowers from both sources were analyzed for volatile organic compounds, it was found that there were many types of volatile substances. Overall, the volatile constituent emission from Persian violet flowers contained alcohols as the main component (approximately 35–45%), followed by terpenes (approximately 20–32%) and aldehydes (approximately 22–23%), and esters were the least detectable aroma components. In addition, the percentages of various volatile organic compounds derived from in vitro and in vivo Persian violet flowers differed (Figure 2). The in vitro flowers produced alcohols more prominently than the in vivo flowers, but terpene synthesis was considerably lower.

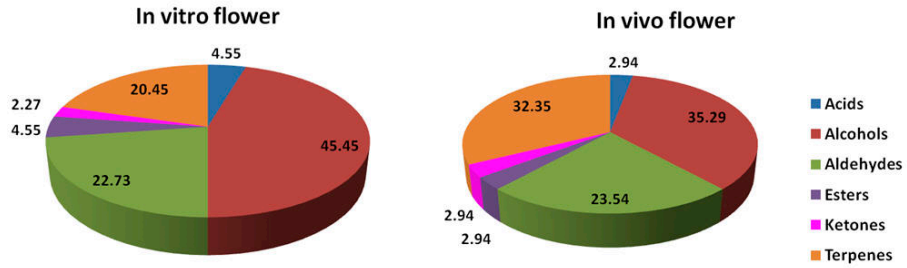

Figure 2. Percentage of volatile organic compounds from in vitro and in vivo Persian violet flowers.

In more detail, the results reveal that different growth conditions affected the relative content of volatile organic compounds generated in Persian violet flowers (Table 1). The most common alcohols in Persian violet flowers obtained from natural plants were 3-hexen-1-ol (a lipid alcohol with a grassy-green odor) and 1-hexanol (a lipid alcohol with a green-fruity odor), while Persian violet flowers obtained from plantlets grown in the aseptic environment contained higher amounts of ethanol (an alcohol that smells of wine) than that in natural flowers, followed by 3-hexen-1-ol and 3-methyl-2-butanol (branched alcohols with a fruity scent).

Table 1. Relative contents, odor activity values, and odor characteristics of volatile organic compounds derived from in vitro and in vivo Persian violet flowers.

Volatile Component	Relative Content (mg Thiophene/gFW)		OAV		Odor Characteristics
	In Vitro	In Vivo	In Vitro	In Vivo	
Acids					
Acetic acid	0.0039	0.0016	0.75	0.1231	Vinegar
tran-2-Pentenoic acid	0.0005	nd	na	na	Sour, caramel

