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BIOLOGY

Gas chromatography-mass spectrometry analysis of variations in the essential leaf oil of 6 Eucalyptus Species and allelopathy of mechanism 1,8-cineole

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ABSTRACT: *The Eucalyptus plant releases allelopathic chemicals into the environment mostly through the essential oils volatilized from the leaves. This study discussed the composition of the leaf oils of few seven-year-old varieties like Eucalyptus pellita (E. pel), Eucalyptus camaldulensis (E. cama), Eucalyptus grandis (E. gra), Eucalyptus dunni (E. dun), Eucalyptus saligna (E. sal), and E. grandis × E. urophylla (E. gra×E. uro) and three-year-old E. grandis × E. urophylla (E. gra × E. uro (three)). It determined the allelopathic mechanism and the types of chemical components playing the leading role. Essential oil was obtained by hydrodistillation and analyzed by the Gas Chromatography-Mass Spectrometry (GC-MS) method. In order to determine the effect of allelopathy, seed germination experiments were carried out at different concentrations (10–100 mL/L) of the E. Gra × E. uro leaf oil (EO) and the major components. The wheat seeds germinated by adding 1,8-cineole were used to determine the activity of α-amylase. Moreover, the mRNA expression of α-amylase in seeds was studied. The major chemical class in the essential oil was oxygenated monoterpene; 1,8-cineole (20.2–67.5%) displayed the highest content. Other substances that were high in content and ubiquitous included α-pinene (0.3–21.8%), α-terpineol (0.44–19.24%), and borneol (0.81–3.05%). The four chemical constituents and EO influenced the germination and growth of the three plants. Among them, 1,8-cineole exhibited the strongest inhibitory effect. The α-amylase activity of the 1,8-cineole-treated wheat seeds had decreased significantly. Molecular evidence suggested that 1,8-cineole decreased the α-amylase gene (AMY) expression.*
Key words: *GC-MS, essential oil, Eucalyptus leaf, allelopathy.*

Análise por cromatografia gasosa-espectrometria de variações no óleo essencial de folhas de seis espécies de eucalipto e alelopatia de componentes principais

RESUMO: *A planta de eucalipto libera substâncias químicas alelopáticas no ambiente principalmente através dos óleos essenciais volatilizados das folhas. Este estudo teve como objetivo discutir a composição dos óleos foliares de algumas variedades com sete anos de idade como Eucalyptus pellita (E. pel), Eucalyptus camaldulensis (E. cama), Eucalyptus grandis (E. gra), Eucalyptus dunni (E. dun), Eucalyptus saligna (E. sal) e E. grandis × E. urophylla (E. gra× E. uro) e E. grandis × E. urophylla (E. gra × E. uro(três)). Também pretendeu-se determinar o mecanismo alelopático e os tipos de componentes químicos que desempenham o papel principal. O óleo essencial foi obtido por hidrodestilação e analisado pelo método de Cromatografia Gasosa-Espectrometria de Massa (GC-MS). Para determinar o efeito da alelopatia, experimentos de germinação de sementes foram realizados em diferentes concentrações (10–100 mL/L) do óleo foliar de E. Gra × E. uro (OE) e dos componentes majoritários. As sementes de trigo germinadas pela adição de 1,8-cineol foram usadas para determinar a atividade da α-amilase. Além disso, a expressão de mRNA de α-amilase em sementes também foi estudada.: A principal classe química do óleo essencial foi o monoterpeno oxigenado; O 1,8-cineol (20,2–67,5%) apresentou o maior teor. Outras substâncias que eram de alto teor e onipresentes incluíam α-pineno (0,3–21,8%), α-terpineol (0,44–19,24%) e borneol (0,81–3,05%). Os quatro constituintes químicos e o OE influenciaram a germinação e o crescimento das três plantas. Entre eles, o 1,8-cineol exibiu o efeito inibitório mais forte. A atividade de α-amilase das sementes de trigo tratadas com 1,8-cineol diminuiu significativamente. Evidências moleculares sugeriram que o 1,8-cineol diminuiu a expressão do gene α-amilase (AMY).*
Palavras-chave: *CG-EM, óleo essencial, folha de eucalipto, a. alelopatia.*

INTRODUCTION

Eucalyptus, an evergreen plant that originates from Australia, is a large genus of the family Myrtaceae that comprises 140 genera and about 3800 species grown in tropical and subtropical parts of the world (WILSON et al., 2001). Because of its rapid growth and wide adaptability, *Eucalyptus* is one of the most important hardwood forestry crops in the world. It offers great economic value by providing

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a major source of pulpwood for high-quality paper production (BEN MARZOUG et al., 2011). Therefore, *Eucalyptus* has now been successfully introduced worldwide and is cultivated extensively in many other countries, including China where it was introduced over 110 years ago (ZHIQUN et al., 2017). However, *Eucalyptus* possesses strong allelopathy. Allelopathy is receiving increasing attention in natural and agro-ecosystems because allelopathic chemicals significantly reduce plant growth and crop yields (INDERJIT & DUKE, 2003). Thus, a growing number of scientists have recognized the ecological impact of the large-scale development of *Eucalyptus* plantations. *Eucalyptus* also releases allelopathic chemicals into the environment by ecological processes, and this could lead to a reduction in species diversity in the understory and severe soil erosion in the *Eucalyptus* forests (INDERJIT & DUKE, 2003). Thus, this is the biggest obstacle against the economic benefits of planting *Eucalyptus*. To solve these problems and achieve maximum economic benefits from *Eucalyptus* planting, the allelochemicals in *Eucalyptus* and the molecular mechanism of allelopathy need to be understood in detail.

It has been confirmed that volatilization (where oil is volatilized from the leaves) is one of the major ways of releasing allelopathic substances. Previous studies have confirmed that the *Eucalyptus* essential oil exerts a variety of biological activities such as insecticidal, weedicidal, and sterilization (BATISH et al., 2006; SETIA et al., 2007; BENCHAA et al., 2018). Some essential oils have certain effects on the seed germination and seedling growth of weeds. For example, the *E. citriodrora* volatile oil has a certain effect on the growth of some weeds like *Amaranthusviridis* L. seeds (BATISH et al., 2004). Thus, the volatile allelopathic compounds of *Eucalyptus* can be employed as the starting point for allelopathic research.

