Transcriptomic Analysis of Fertilization Effects on the Biosynthesis of Sesquiterpenes in Essential Oils from Phoebe Bournei Twigs and Leaves

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Abstract

Background: *Phoebe bournei* is a potential medicinal plant whose essential oil (EO) from leaves has potential inhibitory activities against some bacterium, tumor, and has a certain potential for hypoglycemic activity. Fertilization is a common and effective method to increase plant biomass, which can increase the raw material of essential oil, but has a certain impact on the composition and biological activity of plant essential oil.

Results: The main components are sesquiterpenes in the essential oils from leaves and twigs. The yield of the essential oils and the content of their main components can be modulated by compost and compound fertilizer, to different degrees, and minor differences were registered among the categories of the components in essential oils. However, changes were strongly mirrored in some main components of essential oils. The content of the primary (+) - calarene in the leaf EO were strongly increased by compost, but the opposite happened by compound fertilizer. On the contrary, the effect of compound fertilizer was more significant on the main components of twig essential oil than compost.

The transcriptome sequencing results of *P. bournei* showed that the total number of DEGs in twigs and leaves treated with compost were significantly more than that with compound fertilizer. No change was found in the expression of genes regulating principal components. However, the expression of several key genes regulating the upstream substrates for the synthesis of the sesquiterpenes was significantly changed: the expression of two key speed limiting enzymes genes (DXS and HMGR) and two important branch-point enzyme genes (FPPS and GGPPS) was significantly down regulated, while the expression of gene (HMGS) was significantly up-regulated.

Conclusion: The expression levels of genes (DXS2, HMGR, FPPS and GGPPS) were significantly down regulated in leaves treated with compost, resulting in the changes of the yield and main components of the leaf essential oil. The effect of compost was more significant on the synthesis of the essential oil from *P. bournei* leaves than that of compound fertilizer.

Background

Plant essential oil (EO) has attracted the attention of researchers and the related research in the field of plant essential oil has been increasing (Fig. 1). EO is a secondary metabolite of plants, different from primary metabolites or intermediate metabolites. It plays an important role in plant adaptation to biological and abiotic environment, such as helping plants to resist insect attacks or pathogenic microorganism invasion [1–2], and protecting plants from damages caused by ozone, ultraviolet or drought [3]. EO also has many biological functions, such as antioxidant [4], antibacterial [5], inhibition of tumor growth [6]. Therefore, EO is widely used in the food and cosmetics industry and has great potential application value in the pharmaceutical industry.

EO is mainly composed of a variety of volatile organic compounds, such as terpenoids (mainly including monoterpenes and sesquiterpenes), alcohols, ketones, aldehydes, etc. [2, 7–14]. EO components are complex and volatile, so EO is also called "volatile oil". The composition of the essential oil is closely related to the source of plants, such as family or genus, growth stage, growth region and tissue location. It is quite different for essential oils extracted from herbaceous and woody plants. For example, the essential oils from *Achillea clavennae, A.fragrantissima* and *A.cana Coriandrum sativum* contain a lot of ketones and alcohols [4, 15]. The essential oils from woody plantssuch as cinnamon, lipia and Pistacia chinensis are often composed of aldehydes, sesquiterpenes, monoterpenes and terpenols [16–19]. Sesquiterpenes and monoterpenes are synthesized by terpenoid backbone biosynthesis pathway [20] which consists of two metabolic pathways: mevalonate (MVA) pathway and 2C-methyl-D-erythritol 4-phosphate (MEP) pathway[21].

Phoebe bournei (Hemsl). Yang (*Lauraceae*) is a unique precious timber and ornamental tree species in China. It is mainly distributed in the subtropical regions of China. The wood of *P. bournei* has been considered as a good timber for architecture, furniture, carving technology and shipbuilding. In recent years with the improvement of people's living standards, the demand for forest ecological services and high-grade wood products is increasing. *P. bournei* grows fast with high carbon sequestration capacity and long life. Thus, it has become a priority tree species for forestry development in southern China. The related research work such as *P. bournei* planting and growth environment has also been deepening [22–23]. Scholars are also constantly experimenting in-depth exploration of ecosystem community characteristics, carbon storage, litter decomposition rate of *P. bournei* plantation [24–25]. However, *P. bournei* is also a potential medicinal plant. The wood EO of *P. bournei* has the potential inhibitory activity against leukemia, breast, and colon cancer cell lines, and against *Epidermophyton floccosum* and *Microsporum gypseum* [26].

Fertilization is a common and effective method to increase plant biomass [27–29] and has a certain impact on the composition and biological activity of plant essential oil [30]. Fertilization can not only promote the growth of plants, but also modulate the composition of essential oil, thus changing the EO biological activity, to some extent [31]. A variety of methods and types of fertilization are implemented such as adding a single elements (nitrogen, sulfur, zinc) [32–34], or adding mixed fertilizer such as compost [35]. It is unknown how fertilization impacts the composition and the yield of *P. bournei* essential oil. Therefore, compost and compound fertilizer, which are common fertilizers in practical cultivation management, were used in the present study to investigate how the yield and composition of *P. bournei* essential oils were regulated by fertilization. The effects of the fertilization on the pathway of sesquiterpene biosynthesis were examined by transcriptomic analysis.