Table 1. *Cont.*

Volatile Component	Relative Content (mg Thiophene/gFW)		OAV		Odor Characteristics
	In Vitro	In Vivo	In Vitro	In Vivo	
Aldehydes					
Acetaldehyde	0.1318	0.0729	48.81	27.0	Pungent
3-Methylbutanal	0.013	0.0074	20.312	4.628	Peach-like
2-Butenal	0.0139	0.0057	0.516	0.0521	Sweet
2-Pentenal	0.0004	nd	0.0008	-	Tomato-like
Hexanal	0.0011	0.0038	0.012	0.165	Green apple
2-Hexenal	0.0028	0.004	0.012	0.165	Vegetable-like
Heptanal	0.0008	nd	0.0081	-	Fatty, oily
Octanal	nd	0.001	-	0.0059	Honey-like
Nonanal	nd	0.0044	-	0.419	Fatty
2-Methylbenzaldehyde	0.0005	0.0013	0.115	0.01354	Cherry-like
Benzaldehyde	0.0017	nd	0.049	-	Fruity
Benzeneacetaldehyde	0.0004	nd	158.73	-	Hyacinth-like
Ketone					
Acetone	0.0206	0.0145	0.026	0.0077	Sweet
Alcohols					
Ethanol	0.0865	0.0708	0.349	0.1142	Wine
3-Penten-2-one	0.0077	nd	15.917	-	Fruity
3-Methyl-2-butanol	0.0189	nd	23.049	-	Fruity
4-Methyl-2-pentanol	0.0006	nd	0.0011	-	Pungent
1-Penten-3-ol	0.0023	0.004	0.014	0.0009	Grassy-green
3-Hexanol	0.0004	nd	0.0012	-	Alcoholic
3-Methyl-1-butanol	0.0015	nd	0.623	-	Pungent
4-Methyl-2-pentanol	0.0014	nd	0.003	-	Pungent
3,5-Dimethylphenol	0.0011	nd	67.5	-	Fruity
1-Pentanol	nd	0.0014	-	0.0092	Sweet
1-Hexanol	0.0067	0.031	0.494	0.9118	Green-fruity
2-Heptanol	0.0008	nd	0.02	-	Lemon
2-Penten-1-ol	0.0013	0.0017	3.0	1.544	Green note
2-Hexen-1-ol	0.0026	nd	18.37	-	Fruity-green
3-Hexen-1-ol	0.0365	0.1096	7.015	8.4308	Grassy-green
4-Hexen-1-ol	0.0005	0.0014	12	14	Pungent-oily
3-Methyl-2-heptanol	nd	0.0005	-	0.005	Citrus
1-Octen-3-ol	nd	0.0046	-	0.46	Herbaceous
6-Methyl-5-hepten-2-ol	0.0005	nd	0.6	-	Fruity
2-Ethyl-1-hexanol	0.0008	nd	0.0025	-	Floral
1-Octanol	0.0003	0.0021	0.036	0.0955	Orange-rose
Benzyl alcohol	0.0032	0.0017	0.0031	0.0007	Fruity
Phenylethyl alcohol	0.0017	0.0005	0.2048	0.0238	Rose
Esters					
Geranyl acetate	0.0006	nd	0.015	-	Floral
Methyl salicylate	0.001	0.0015	0.00002	0.00003	Minty, wintergreen
Terpenes					
γ-Terpinene	0.0013	nd	0.000049	-	Lemon
2-Carene	0.0003	0.0008	0.2	0.2	Floral
3-Carene	nd	0.0018	-	0.0005	Citrus
Caryophyllene	nd	0.0045	-	0.0571	Floral
Humulene	nd	0.0008	-	5.0	Woody
β-Ionone	nd	0.0004	-	0.025	Floral
Eucalyptol	0.0033	0.0052	0.0041	0.0026	Camphoraceous
Terpinen-4-ol	0.0015	0.0006	3.17	0.5	Spicy
α-Terpineol	0.0007	0.006	0.0198	0.0007	Floral
β-Citronellol	0.0392	0.0117	0.035	0.0042	Rose
Linalool	0.0013	0.0006	0.0005	0.0001	Floral
β-Farnesene	0.0003	nd	9.195	-	Woody
α-Farnesene	0.0005	0.0016	14.94	18.3908	Woody

Note: nd = not detected, na = not available, and - = unable to calculate.

Regarding aldehydes, the acetaldehyde (an aldehyde that produces a pungent smell) content of in vitro Persian violet flowers was approximately 0.132 mg thiophene/gFW, which was almost twice as high as that in natural flowers (0.073 mg thiophene/gFW), followed by 2-butenal (an aldehyde that produces a sweet smell) and 3-methylbutanal (an aldehyde that produces a peach-like scent). The principal terpene constituent in both in vitro and in vivo flowers was β-citronellol (a terpene alcohol with a rose scent). However, the minor terpenes 3-carene (a monoterpene with a citrus scent), caryophyllene (a sesquiterpene that produces a floral scent), humulene (a sesquiterpene with a woody scent), and β-ionone (a monoterpene that produces a floral scent) were only found in natural flowers, while the minor terpenes in flowers grown in sterile conditions were γ-terpinene (a monoterpene that smells like lemon) and β-farnessence (a sesquiterpene with a woody scent) (Tables 1 and 2).

Table 2. Types and amounts of terpenes derived from in vitro and in vivo Persian violet flowers.