Currently, studies on the volatile allelopathic components of *Eucalyptus* have some limitations because they mainly focused on the overall activity of the volatile oil. Several studies have reported the composition of volatile oils in different varieties of *Eucalyptus* leaves (ELAISSI et al., 2010a; ELAISSI et al., 2010b; ELAISSI et al., 2010c; ELAISSI et al., 2011a; ELAISSI et al., 2011b; NASR et al., 2019), and many have reported the activities of these volatile oils (INDERJIT & DUKE, 2003; BATISH et al., 2006; ZHANG et al., 2014; BEN GHNAYA et al., 2016; ZHIQUN et al., 2017; BENCHAA et al., 2018; IBANEZ & BLAZQUEZ, 2019). These studies investigated the herbicidal, antibacterial, and antifungal activities of the volatile oils (GHAFFAR et al., 2015; DOGAN et al., 2017). However, studies screening the main allelopathic components present in the volatile oils and their mechanisms of action are relatively few. As a result, *Eucalyptus* planting has caused a series of ecological imbalances that have not been solved. The low biodiversity of undergrowth in *Eucalyptus* forests is a problem in many countries (FORK et al., 2015; WU et al., 2015). While *Eucalyptus* trees produce economic benefits, the ecological balance and sustainable development of *Eucalyptus* forests cannot be guaranteed. Previous studies have shown that an appropriately mixed forest can improve the light efficiency, water, and nutrient absorption as well as the comprehensive productivity of the stands (JOSE et al., 2006; NOUVELLON et al., 2012). However, this is not a fundamental solution to the problem of biodiversity loss. Because of the lack of research on allelopathy mechanisms, the current literature is unable to link the plant's physiology, biochemistry, and ecology effectively and thus cannot form a systematic study. Therefore, problems such as low biodiversity and serious soil erosion in the *Eucalyptus* forest cannot be solved fundamentally.

In this paper, we reported the results of our research on the similarities and differences in the components of the essential oils of six common species of *Eucalyptus* collected from Guangxi in the south of China. By comparing with the literature, several common and high content components in different *Eucalyptus* varieties were screened out. In addition, wheat, green bean, and white radish were selected as the experimental subjects for the seed germination experiment. The three experimental subjects belong to the Gramineae, Leguminosae, and Cruciferae families, respectively, and are common families and genera of undergrowth vegetation. The allelopathic constituents were further screened by seed germination experiments. This paper studied the allelopathy and mechanism of the main allelopathic component, 1,8-cineole. Previous studies have demonstrated the antibacterial potential of 1,8-cineole (SIMSEK & DUMAN, 2017). The germination of seeds is mainly related to amylase, lipase, and protease enzymes, which hydrolyze the starch, fat, and protein stored in the seeds to provide energy for germination (CENTENO et al., 2001; FASHUI et al., 2003; SIBIAN et al., 2017; YU & LIU, 2019). The starch content in the wheat seeds is high (KUMAR et al., 2018). Hence, we also investigated the activity and the mRNA expression of the amylase. The purpose of these studies was to facilitate the next step of research on the influence of one or more essential substances on other plants in the *Eucalyptus* forest, to lay a foundation for the study of *Eucalyptus's* allelopathic adaptation to ecological balance, and to provide a novel basis for the rational management and development of *Eucalyptus* plantations. This exercise was performed to ensure that *Eucalyptus* planting not only yielded economic benefits but also maintained the ecological balance and thereby sustainable development.

MATERIALS AND METHODS

Reagents andmaterial

The samples of fresh leaves were collected from three trees each of *E. pel*, *E. cama*, *E. gra* × *E. uro*, *E. gra* × *E. uro* (three), *E. gra*, *E. dun*, and *E. sal* from the Bozhai Tree Farm near the city of Liuzhou in the Guangxi Province of China. They were identified by Professor Wei Fanan of the Guangxi Institute of Botany. Except for *E. gra* × *E. uro* (three), all other samples were seven years old.

The standard mixture C6–C24 used in the GC-MS analysis was purchased in Shanghai, China. The purity of α-pinene ($C_{10}H_{16}$), α-pinoleol ($C_{10}H_{18}O$), 1,8-cineole ($C_{10}H_{18}O$), and borneol ($C_{10}H_{18}O$) was 99%, 98%, 99%, and 97%, respectively. All products were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. All reagents were of analytical grade. The amylase activity assay kit was purchased from Nanjing Jiancheng Bioengineering Inc., while the RNA extraction kit, RNA reverse transcription kit, and fluorescence quantitative kit were purchased from Takara Biomedical Technology Co., Ltd. Primers were synthesized by Wuhan Servicebio Technology Co., Ltd.

Steam distillation extraction

The essential oil was extracted from 200 g of crushed leaves boiled for 4 h using the steam distillation method (ELAISSI et al., 2011a; SOLIMAN et al., 2014). When the extraction mixture was cooled, the oil remained on the water surface. The essential oil was then collected by extracting thrice with n-hexane. Anhydrous sodium sulphate was added, and the solution was left to stand for 6 h. Next, the n-hexane was recovered, and the essential oil was stored at 4 °C until further analysis.

GC-MS conditions

Gas chromatography analysis was conducted with an Agilent (USA) 7890A gas chromatograph equipped with an HP–5 ms capillary column (30 m × 250 μm × 0.25 μm) using a 5% Phenyl Methyl Silox phase. The column oven temperature was programmed at 70 °C and increased to 100 at 1 °C/min, then to 180 °Cat 2 °C/min; it was maintained for a final time of 5 min at 250 °C. The injector was at 250 °C. The carrier gas was helium (99.999%) with a flow rate of 1 mL/min. The injected volume was 1 μL with a split ratio of 1/30. The MS analyses were performed using an Agilent (USA) 5975C with ionization energy of 70 eV, and the ion source temperature was 230 °C. The compounds of the oil were identified by comparison of the mass spectra of the compounds with those in the NIST05 database. The retention indices (RI) were calculated by a standard mixture of C6–C24 under the chromatographic conditions of the samples analyzed.

