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Localization of a defensive volatile 4-hydroxy-4-methylpentan-2-one in the capitate glandular trichomes of *Oenothera glazioviana*

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ABSTRACT

Glandular trichomes of plants produce a wide variety of secondary metabolites which are considered as major defensive chemicals. The capitate glandular trichomes of *Oenothera glazioviana* (Onagraceae) were collected with laser microdissection and analyzed by gas chromatography–mass spectrometry. The volatile compound 4-hydroxy-4-methylpentan-2-one (**1**) was identified. We found that compound **1** displays antimicrobial, insecticidal, and phytotoxic activities. These results suggest that compound **1** might function as a defensive compound in the capitate glandular trichomes of *O. glazioviana* against pathogens, insect herbivores, and presumably competitive plants as well.

1. Introduction

Glandular trichomes (GTs) populate the aerial surface of many terrestrial vascular plants (Wang, 2014), and can be subdivided into peltate glandular trichomes (PGTs) and capitate glandular trichomes (CGTs) based on their respective morphological characteristics (Wagner et al., 2004). GTs synthesize, store, and release a wide variety of secondary metabolites such as terpenoids, alkaloids, and phenolic compounds, and thus have been regarded as chemical factories of plants (Schilmiller et al., 2008; Wagner et al., 2004). It is well known that many GT-produced chemicals have important pharmaceutical values, as exemplified by artemisinin and Δ^9-tetrahydrocannabinol (Happyana et al., 2013; Kim and Mahlberg, 1997; Wang, 2014; Wang et al., 2015). In nature, these special chemicals have been generally considered as defensive compounds in antagonistic interactions among organisms (Benlarbi et al., 2014; Nyasembe and Torto, 2014). For instance, pyrethrin, which is located in the GTs of *Tanacetum cinerariifolium*, has been recognized as a potent botanical insecticide (Ramirez et al., 2013; Wang, 2014); sclareol, excreted by the GTs of *Salvia sclarea*, shows antimicrobial activity and also affects *Arabidopsis thaliana* seed germination (Caissard et al., 2012; Campbell and Manners, 2003; Jasiński et al., 2001).

Volatile compounds (including terpenoids and phenylpropenoids) produced by the GTs have also been reported to contribute to the defensive properties of many plant species (Gang et al., 2001; Veronese et al., 2001). For example, mint oils are synthesized and stored in the GTs of *Mentha arvensis* (Ohloff, 1994; Sharma et al., 2003), and play valuable roles in plant defense against herbivores (Harborne, 1993) and attracting predatory and parasitic insects to attack phytophagous pests (Turlings et al., 1995); methylketones in the GTs of the wild tomato *Lycopersicon hirsutum* serve as defensive compounds against various insects (Williams et al., 1980; Yu et al., 2010); (−)-5,6-dehydrocamphor in the GTs of *Zuccagnia punctata* shows antifungal activity (Álvarez et al., 2012).

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In order to investigate the bioactive compounds in plant GTs as well as their defensive functions, we previously developed an approach which uses laser microdissection (LMD) coupled with sensitive analytical technology including cryogenic nuclear magnetic resonance and ultra-performance liquid chromatography-tandem mass spectrometry (Li et al., 2013). This new approach has identified several defensive plant compounds. We discovered two unique classes of defensive sesterterpenoids with insect antifeedant and antifungal activities in the PGTs of two Lamiaceae plants, *Leucosceptrum canum* and *Colquhounia coccinea* var. *mollis* (Li et al., 2013; Luo et al., 2010), respectively. Three defensive clerodane diterpenoids, which exhibited significant insect antifeedant activity, were also found in the PGTs of another Lamiaceae plant *Colquhounia seguinii* (Li et al., 2014). In addition, five phytotoxic labdane diterpenoids were identified in the CGTs of *Paragutzlaffia henryi* (Acanthaceae), which might serve as allelochemicals against other neighboring competitive plants (Wang et al., 2015). This research indicates that the secondary metabolites of plant GTs and their defensive roles may vary greatly among different species.