Terpene	In Vitro	In Vivo	Terpene Type
γ-Terpinene	++	-	Monoterpene
2-Carene	+	+	Monoterpene
3-Carene	-	++	Monoterpene
Caryophyllene	-	++	Sesquiterpene
Humulene	-	+	Sesquiterpene
β-Ionone	-	+	Monoterpene
Eucalyptol	+	+	Monoterpene
Terpinen-4-ol	++	+	Terpene alcohol
α-Terpineol	+	++	Terpene alcohol
β-Citronellol	+++	++	Terpene alcohol
Linalool	++	+	Terpene alcohol
β-Farnesene	+	-	Sesquiterpene
α-Farnesene	+	++	Sesquiterpene

Note: - = not detected, + = small amount, ++ = medium amount, and +++ = large amount.

In the case of esters, Persian violet flowers that grow in nature have minimal ester synthesis, the only detectable ester of which is methyl salicylate (an ester that smells like mint or wintergreen), while Persian violet flowers grown in a culture container synthesized geranyl acetate (an acetate ester that produces a floral scent), as well as methyl salicylate (Table 1).

4. Discussion

In the present research, a tissue culture system realized floral induction from Persian violet. During in vitro propagation using a Persian violet shoot tip explant, flowers were produced at week 4 and were available for further application. This point is the advantage of Persian violet tissue culture for in vitro flower induction over growing this decorated plant from seedsthat require more time to obtain flowers.

In vitro technology is crucial for micropropagation and assists in producing various phytochemicals [26,27]. For over three decades, this technology has overcome the problem of fertility reduction in Persian violet. However, effective micropropagation via microshoots and in vitro flower initiation has been achieved in the last six years. Recently, UV-C irradiation has been used to enhance root initiation and flower mutation in vitro [4,9,28,29].

Plants generally emit volatile organic compounds (phytochemicals) from leaves, flowers, fruits, and roots. In addition to the colors of flowers, humans are also attracted to the perception of smell. Odors or volatile substances are perceived via smell and can influence a human's memory and emotions [30–32]. However, in nature, many kinds of flowers, including Persian violet, produce different volatile compounds; therefore, are the odors produced in sterile environments different from those produced in natural environments? In this study, Persian violet flowers derived from both sources were examined using GC-MS, and it was revealed that Persian violet flowers grown in vitro showed a higher synthesis of

ethanol and acetaldehyde than those grown in natural conditions. This outcome implies that hypoxia may occur during culture under aseptic conditions, causing the fermentation process, which begins various other processes, as shown in Figure 3.