Laboratory biological simulation experiments

The four chemical components and the oil of *E. gra* × *E. uro* were prepared into three solutions at concentrations of 10, 50, and 100 mL/L. They were then dissolved with a small amount of anhydrous ethanol (1/100th of the solution) and diluted with distilled water to the required concentration.

Seeds of green beans, wheat, and white radish of almost the same size, color, and shape were selected. After cleaning with distilled water, these seeds were soaked in cold water for 2 h in a culture room at about 25 °C and then dried for sowing. The biological method of an indoor Petri dish was adopted (BATISH et al., 2006). Seven groups of parallel experiments were conducted in the incubator with the same chemical composition and the same concentration of solutions. Fifty pre-treated seeds were put into each culture box. The incubator's constant temperature was set as 25 °C with 80% humidity and dark culture conditions. The number of germinations was recorded daily, and a 2 mL solution was added to the incubator. The rate of seed germination on the third day was recorded as the germination potential. From the 5th day, the germination rates were recorded, and 20 seedlings with normal growth were randomly selected. The bud length and root length were then measured to determine the average value. Next, the germination rate and germination potential were calculated. The Microsoft Excel 2010 and SPSS 19.0 software were used for the statistical analysis of the data.

Determination of the α-amylase activity

Each group of wheat seeds (1 g) was ground in a mortar. A total of 0.3 g powder was placed in a centrifuge tube, and 1.8 mL of normal saline was added for centrifugation at 3000 rpm for 10–15 min. The supernatant was the solution used for testing. The α-amylase activity was determined by the starch–iodine colorimetry following the kit's instructions.

Method for analysis of the α-amylase gene expression

RNA extraction was carried out by the Trizol method (Takara Bio). cDNA synthesis was performed by the Prime ScriptTM RT reagent Kit with gDNA Eraser (Takara Bio), and real-time qPCR was performed using the TB Green TM Premix Ex TaqTM II (Takara Bio). The primer sequences are shown in table 1. Statistical significance was calculated using an unpaired T-test with significance at $P < 0.05$.

RESULTS

The oil yield

The oil yields of the seven samples of Eucalyptus leaves are shown in figure 1. The analysis of variance (ANOVA) indicated that the oil yields were significantly different between the species ($P < 0.05$). The average volatile oil production of *E. cama*, *E. gra*, *E. dun*, and *E. gra* × *E. uro* was 1.80%, 1.60%, 1.37%, and 1.13%, respectively. These values were significantly higher than the 0.23%, 0.33%, and 0.63% found in *E. pel*, *E. sal*, and *E. gra* × *E. uro* (three). The oil content of all *Eucalyptus* species except *E. pel* was consistent with the results of the previous studies (CIMANGA et al., 2002; ELAISSI et al., 2011b; MOSSI et al., 2011; SALEM et al., 2015).

Chemical composition

As seen in figure 2, the comparison of the number of peaks demonstrated that the leaf oil of *E.gra* × *E.uro* was the richest in terms of its contents, while *E.pel* leaf oil was the poorest in content. The chromatographic analyses of the essential oils allowed the identification of 70 compounds that represented 82.6–93.5% of the total oil content. The identified components were divided into five chemical classes. Table 2 lists the contents of these five chemical classes; also depicted are 27 of the 70 components that are high in content and relatively more prevalent in the studied *Eucalyptus* species. The content of other components was relatively low and appeared only in individual *Eucalyptus* species so they were not studied and listed here.

The major class included the oxygenated monoterpenes (26.4–86.6%), which exhibited the highest content in the leaf essential oil of *E.sal*. In this group, the main compounds were 1,8-cineole (20.2–67.5%), borneol (0.8–3.1%), α-terpineol (0.4–19.2%), and erpineol-4-ol (0.4–3.4%) with the highest content in *E.sal*, *E. gra* × *E. uro* (three), *E. pel*, and *E. dun*, respectively, compared to the other species. The monoterpene hydrocarbons constituted the second-highest class (1.9–45.5%) and were represented by α-pinene (0.3–21.8%), *p*-cymene (0.3–21.3%), and α-terpinene (0.1–18.0%), with the highest mean percentages in *E. gra* × *E. uro*, *E. cama*, and *E. dun*, separately. Amongst the species, the highest amount of this group was reported in the essential oil of *E. gra*. Sesquiterpene hydrocarbons (0.9–21.5%), with the highest levels in the leaf oil of *E.pel*, represented the third major class. They were essentially constituted by α-cubebene (0.2 –9.9%), β-caryophyllene (0.3–12.0%), and α-guaiene (0.1–6.3%) with the highest amount in *E. gra* × *E. uro*, *E. pel*, and *E. dun*, respectively. The oxygenated sesquiterpenes (0.3 –12.8%) formed the class with the fourth-highest contents. Its contents were globulol (0.23 –10.05%) and spathulenol (0.5–8.8%), with the highest percentages in *E. dun* and *E. cama*. *E.dun* was the richest species for this chemical type. The other components were minor compound classes and were not discussed in further detail.

The leaf oil of all samples except *E. cama* presented the highest mean percentage of 1,8-cineole. Among these samples, *E. sal*, *E. pel*, *E. gra* × *E. uro* and its three-year-old sample, were the species richest in oxygenated monoterpenes. *E. sal* was distinguished by the highest levels of oxygenated monoterpenes but was poor in other chemical types. However, in the *E. pel* oil, the content of sesquiterpene hydrocarbons such as β-caryophyllene was also very high. The two samples showed high levels of 1,8-cineole and α-terpineol. Except for the oxygenated monoterpenes, the monoterpene hydrocarbon content of *E. gra* × *E. uro* and its three-year-old sample was also relatively high. These samples were characterized by high levels of 1,8-cineole and α-pinene. Compared with its three-year-old sample, the oxygenated monoterpene content of *E. gra* × *E. uro* was significantly reduced; borneol and α-terpineol decreased the most and the other compounds experienced a small increase. *E. dun* and *E. gra*contained the highest percentage of the monoterpene hydrocarbons with *p*-cymene and α-terpinene as the major compounds. In *E. gra*, the second-highest component was α-pinene but *E. dun* contained a low yield. *E. cama* was very rich in both oxygenated monoterpenes and monoterpene hydrocarbons; *p*-cymene was the compound with the highest percentage, followed by 1, 8-cineole, *cis*-piperito, and spathulenol.