Oenothera glazioviana Mich., a member of the Onagraceae family, is commonly known as Evening Primrose, and has been an excellent model for studying molecular mechanisms of speciation (Greiner et al., 2008; Massouh et al., 2016). *O. glazioviana* is widely distributed in subtropical areas throughout the world and is commonly cultivated in northeast and southwest China due to its outstanding commercial value. An essential oil, whose major component is γ-linolenic acid, is produced from the seeds of *O. glazioviana* and has been used as a nutritional and medicinal supplement due to its anti-inflammatory and antithrombotic properties. In addition, previous reports have shown that this oil reduces lipids as well as enhances smooth muscle relaxation and vasodilation (Guo et al., 2014; Rauwolf et al., 2008; Stonemetz, 2008). We observed that in the wild the pedicels and calyxes of *O. glazioviana* are covered with abundant CGTs, and that their number gradually declines during the flowering stage. Curiously, the taste of *O. glazioviana* CGTs is pungent, which prompted us to investigate the identity, chemistry and function of putative chemical compounds of the CGTs. Herein, we report the localization of a volatile compound, 4-hydroxy-4-methylpentan-2-one (= diacetone alcohol) (**1**), in the CGTs of *O. glazioviana*, using LMD coupled with gas chromatography-mass spectrometry (GC–MS). We also evaluated the antimicrobial, phytotoxic and insecticidal activities of compound **1**.

2. Materials and methods

2.1. Plant material

O. glazioviana GTs were collected for microscopic study and metabolic analysis by LMD from plants grown in Kunming Botanical Garden in July 2015. *A. thaliana* wild-type seeds (ecotype Colombia) were maintained in our laboratory.

2.2. Microscopy

Fresh pedicels and flowers of *O. glazioviana* were examined under a Leica S8 APO light stereo microscope (Leica Microsystems, Wetzlar, Germany) with bright-field optics. For scanning electron microscope (SEM), samples were processed according to our previously reported protocol (Li et al., 2013), and the specimens were observed using a Hitachi S-4800 scanning electron microscope (SEM) (Hitachi Ltd., Tokyo, Japan) at 10.0 keV accelerating potential. Magnifications ranged from 30× to 500× (Wang et al., 2015).

2.3. Laser microdissection of CGTs and metabolic analysis by GC–MS

CGTs were microdissected and collected from fresh pedicels of *O. glazioviana* with a Leica LMD 7000 system (Leica Microsystems, Wetzlar, Germany) using a procedure similar to one we have previously described (Li et al., 2013). Approximately 800 CGTs were collected in the cap of a microtube. The collected samples were centrifuged at 4 °C (12,000 g, 10 min) to settle the contents, and extracted with acetone (500 μL) by ultrasonication for 10 min. The extract was directly analyzed by Agilent GC model 7890A coupled to a 5975C mass spectrometer (Aglient Technologies, Santa Clara, USA) equipped with a HP-5MS capillary column (50 m × 0.32 mm i.d, 0.52 μm film thickness). A split injection and diversion ratio (10:1) mode was used and 1 μL of sample was injected. Helium carrier gas was used at a constant flow rate of 3 mL/min. The temperatures of injector and mass transfer line were at 250 °C and 230 °C, respectively. The initial temperature was held at 50 °C with a programmed increase at 5 °C/min to 250 °C followed by 10 min at 250 °C. For mass spectral detection, an electron impact (EI) mode with ionization energy of 70 eV was used. Total time of the program running was 50 min. Spectra were obtained over an *m/z* range of 45–450. Total ion chromatogram (TIC) acquired via GC–MS was used for peak area integration. MSD ChemStation software was used for data acquisition.