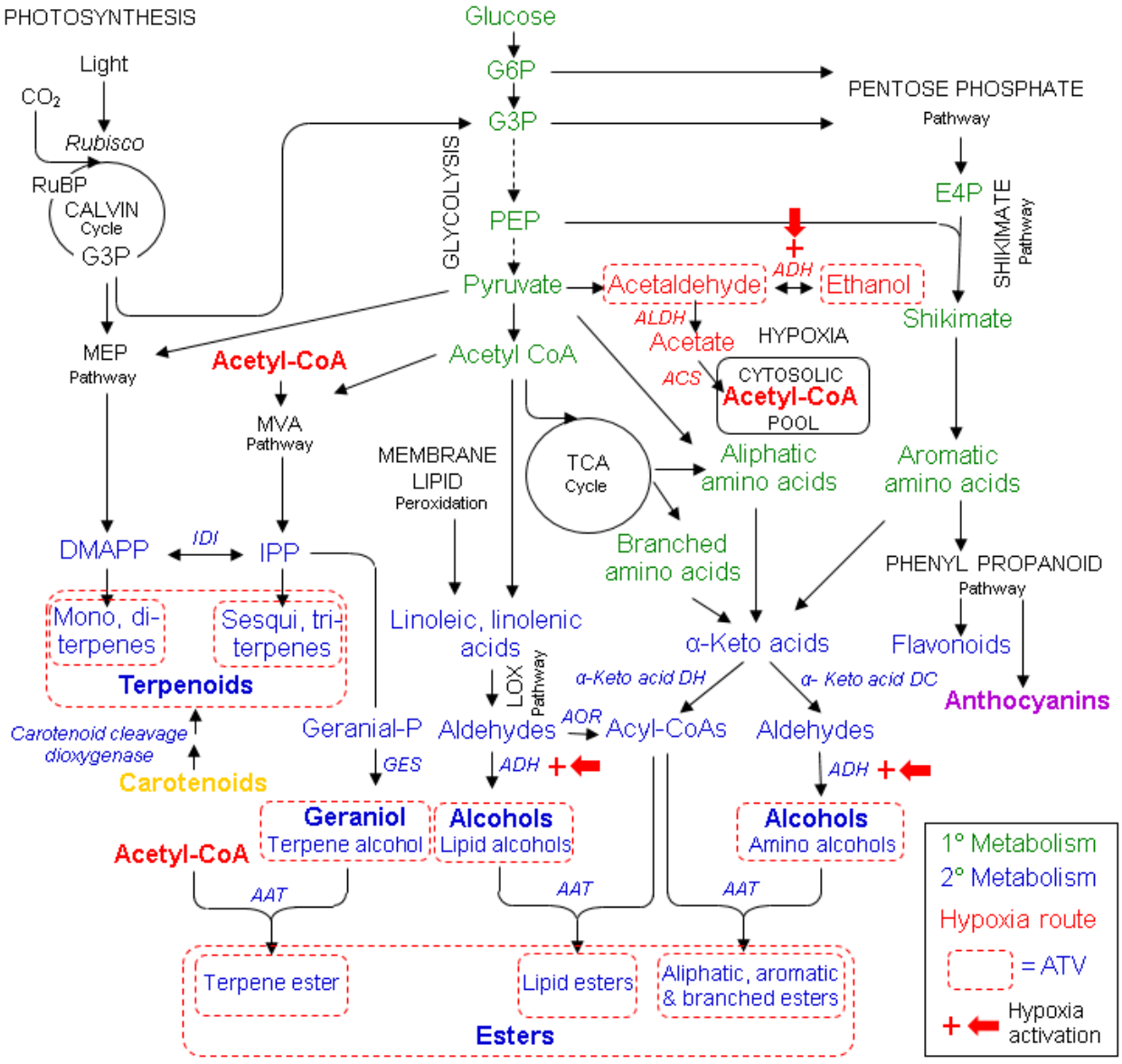

Figure 3. The proposed effects of hypoxia occurring under aseptic conditions on volatile compound production. (AAT = alcohol acyltransferase; ACS = acetyl-CoA synthetase; ADH = alcohol dehydrogenase; ALDH = aldehyde dehydrogenase; AOR = aldehyde oxidoreductase; ATV = able to be volatile; GES = geraniol synthase; IDI = isopentenyl diphosphate isomerase; α-keto acid DC = α-keto acid decarboxylase; α-keto acid DH = α-keto acid dehydrogenase).

The way plants survive in hypoxia is primarily through fermentation. The function of the alcohol dehydrogenase (ADH) enzyme is, therefore, essential [33,34]. In this circumstance, a higher frequency of fermentation cycles is required to supply plants with enough energy to survive, leading to more ethanol accumulation. However, the presence of large amounts of ethanol is harmful to plants. Ethanol must, therefore, be converted into acetaldehyde, but acetaldehyde is also highly toxic to plants. To reduce toxicity, plants must convert acetaldehyde into acetate and acetyl-CoA, which induces the accumulation of acetyl-CoA in the cytosol and is linked to terpene synthesis [35–37].