Table 1 - Chemical composition of the essential oils extracted from the leaves of six *Eucalyptus* species.

Compound class and name	RI	---Composition (%)---						
		E.dun	*E. cama*	*E. pel*	*E. sal*	*E. gra*	*E.gra×E.uro*	*E. gra×E. uro* (three)
Monoterpene hydrocarbons		34.43	27.43	3.15	1.86	45.48	17.63	13.72
α-Pinene	941	0.55	0.33	0.53	1.25	21.77	15.55	12.31
β-Pinene	977	tr	0.25	-	-	0.11	0.10	0.10
B-Myrcene	992	0.33	0.44	0.18	0.33	0.04	0.19	0.17
α-Phellandrene	1011	0.34	2.98	-	-	0.15	0.39	-
p-Cymene	1025	14.74	21.32	0.25	-	13.38	-	-
α-Terpinene	1056	17.96	0.93	-	0.13	9.35	0.58	0.12
Terpinolene	1088	0.40	0.32	-	-	0.56	0.31	0.23
Oxygenated monoterpenes		26.31	36.51	50.06	86.56	36.62	57.35	70.51
1,8-Cineole	1030	20.22	17.37	20.34	67.48	23.64	48.21	46.02
Linalool	1098	0.50	0.78	0.40	0.54	0.37	0.32	0.28
Fenchol	1117	-	-	0.79	0.60	0.43	0.32	0.96
Carvotanacetone	1160	-	-	0.33	0.31	0.17	0.36	1.02
Borneol	1165	-	-	2.31	1.59	1.49	0.81	3.05
Terpinen-4-ol	1176	3.44	-	-	0.47	1.71	0.41	0.52
cis-Piperitol	1185	-	9.70	-	-	-	0.61	0.25
α-Terpineol	1189	1.65	0.44	19.24	11.54	6.42	3.26	12.28
trans-Carveol	1217	tr	-	1.75	0.57	0.23	0.40	0.72
Phellandral	1276		3.61				0.25	0.11
Carvacrol	1293	0.18	1.63	0.24		0.35	0.13	0.10
Sesquiterpene hydrocarbons		13.07	4.74	21.45	1.40	0.92	11.70	6.69
α-Cubebene	1341	4.62	0.99	7.78	1.33	0.23	9.86	4.65
β-Caryophyllene	1412	tr		12.02		0.26	0.45	1.26
α-Guaiene	1433	6.32	0.29			0.12	0.26	tr
α-Humulene	1458	tr		1.31		0.05	0.11	0.18
Alloaromadendrene	1461	1.06	0.70	0.34		0.15	0.20	0.10
Oxygenated sesquiterpenes		12.83	10.16	7.99	0.32	1.01	2.67	2.01
Spathulenol	1576		8.83	2.34		0.48	0.80	0.71
Globulol	1578	10.05	1.33	4.17	0.23	0.23	0.84	0.69
Viridiflorol	1585			1.48		0.18	0.22	0.20
Others		0.07	3.71	4.61	0.89	0.23	0.57	0.56
p-Menth-1-en-3-one	1258	-	3.53	-	-	-	-	-
Benzeneacetaldehyde	1046	tr	tr	0.17	0.12	tr	tr	tr
Jacksone	1693	tr		3.10			tr	tr
Total		86.71	82.55	87.26	91.03	84.26	89.92	93.49

RI: retention index of HP-5MS column; tr: trace amounts (<0.1%). The values in the five compound classes are the sum of the contents of all the compounds identified in the class. The names and contents of some compounds with higher content that are commonly reported in six species of *Eucalyptus* are listed below the five compound classes.

Effects of four main chemical constituents on the seed germination and seedling growth of plants

In this section, the effects of 1,8-cineole, α-pinene, α-terpineol, borneol, and EO on the seed germination and seedling growth of three plants, including wheat, white radish, and green bean, were preliminarily studied. The experimental results are shown in figure 3 and figure 4. Figure 3 depicts the effects of 1,8-cineole, α-pinene, α-terpineol, borneol, and EO on the germination rate (Figure 3a, b, c) and germination potential (Figure 3d, e, f) of these three plants. Germination potential refers to the percentage of the germinated seeds and the number of tested samples during the germination process when the daily number of germinated seeds reached a peak (the third day in this experiment); it indicates the strength of seed vitality. Figure 4 depicts the effects of the four substances and EO on the root length (Figure 4a, b, c)

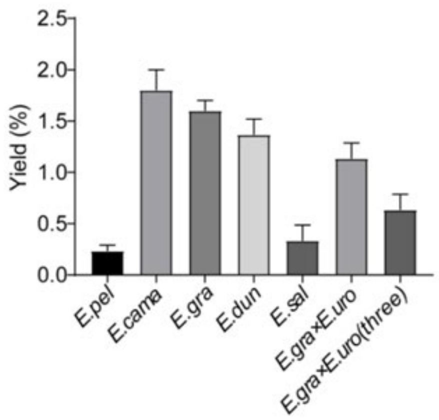

Figure 1 - Essential oil yields of six *Eucalyptus* samples by hydrodistillation (each sample was studied in triplicate). Values are mean percentages ± SD.

and bud length (Figure 4d, e, f) of the three tested plants after germination. The experimental results of the germination rate, germination potential, root length, and bud length after germination demonstrated that the four constituents and EO could inhibit the growth of the three plants to some extent. After the statistical analysis, the germination rate, germination potential, and the bud and root length of the three plants treated by 1,8-cineole, α-pinene, α-terpineol, borneol, and EO were significantly different from those of the control group ($P < 0.05$).

The comprehensive analysis of figure 3 and figure 4 showed that the inhibitory action of α-pinene, α-pinoleol, 1,8-cineole and EO on the seed germination rate, germination potential, root length, and bud length of the three plants increased with an increase in the concentration. 1,8-cineole and EO had the strongest inhibitory effect, and the inhibitory activity of 1,8-cineole was inferior to that of EO at 10 mL/L in terms of the germination rate and germination potential of wheat and radish, the germination potential of green beans, and the bud and root length of radish ($P < 0.05$). However, at a concentration of 50 mL/L, the inhibition exerted by 1,8-cineole on the germination of the three plants was 100%; EO caused 100% inhibition of the germination of only radish and green beans. Thus, 1,8-cineole was stronger than EO. The inhibition rate of 1,8-cineole and EO was 100% at 100 mL/L.