2.4. Antimicrobial assay

We tested the antimicrobial properties of compound **1** against bacteria and fungi. Specifically, we used three strains of gram positive bacteria (*Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*) obtained from the Research Institute of Resources Insects, Chinese Academy of Forestry; three strains of pathogenic fungi (*Rhizoctonia solani, Colletotrichum litchi*, *C. gloeosporioides*) provided by the Department of Plant Pathology, South China Agriculture University; and a fungus that was isolated from diseased leaves of *O. glazioviana* and identified as *Aspergillus niger* HQ170509 by sequencing the internal transcribed spacers (ITS) of the ribosomal RNA gene region, were used in this study. The antimicrobial activities were evaluated using a broth dilution method. All experiments were performed in a 96-well microtitre plate with three replicates. The strains were prepared from 24-h broth cultures, and each suspension was standardized to 0.4 McFarland standard turbidity. Then, the test compound was dissolved into methanol to obtain a high concentration (2.20 mM) and series concentrations were acquired (1.10, 0.55, 0.28, 0.14, 0.07 mM) by two-fold dilution. Each well containing 90 μL of Luria–Bertani (LB) broth and 10 μL of the diluted test compound was inoculated with the microbial fluids to a final volume of 200 μL (Peng and Don, 2013). The 96-well microtire plates were further incubated for 6 h at 37 °C (bacteria) or 24 h at 28 °C (fungi), until McFarland standard turbidity reached the value between 0.6 and 0.8. The results were recorded with a microplate reader (Molecular Devices, USA). Ampicillin and nystatin were used as positive controls for antibacterial and antifungal assays, respectively, and methanol was used as negative control.

$$Inhibitory\ rate\ \% = \left(1 - \frac{N_T}{N_c}\right) \times 100$$

here, N_T is the value of optical density at 600 nm (OD_{600}) at each treatment, and N_C is the value of OD_{600} in control. The half maximal inhibitory concentration (IC_{50}) was obtained by probit analysis using SPSS.

2.5. Insecticidal assay

Larvae of *Myzus persicae* were obtained from Key Laboratory of Economic Plants and Biotechnology in West China, Kunming Institute of Botany, Chinese Academy of Sciences, and reared on *Nicotiana tabacum* plants. The test compound was dissolved in methanol at a concentration of 0.52 mM, and water containing Triton X-100 (0.1 mg/L) was added to dilute the compound to a series of concentrations (0.26, 0.13, 0.06, 0.03 and 0.015 mM). Leaves of *N. tabacum* with 20 healthy larvae were dipped into the chemical solutions for 5 s, and the excess liquid was removed with filter paper. After that the leaves were placed on a Petri dish (9.0 cm in diameter). Water containing Triton X-100 (0.1 mg/L) was used as a control. The mortality rate was evaluated at 24 h after treatment. Each treatment was repeated three times.

$$\textit{Corrected mortality } \% = \left(\frac{N_T - N_c}{20 - N_c}\right) \times 100$$

here, N_T is the number of deaths in each treatment, and N_C is the number of deaths in control. The half maximal lethal concentration (LC_{50}) was estimated by probit analysis using SPSS.

2.6. Seed germination assay

A. thaliana seeds were vernalized in a refrigerator at 4 °C for 3 days before using. The test compound was assayed at 0.86, 0.43, 0.22, 0.11, and 0.055 mM, respectively. To avoid toxic effects of the organic solvent, the final concentration of methanol did not exceed 0.2%. Fifty sterilized seeds were equidistantly sown on Murashige and Skoog medium [0.8% agar (w/v), pH 5.8] supplemented with the test sample at various concentrations in a Petri dish (9.0 cm in diameter). The same volume of methanol was used as a blank control. Three replicates of each treatment were carried out. The seeds were left under 24 h dim light at 25 °C in a growth chamber for 4 days, allowing germination. The number of germinated seeds was recorded daily and the inhibition index was calculated until most seeds (≥95%) in the control Petri dishes germinated. The seed germination inhibition rate (I_G) was evaluated by the following equation:

$$\textit{Inhibitory rate } \% = \left(1 - \frac{N_T}{N}\right) \times 100$$

here, N_T is the number of germinated seeds at each treatment, and N is the number of seeds used in the bioassay. The half maximal effective concentration (EC_{50}) was then determined by probit analysis using SPSS.