Many types of terpenes, such as monoterpenes, sesquiterpenes, and terpene alcohols, that can cause a variety of scents from citrus to rose were synthesized in flowers from both sources. Both monoterpenes and sesquiterpenes are noticeable odor-active compounds that are constituents of the Persian violet flower odor. Plants commonly synthesize monoterpenes using precursors (G3P) in the Calvin cycle, entering the methylerythritol phosphate pathway (MEP pathway) to form dimethylallyl pyrophosphate (DMAPP), which combines to form monoterpene and diterpene in the chloroplasts. Meanwhile, sesquiterpene and triterpene are synthesized in the cytosol from acetyl-CoA via the mevalonic acid pathway (MVA pathway), thereby producing isopentenyl pyrophosphate (IPP), which is used to form sesquiterpene and triterpene. However, the amounts of IPP in the cytosol and DMAPP in the chloroplasts can be interchangeable with each other. In addition, the amount of acetyl-CoA in the cytosol is vastly increased due to fermentation, thus causing more IPP synthesis. Then, IPP can be converted into terpene alcohol. Many terpene alcohols are thus synthesized. However, terpene alcohols are also used as precursors along with acetyl-CoA to synthesize terpene esters based on alcohol acyltransferase (AAT) activity.

During hypoxia, glycolysis adds substrates (G6P and G3P) to the pentose phosphate pathway to generate other energy metabolites [38]. In addition, the pentose phosphate pathway facilitates the synthesis of intermediate substrates used as precursors for synthesizing amino acids and fatty acids. However, to obtain enough energy to support plant life, increasing fermentation frequency results in the formation of more intermediate substrates that can be converted into aldehydes and alcohols, which are volatile substances with particular characteristic odors. The conversion of aldehydes into alcohol depends on the activity of ADH enzymes, and it was found that hypoxia can induce ADH activity.

Moreover, many aromatic alcohols are synthesized from aromatic amino acids via the shikimate pathway using precursors derived from the pentose phosphate pathway. These aromatic amino acids are not abundant in plants in typical environments, but they are induced via the occurrence of stresses. Therefore, fermentation is considered one of the stimuli for synergistic compartmentation. Furthermore, despite the presence of various alcohols, ester was the least detectable odor component in both conditions. This may have been caused by the lack of an enzyme to synthesize an ester, or it may not be the preferred alcohol of AAT.

The volatile compositions of Persian violet cv. 'Blithe Spirit', which has white flowers (obtained from the previous report), were compared with the present results for purple flowers, and dissimilar constituents were found. The main volatile organic compounds in white Persian violet flowers, which were grown in nature, were terpenes, which produced a pine scent mixed with a lemon odor, followed by alcohol derived from the degradation of fatty acids and amino acids. At the same time, the ester aroma was not prominent compared with the other volatile components. The different volatile constituents in various Persian violet flower colors may depend on the different variants of the gene, as the violet flower color is dominant compared to the white one. Moreover, the locus that conditions the flower color also pleiotropically controls the full expression of the Persian violet stem color. Thus, different genes for violet and white flower colors may reflect the different expressions of volatile production [39,40].

For future aspects, assessing whether Persian violet flowers, after transplantation, still produce the same volatile compositions as in sterile conditions is essential for evaluating the variations in volatile production during tissue culture. Moreover, the timing for the highest volatile emission during culture in vitro is an exciting topic to compare the similarity pattern of this incident. The effect of plant growth regulators, for example, ethylene, on volatile compound production in vitro is also interesting. This prospective research will provide more knowledge regarding volatile compound production in vitro environments.

5. Conclusions

It is challenging to smell a flower's fragrance in a tissue culture container. However, the present research has shown that Persian violet flowers can also produce a scent in sterile environments. Persian violets grown in vitro showed almost twice as much acetaldehyde content as those grown in natural conditions, followed by 2-butenal, an aldehyde with a sweet odor, and 3-methylbutanal, an aldehyde with a peach-like odor. This occurred due to hypoxia. Therefore, the environment during plant growth affects the substrate for volatile organic compound synthesis. Thus, the scent of the Persian violet flowers that grew in nature was a mix of rose and floral, while the scent of the Persian violet flowers that grew in a tissue culture container was a mix of rose, floral, and lemon.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae9090981/s1. Figure S1: Persian violet flowers grown in the in vitro environment; Figure S2: Persian violet flowers grown in the in vivo environment.

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