The phytotoxic activity of α-pinene and α-pinoleol was significantly weaker than that of 1,8-cineole and EO. At concentrations of 10 mL/L, the inhibitory effects of α-pinene and α-pinoleol on the germination rate and germination potential were not as strong as those of 1,8-cineole and EO except the germination potential experiment of radish by 1,8-cineole compared with α-pinene. There were significant differences among each group ($P < 0.05$). Overall, the inhibitory effects of α-pinene and α-pinoleol on the root length and bud length after germination were also weaker than that of 1,8-cineole and EO. Compared with 1,8-cineole, α-pinoleol displayed a weaker inhibitory effect on the root and bud length of the three plants. Moreover, the inhibition of α-pinene on the root and bud length of wheat and the bud length of green beans was also poor. All these differences were statistically significant ($P < 0.05$). There was a weaker inhibitory effect of α-pinoleol on the root and bud length of the three plants, compared with that of EO ($P < 0.05$). Similarly, α-pinene demonstrated a weaker inhibition on the root length of wheat and radish and the bud length of radish and green beans compared with that of EO ($P < 0.05$). At concentrations of 50 mL/L and 100 mL/L, the inhibition of α-pinene and α-pinoleol on the germination rate and germination potential was also less effective than that exerted by 1,8-cineole and EO. In addition to the effect of EO on wheat at 50 mL/L, the effect of 1,8-cineole and EO on the germination rate of the three plants was zero.

The allelopathic activity of borneol decreased upon increasing its concentration. When its concentration was 10 mL/L, all the four indices showed a stronger inhibitory effect on wheat and green beans compared to that shown by EO at the same concentration. Compared with EO, borneol significantly inhibited the germination rate and germination potential of wheat and green beans, the bud length of wheat, and the root length of wheat and green beans ($P < 0.05$). Meanwhile, the inhibition exerted by borneol on the germination rate and germination potential of the three plants, as well as on the root length of white radish and green beans, and the bud length of white radish were stronger than that exerted by 1,8-cineole. Compared with 1,8-cineole, borneol conspicuously inhibited the germination rate and germination potential of the three plants, the bud length of radish, and the root length of radish and green beans ($P < 0.05$). However, at concentrations of 50 mL/L and 100 mL/L, the inhibitory effect of borneol on the three plants was weaker than that of 1,8-cineole and EO.

The Mechanism of 1,8-cineole in inhibiting wheat seed germination

As shown in figure 5(a), the α-amylase (AMY) activities of wheat seeds under the effect of

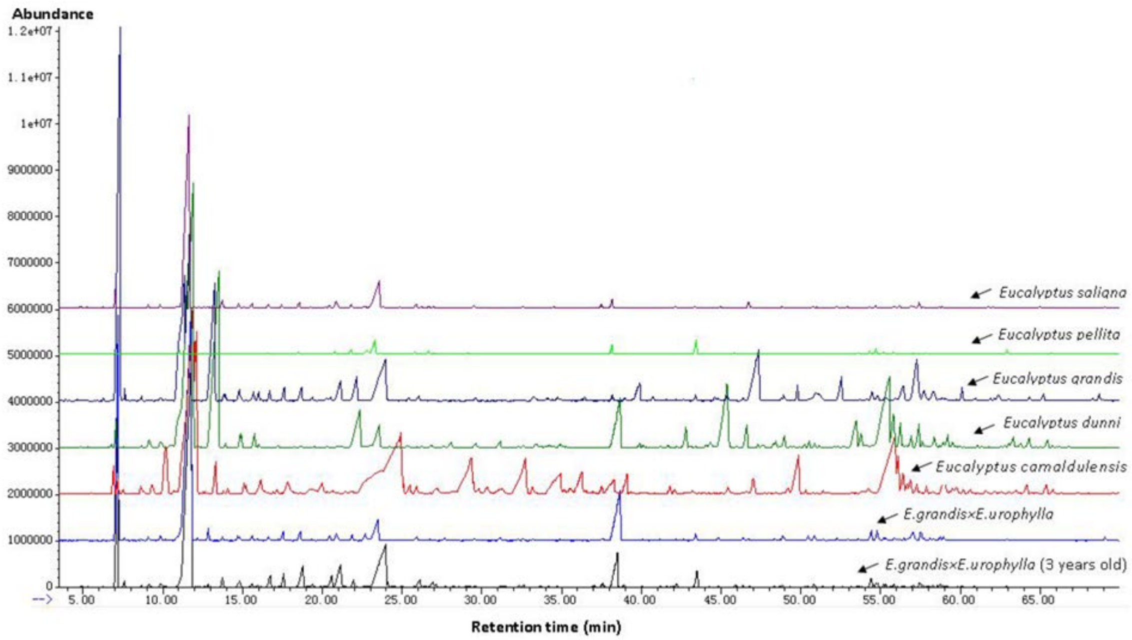

Figure 2 - The GC-MS total ion chromatograms of essential oils from six *Eucalyptus* samples.

three different concentrations of 1,8-cineole were significantly weaker than the α-amylase activities in the control group ($P < 0.05$). The α-amylase activity decreased gradually with an increase in the 1,8-cineole concentration. Meanwhile, based on the gene expression experiment (Figure 5(b)), it was demonstrated that the expression of AMY under the action of 1,8-cineole in the treated groups was lower than the expression in the control group (for all three concentrations). Although, the AMY expression of the 10 mL/L-treatment group decreased slightly compared with the control group, the expression in the higher-dose groups reduced significantly ($P < 0.05$).

DISCUSSION

The oil yield

A study has shown that allelopathic substances have a concentration-dependent inhibitory effect on the growth parameters in rice (ISMAIL & SIDDIQUE, 2011). Therefore, differences in the volatile oil yield of *Eucalyptus* leaves of different varieties may also lead to differences in their allelopathic intensities. This paper discussed the influence of the difference and quantity of the components in the volatile oil on allelopathy. Among these species, the oil yields (analyzed by GC-MS) of

Table 2 - Primer sequences applied in qPCR.