3. Results and discussion

3.1. Morphology and distribution of trichomes on O. glazioviana

To study the morphology and distribution of *O. glazioviana* trichomes, flowers, leaves and young stems were analyzed by stereomicroscope and SEM. CGTs were only present on the pedicels and calyxes, especially on young pedicels (Fig. 1A–B), and the number of CGTs on the pedicels gradually declined with the flowering period. CGT length was about 100–200 μm (Fig. 1C–D). Typical CGTs had a common globular-like storage cavity with 20–25 μm in diameter atop a short stalk (Fig. 1E). Interestingly, double globular-like storage cavities were also occasionally found at the top of one stalk (Fig. 1F). Also, we commonly observed the breakdown of the storage cavities, especially after rain.

3.2. Collection of CGTs by LMD and secondary metabolite analysis by GC–MS

In order to analyze the secondary metabolites present in *O. glazioviana* CGTs, intact CGTs were carefully collected under a

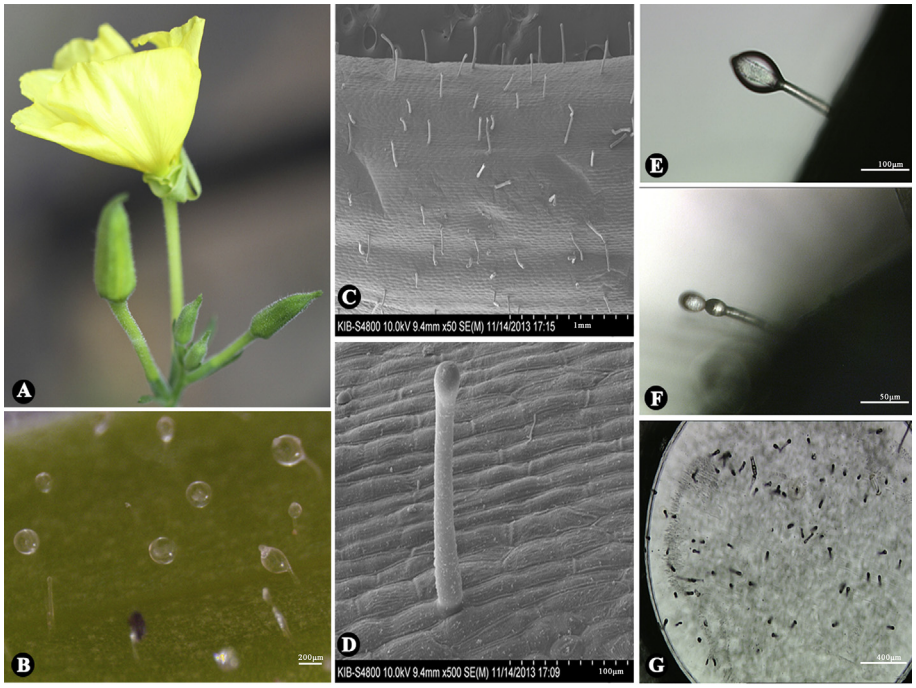


Fig. 1. Morphology and distribution of glandular trichomes (GTs) of *Oenothera glazioviana* and laser microdissection of capitate glandular trichomes (CGTs). (A) *O. glazioviana* in flowering. (B) CGTs on the pedicels under optical microscope. Scale bar: 200 μm. (C–D) CGTs on the pedicels under scanning electron microscope. Scale bar: 1.00 mm (C), 100 μm (D). (E) Intact CGTs before dissection. Scale bar: 100 μm. (F) Intact CGTs with double globular-like storage cavities before dissection. Scale bar: 50 μm. (G) Collected CGTs by laser microdissection. Scale bar: 200 μm.