Gene	Primer sequences(5' –3')
β-actin-F	TCATCCTGTGTTGCTGACTGAG
β-actin-R	ACAGAACGGCCTGGATTGC
α-AMY-F	GCCTGGCTCAACTGGCTCAA
α-AMY-R	ACGAACGACGGCTTGCTGTT

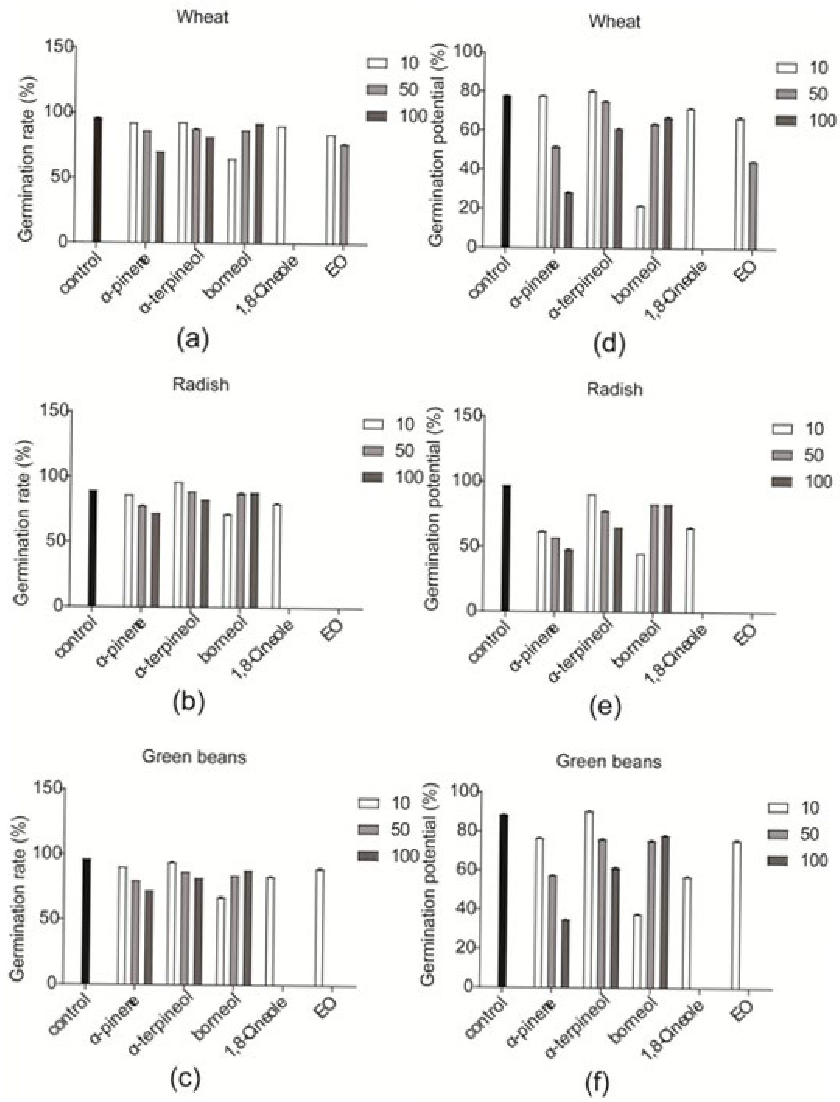

Figure 3 - Effect of chemicals from *Eucalyptus* on the seed germination rate of wheat (a), radish (b), and green beans (c), and the germination potential of wheat (d), radish (e), and green beans (f). Values are mean percentages ± SD of seven replications. The germination potential was recorded after three days of incubation, and the germination rate was recorded after five days of incubation. The doses were 10–100 mL/L.

the two samples of *E. gra* × *E. uro* of different ages were significantly different too ($P < 0.05$). The oil yields of *E. gra* × *E. uro* (three) were less than those of *E. gra* × *E. uro* (seven years old). It was previously reported that the content of the volatile oil in the leaves increases with age over a certain period(MAFFEI et al., 1986). The oil yield results demonstrated that the volatile oil extracted was consistent with the literature.

Chemical composition

In this part, the composition and content of volatile oil in the leaves of *Eucalyptus* species cultivated widely in China were analyzed. The literature contains very few studies on the volatile oil composition of different *Eucalyptus* varieties investigated in the current study except for *E. cama*. Therefore, the experimental results in this section are slightly different from those in the literature; this may be due to the differences in the growth environments and climates between these studies (GRULOVA et al., 2015). Oxidized monoterpenes have high herbicidal properties, and *Eucalyptus* species possess a high content of oxidized monoterpenes. Therefore, oxidized monoterpenoids are the research focus of the allelochemicals from *Eucalyptus*. The main components in all varieties were 1,8-cineole, α-pinene, α-terpineol, and borneol; they were consistent with the results in the literature (SARTORELLI et al., 2007; LIU et al., 2008; ELAISSI et al., 2010b; ELAISSI et al., 2011b; DOGAN et al., 2017). These results can help estimate the allelochemical types, and identify the main substances with possible allelopathic activity. These results can be used to screen the allelopathic substances from the volatile oil in detail through biological activity experiments.

Effects of four main chemical constituents on the seed germination and seedling growth of plants

Many studies have reported that essential oils with the greatest phytotoxic potential contain

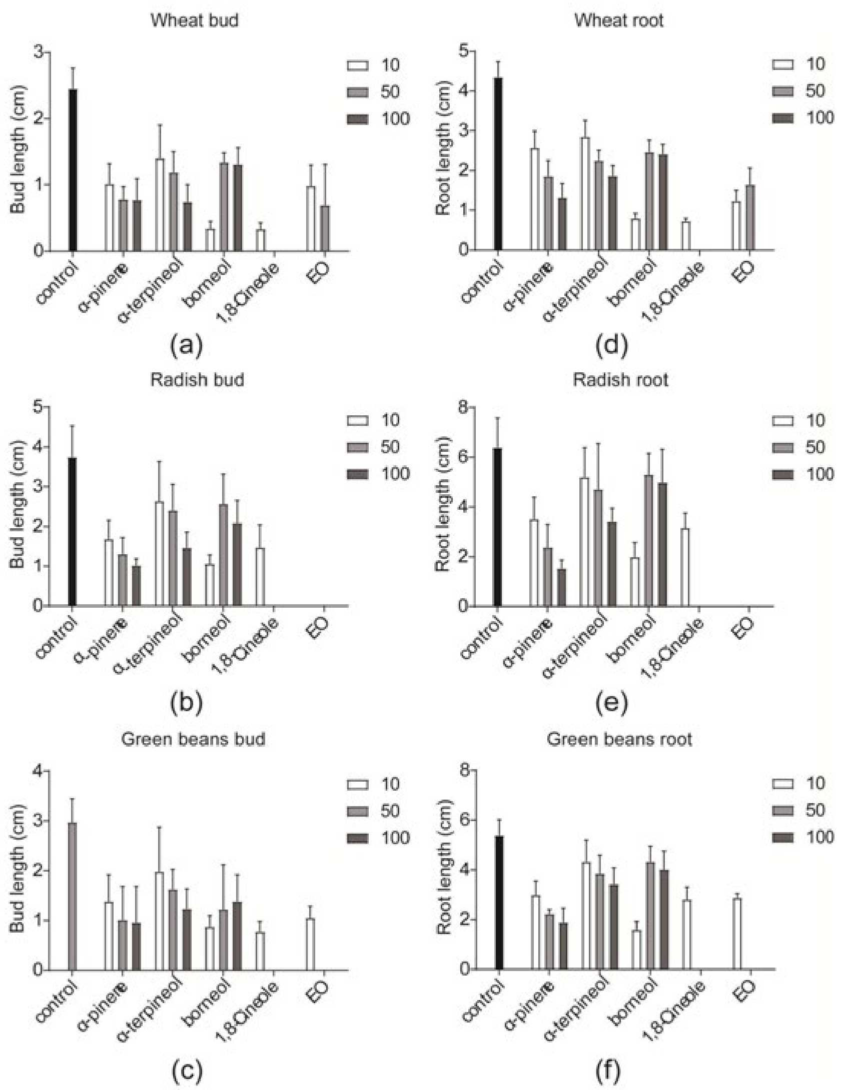

Figure 4 - Effect of chemicals from *Eucalyptus* on the bud length of wheat (a), radish (b), and green beans (c), and the root length of wheat (d), radish (e), and green beans (f). Values are mean length ± SD of 20 seeds after five days of germination. The doses were 10–100 mL/L.

oxygen-containing compounds as their main components (ANGELINI et al., 2003; VOKOU et al., 2003; DE MARTINO et al., 2010; SYNOWIEC et al., 2017; SARIĆ-KRSMANOVIĆ et al., 2019). In several papers, monoterpene hydrocarbons were shown to exert a lower inhibitory activity than oxygenated compounds (VOKOU et al., 2003; KORDALI et al., 2007). The solubility of monoterpenoids may be the main factor behind these differences (ABRAHIM et al., 2000). There are many oxygenated monoterpenes, such as 1,8-cineole in the *Eucalyptus* oil. The GC-MS analysis also demonstrated a relatively higher content of 1,8-cineole in the volatile oil.

In the experiment on seed germination, the inhibition of 1,8-cineole was 100% in several test indices of the three plants; 1,8-cineole had the strongest inhibitory effect. α-pinene and α-pinoleol did not play a major role in the volatile oil. Previous literature also showed that the allelopathy activity of α-pinene was lower than that of 1,8-cineole (ABRAHIM et al., 2000). 1,8-cineole and EO did not show strong inhibition at low concentrations, Likely, the presence of certain allelopathic substances such as borneol (that are stronger than 1,8-cineole) may play a role at lower concentrations and lead to a weaker inhibitory activity of 1,8-cineole than that of EO. However, with an increase in concentration, the activity of these components decreases, while the allelopathic effect of 1,8-cineole increases. Further, at 50 mL/L 1,8-cineole was stronger than borneol and played a key role; hence, the inhibitory effect of 1,8-cineole was stronger than that of EO. At 100 mL/L, the content of 1,8-cineole already increased to a certain extent. Therefore, the inhibition rates of 1,8-cineole and EO were 100%.Thus, it can be concluded that 1,8-cineole may play an important role in the allelopathy of the *Eucalyptus* volatile oil.

Borneol showed strong inhibition at seed germination, which is consistent with the result of a study (SEKINE et al., 2020). However, in our study, at low concentrations, borneol showed stronger allelopathy than that at high concentrations probably due to its concentration-dependent dual effect. In addition, borneol was reported to promote the absorption or transportation of carbohydrates, and as an antioxidant, to prevent auxin or cell damage resulting from noxious oxidation to promote root growth (TIAN et al., 2014). Therefore, the concentration-

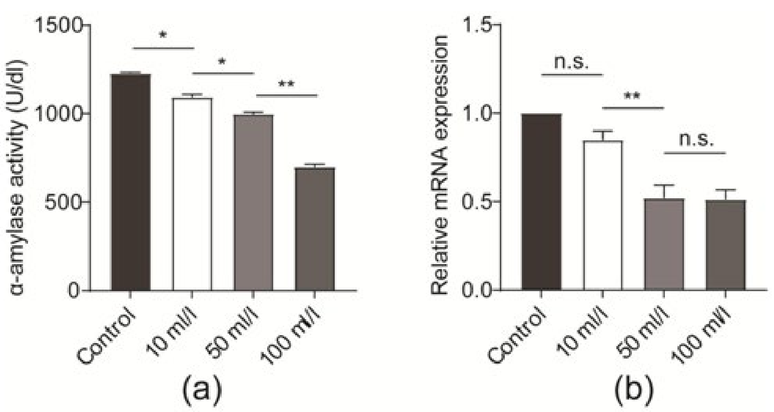

Figure 5 - (a) α-amylase activity under different concentrations of 1,8-cineole (U/dL). The α-amylase activity was determined by the starch–iodine colorimetry method following the kit's instructions. *: statistically significant differences, $P < 0.05$, **: statistically significant differences, $P < 0.01$ (Kruskal-Wallis ANOVA with Mann-Whitney test for between-group comparison). The values are the means ±SD of seven independent experiments. (b) The effects of different concentrations of 1,8-cineole on the relative expression levels of AMY in the wheat seeds. The relative expression levels of AMY genes were measured by RT-PCR. β-actin served as the internal control. The relative gene expression was calculated with respect to the levels at 0 h, which was set to 1. **: statistically significant differences, $P < 0.01$, $n = 4$, (One-Way ANOVA with Student-Newman-Keuls test for between-group comparison). The values are the means ± SD of seven independent experiments. n.s.: no significant difference.

dependent dual effect of borneol on seed germination and growth is obtained probably because at only high concentrations, borneol activates the aforementioned positive mechanisms.