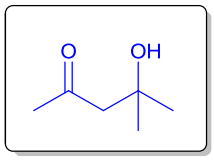
microscope and dissected using LMD, as illustrated in Fig. 1G (Li et al., 2014; Wang et al., 2015). Approximately 800 CGTs were collected in the caps of a 0.5 mL Eppendorf microcentrifuge tubes, which were stored at −80 °C immediately after LMD to avoid degradation of secondary metabolites.

The collected CGT samples were extracted with 500 μL of acetone by ultrasonication for 10 min. Since *O. glazioviana* has been reported to be rich in essential oil (Liu, 1997; Pelc et al., 2005), the CGT extract was analyzed directly by GC–MS. One predominant peak (compound **1**) with a retention time of 11.73 min was detected in the chromatogram (Fig. 2A).

3.3. Structural elucidation of secondary metabolites in CGTs

Compound **1** had a molecular weight of 116 according to its mass spectrum. The characteristic fragmental ions were clearly present at *m*/*z* 101 and 59 in the mass spectrum, which was very similar to that of a volatile compound, 4-hydroxy-4-methylpentan-2-one, in the NIST library (version 2.0), with a high match value of 968 (out of 1000). We therefore speculated that compound **1** detected in *O. glazioviana* CGTs might be 4-hydroxy-4-methylpentan-2-one.

Since the CGTs collected by LMD were insufficient for direct isolation of compound **1** for structural elucidation, a commercial sample of 4-hydroxy-4-methylpentan-2-one was obtained as a standard for verification. Through comparison of their retention times and MS spectra (Fig. 2B–E), the identity of compound **1** in the CGT extract of *O. glazioviana* was confirmed (Fig. 3). In addition, a series of minor peaks with retention times ranging from 32 to 48 min were detected in the total ion chromatogram of the CGT extract. However, these compounds were not identified due to the low match values between their MS spectra and those in the NIST data library.



4-Hydroxy-4-methylpentan-2-one (**1**)

Fig. 3. The chemical structure of 4-hydroxy-4-methylpentan-2-one (**1**).

3.4. Antimicrobial assay

Since the secondary metabolites of plant GTs have been generally considered to contribute to plant defense, we were interested in discovering whether compound **1** played a role in plant defense. Compound **1** was tested for its antimicrobial activity against three strains of gram positive bacteria (*B. subtilis*, *M. luteus*, and *S. aureus*), and three strains of pathogenic fungi (*R. solani, C. litchi*, and *C. gloeosporioides*), using a modified broth dilution method (Luo et al., 2010; Peng and Don, 2013; Wiegand et al., 2008). Compound **1** showed possible growth inhibitory activities against *B. subtilis*, *M. luteus*, and *S. aureus*, with IC_{50} values of 0.51 ± 0.29, 0.16 ± 0.80, and 0.13 ± 0.89 mM, respectively. In addition, compound **1** exhibited potent antifungal activity against the pathogenic fungus *R. solani*, with an IC_{50} value of 0.15 ± 0.82 mM (Table 1). The growth of associated fungus *A. niger* HQ170509 was also significantly inhibited by compound **1**, with IC_{50} value of 0.10 ± 0.99 mM. These results suggest that compound **1** may serve as an

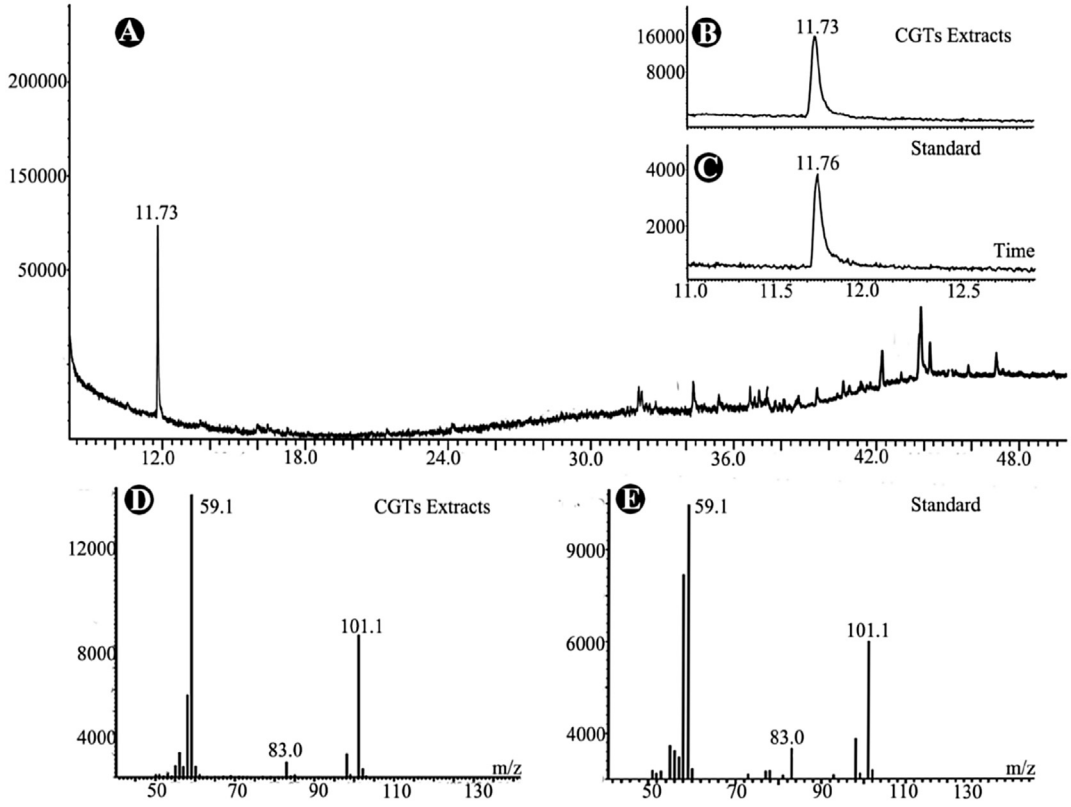


Fig. 2. GC–MS analysis of secondary metabolites in the capitate glandular trichomes (CGTs) of *Oenothera glazioviana*. (A) Total Ion Chromatogram (TIC) spectrum of CGT extracts. (B) and (C) Comparison of retention times of 4-hydroxy-4-methylpentan-2-one in TICs of CGT extract (B) and commercial standard (C). (D) and (E) MS spectra of 4-hydroxy-4-methylpentan-2-one in CGT extract (D) and commercial standard (E).

Table 1
Antimicrobial activities of 4-hydroxy-4-methylpentan-2-one.

Test organism	Antimicrobial activities/IC_{50} (mM)	
	4-Hydroxy-4-methylpentan-2-one	Positive control
Bacteria[a]		
Bacillu subtilis	0.51 ± 0.29	<0.0005
Micrococcus luteus	0.16 ± 0.80	<0.0005
Staphylococcus aureus	0.13 ± 0.89	<0.0005
Fungi[b]		
Rhizoctonia solani	0.15 ± 0.82	0.06 ± 0.008
Colletotrichum litchi	NA	<0.5
C. gloeosporioides	NA	<0.5
Aspergillus niger	0.10 ± 0.99	0.004 ± 0.19

[a] Ampicillin was used as a positive control.
[b] Nystatin was used as a positive control; NA = not active.

antimicrobial chemical for the plant against pathogenic microbes such as bacteria and fungi.