Regarding the seeds of the three tested plants, EO and the four chemical constituents exhibited the greatest influence on the germination and growth of wheat and white radish seeds; this was followed by a greater influence on the green beans. The plants were affected differently by allelopathy. This difference might be related to (i) the fact that the seeds of the three plants were monocotyledons or dicotyledons and belonged to different families and genera (MEINERS et al., 2012) or (ii) the presence of different components contained in the seeds of these three plants. Generally, the undergrowth of *Eucalyptus* is sparse and features mostly grass family plants (the wheat in the experiment was also a part of the grass family). *Eucalyptus* may also exert a strong inhibition on the germination and growth of the Graminacea plants, as reflected in the literature (MEINERS et al., 2012). In addition, the higher the concentration of EO, the stronger was the inhibition of the three plants. This observation is consistent with the conclusion that the higher the yield of the volatile oil, the stronger is the allelopathic activity (MAFFEI et al., 1986).

Based on the overall data analysis, the allelopathy of *Eucalyptus* leaf oil was related to the type and content of its constituent substances and was the result of the interaction between different concentrations and different kinds of components. In the present experiment, we suspected that 1,8-cineole was one of the allelopathic substances that played a major role in the volatile oil of *Eucalyptus*. However, in this paper, only four main components were compared with the volatile oil of *Eucalyptus* leaves. The other substances with a lower content may also play a stronger allelopathic role, and they need to be studied further.

The Mechanism of 1,8-cineole in inhibiting wheat seed germination

The allelopathy of 1,8-cineole was the strongest and exerted a huge effect on wheat germination. For further investigation of the allelopathic mechanism, wheat seeds that were germinated by adding 1,8-cineole in the above experiments were selected (i) to determine the differences in the α-amylase activity (amylase strongly influences seed germination) (SETHI et al., 2016) and (ii) to study the differences in the mRNA expression that affected the synthesis of amylase in the seeds. Simultaneously, the inhibition of 1,8-cineole was evaluated in further detail. Results of the previous experiments illustrated that the inhibitory effect of 1,8-cineole on the wheat seeds was enhanced with an increase in its concentration. The results of this experiment corresponded to the changes in seed germination.

The overall analysis showed that the trend of variation in the amylase activity and AMY expression at different concentrations of 1,8-cineole was the same, and it corresponded to the phenomenon of the seed germination experiment. In other words, with an increase in the 1,8-cineole concentration, the germination rate, root and bud length after germination, amylase activity, and AMY expression decreased and were lower than the values of the control group. Previous studies have shown that 1,8-cineole inhibited seed germination (MULLER & MULLER, 1964) by inhibiting DNA synthesis in the roots (KOITABASHI et al., 1997). This was also an important factor in the volatile allelopathy of star anise (KANG et al., 2019). Therefore, 1,8-cineole may be one of the main substances with allelopathic activity in the volatile oil of *Eucalyptus* leaves that reduced the expression of the amylase gene and the activity of amylase. Thus, it reduced the germination rate of the wheat seeds and inhibited the growth of the seedlings. Here, only the amylase enzyme was studied to prove the allelopathy of 1,8-cineole. Inhibition of the amylase activity and AMY expression was one of the factors that 1,8-cineole affected in the wheat seed germination and growth. However, amylase is not the only enzyme involved in germination. Several other enzymes are involved in seed germination; the process is also related to nutrient concentration in the soil (MOHAMMADKHANI & SERVATI, 2018). There are many other mechanisms of 1,8-cineole on wheat seed germination and growth that need further study.

In this study, several components of the *Eucalyptus* volatile oil with possible inhibitory effects on seed germination and growth were revealed. Moreover, the inhibitory mechanism of 1,8-cineole, the main allelopathic component, was studied. This investigation illustrated the possible allelopathic substances in the volatile oil of *Eucalyptus* and laid the foundation for further research on the allelopathy of *Eucalyptus*. However, whether 1,8-cineole plays an allelopathic role in the volatile oil needs to be verified by other means such as those, which reduce the secondary metabolism yield of 1,8-cineole in the plants. The concentration ratio of the allelochemicals in the volatile oil can be modified, and the content of active allelopathic substances such as 1,8-cineole can be reduced with genetic improvements and genetic breeding, combined with plant physiology and phytochemistry (TSUSAKA et al., 2019). This can help to weaken the plant's allelopathy, improve the ecological balance in the *Eucalyptus* forests to adapt to the requirements of the surrounding ecology. It will also allow the *Eucalyptus* plants to produce economic benefits without damaging the ecological balance. The scholarly exploration of botany, ecology, chemistry, and other fields is required to achieve these goals.

CONCLUSION

This paper mainly studied the volatile oil composition of six kinds of *Eucalyptus* leaves that are commonly cultivated and used in China to analyze the similarities and differences in the contents of the main components. The major chemical class was oxygenated monoterpenes. Here, 1,8-cineole (20.2–67.5%) displayed the highest content and exhibited the strongest allelopathy activity. With an increase in the 1,8-cineole concentration, the germination rate, amylase activity, and AMY expression of the wheat seeds decreased to values lower than those of the control group. One of the factors that 1,8-cineole inhibits is the amylase activity and AMY expression during wheat seed germination and growth. Further, we can conclude that 1,8-cineole may be one of the allelochemicals in the *Eucalyptus* essential oil.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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AUTHORS' CONTRIBUTIONS

ZYL and JW contributed equally to the manuscript. ZYL, JW and WJG conceived and designed experiments. JW and MJY performed the experiments, MSL and YMJ performed statistical analyses of experimental data. ZYL and JW prepared the draft of the manuscript. All authors critically revised the manuscript and approved of the final version.

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