3.5. Insecticidal assay

Compound **1** has been used as a dispersant solvent contributing to the acute toxicity of insecticides, and has been reported to have a poisonous effect on the skin, eyes, and the pulmonary system (Kitulagodage et al., 2008; Lewis, 2004). Given that the above work has shown that *O. glazioviana* GTs are solely distributed on the pedicels and calyxes, and that the insect *M. persicae* feeds preferentially in or near plant inflorescences (Ibbotson and Kennedy, 1950; Metcalfe, 2005; Sholes, 1984), we tested the insecticidal activity of compound **1** against the larvae of *M. persicae*. Interestingly, compound **1** exhibited significant insecticidal activity, with LC_{50} value of 16.2 ± 0.18 μM.

3.6. Seed germination assay

Previous studies have reported that compound **1** inhibits germination in corn and wheat (Zolotovich et al., 1974). We therefore tested the phytotoxic effect of compound **1** on *A. thaliana* seed germination. We found that compound **1** was capable of effectively inhibiting seed germination (EC_{50} = 0.73 ± 0.13 mM).

In summary, we have identified a volatile compound, 4-hydroxy-4-methylpentan-2-one, in the CGTs of *O. glazioviana* using LMD coupled with GC–MS. Although 4-hydroxy-4-methylpentan-2-one has been frequently detected in plants (e.g. *Phlomis frutic*, *Juniperus phoenicea*) and microorganisms (e.g. *Mycobacterium tuberculosis*) by GC–MS (Elsawi et al., 2007; Mgode et al., 2012; Özcan et al., 2011), and has also been isolated from *Lonchocarpus laxiflorus* and *Stipa vaseyi* (Igoli et al., 2008; Epstein et al., 1964), this is the first time it has been localized in the GTs of a plant. 4-Hydroxy-4-methylpentan-2-one has been used as a synthetic material in industry and as a wood preservative (Gong and Gong, 1990). Oral transmission of 4-hydroxy-4-methylpentan-2-one to rats (LD_{50} of 4 g/kg) has been shown to be toxic (Smyth and Carpenter, 1948). However, the function of 4-hydroxy-4-methylpentan-2-one in plants, especially in the GTs, has until now remained unclear. GTs are considered an apparent first defense line for plants, and compounds synthesized and accumulated in GTs usually contribute to plant defense against herbivores and pathogens. As we predicted, 4-hydroxy-4-methylpentan-2-one showed obvious antimicrobial, insecticidal and phytotoxic activities. These results suggest that 4-hydroxy-4-methylpentan-2-one in the GTs of *O. glazioviana* may have defensive functions for the plant against pathogens and insects. Considering the uneven distribution pattern of GTs on *O. glazioviana*, 4-hydroxy-4-methylpentan-2-one might be a constitutive defensive chemical specially for the plant to protect its reproductive organs from pathogens and insects. In addition, as breakdown of the storage cavities of *O. glazioviana* GTs were commonly observed especially after raining, 4-hydroxy-4-methylpentan-2-one may also be released into the environment to function as an allelochemical against the surrounding competitive plants.

4. Conclusions

In our study, the volatile compound 4-hydroxy-4-methylpentan-2-one was localized in the CGTs of *O. glazioviana* using LMD coupled with GC–MS. 4-Hydroxy-4-methylpentan-2-one exhibited significant inhibitory activities against three strains of gram positive bacterium (*B. subtilis*, *M. luteus*, *S. aureus*), a strain of pathogenic fungus (*R. solani*) and a strain of associated fungus of *O. glazioviana* (*A. niger*). Moreover, 4-hydroxy-4-methylpentan-2-one displayed strong phytotoxic activity against *A. thaliana* seed germination and insecticidal activity against *M. persicae* larvae. These findings suggest that 4-hydroxy-4-methylpentan-2-one in the GTs of *O. glazioviana* may have defensive functions for the plant against pathogens, insect herbivores, and presumably competitive plants as well.

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