Results

Characteristics of main components in *Phoebe bournei* EOs after fertilization

The yield of EOs from leaves and twigs of *P. bournei* was 0.102% and 0.135% respectively. Only the yield of the leaf EO was decreased by the compost treatment (**Table 1**). The results of GC / MS showed that the components are mainly sesquiterpenes in EOs from *P. bournei* leaves and twigs (**Fig. 2-A and B**). As shown in **Fig. 2-C and D**, the dominant component in the leaves is (+)-calarene), and the relative content is more than 20%; the primary component in the twigs is (+) - δ-cadinene, with a relative content of more than 14%. The other main components (relative content > 3%) in EOs of *P. bournei* are as follows: δ -

cadinene (leaf9.21%, twigs 14.54%), copaene (leaf 5.72%, twigs 12.70%), caryophyllene (leaf 3.09%, twigs 10.11%) and α- caryophyllene (leaf 3.46%, twigs 3.52%). (−) – Allo - aromadendrene (6.54%) was only detected in leaves, while β - eudesmol (6.20%), γ - maaliene (6.37%) and α - bergamotene (6.73%) were merely detected in twigs. After fertilization, the categories of components in *P. bournei* EOs were not changed significantly, but the content of some main components was changed. After the application of the compost, the content of (+) - calarene in the leaf EO was increased significantly, while the content of other main components was not changed significantly (**Fig. 2-C**); the contents of α - bergamotene and γ - maaliene in the twig EO were decreased significantly, and other main components were not significantly changed (**Fig. 2-D**). After the application of compound fertilizer the contents of (+) - calarene, (+) - δ - cadinene, (−) - allo aromadendrene and α- caryophyllene in the leaf EO were significantly decreased, only germacrene B was significantly increased **(Fig. 2-C)**; the content of copaene in the twig EO was significantly increased, while the contents of β - eudesmol, γ - maaliene and α - bergamotene were significantly decreased (**Fig. 2-D**). In general, compost increased the content of the primary component in the leaf EO, but had no significant effects on other components, while compound fertilizer decreased the content of most main components in leaf and twig EOs.

Table 1 The yield of EOs from leaves and twigs of *Phoebe bournei*

Samples	Yield (%)		
	Compost	Compound fertilizer	Control
Leaves	0.089494±0.00313[b]	0.095891±0.01457[a]	0.112113±0.00548[a]
Twigs	0.128376±0.00629[a]	0.147014±0.03066[a]	0.134758±0.01255[a]

Note: 1) LCK — leaf control group; LOF — leaves treated with compost; LCF — leaves treated with compound fertilizer; TCK — twig control group; TOF — twigs treated with compost; TCF — twigs treated with compound fertilizer, the same below.

2) The letters a, b, c represents the significance of the difference.

Establishment and annotation of *Phoebe bournei* transcriptomes

The functional annotation of unigenes was conducted using BLASTx against public datebases (NR, Swiss-Prot, Pram, COG, GO and KEGG datebases). A total of 226819 unigenes were annotated from the public datebases. Different numbers of unigenes from all the libraries were annotated against NR, Swiss-Prot, Pram, COG, GO and KEGG database, respectively (**Table 2**). The results indicated an extensive coverage of ***Phoebe bournei*** transcriptomes. BLASTX results against NR database showed that the annotated unigenes (26615, 30.65%) in ***Phoebe bournei*** is largely similar to ***Quercus suber*** assembled unigenes (**Fig. 3**). The correlation coefficients of the unigenes expression in all the sample were between 0.78 ~ 1, indicating that the biological repeatability of this experiment was good, and sequencing data were accurate and reasonable (**Fig. 4**).

Table 2 The number of unigenes annotated against six databases

	Total	NR	Swiss-Prot	Pfam	COG	GO	KEGG
Unigene number	226819	87595	64362	62401	10938	54370	42768
Percentage	100%	38.62%	28.38%	27.51%	4.82%	23.97%	18.86%

In comparison with the control many **DEGs (different expression genes)** were annotated in twigs and leaves after the treatment of compost and compound fertilizer. The total number of DEGs in twigs and leaves treated with the compost were significantly larger than that with the compound fertilizer. The number of DEGs in leaves was noteworthy more than that in twigs treated with the compost. However, the number of DEGs in twigs was significantly higher than that in leaves treated with the compound fertilizer. After the two fertilization treatments, there were many common DEGs in twigs and leaves, but the number of common DEGs in twigs was less than that in leaves (**Fig. 5**).

Among the DEGs the number of genes significantly up-regulated or down-regulated was as shown in **Fig. 6** (p-ajust < 0.05). The number of up-regulated genes and down-regulated genes in twigs and leaves treated with the compost was more than those in twigs and leaves treated with compound fertilizer. The number of up-regulated and down-regulated genes in leaves was significantly higher than that in twigs treated with the compost. After adding the compound fertilizer the number of up-regulated genes was significantly higher than that of down-regulated genes in twigs; the number of up-regulated genes was lower than that of down-regulated genes in leaves; the number of up-regulated genes in twigs was more than that in leaves, and the number of the down-regulated genes in twigs was lower than that in leaves.

Gene ontology annotation and KEGG pathway analysis of *Phoebe bournei*

***Phoebe bournei* GO and KEGG classification**

Knowledge of the DEGs function of *P. bournei* would be important for us to understand the changes in yields and components of *P. bournei* EOs. All unigenes were annotated and classified by web gene ontology annotation plot (WEGO). WEGO annotation results showed that all the assembled unigenes were classified into 54 functional categories, as shown in **Fig. 7**. Among all the GO functional categories the dominant categories were 'binding' and 'catalytic activity' (> 56%), followed by 'cellular process' and' metadata process'. In addition, "cell", "cellular part" and "membrane" also accounted for a high proportion.

Out of the total identified unigenes 21713 unigenes (39.94%) were categorized as "metabolic process", among which 1343 unigenes were involved in “secondary metabolism process”.

The functional biological pathway of *P. bournei* was indenetified by 34368 unigenes mapped into the canonical pathways reference in KEGG using KOBAS (**Fig. 8**). Among them 674 unigenes were assigned as "terpenoid and polyketide metabolism", and 5459 genes were classified as "carbohydrate metabolism". The pathway hierarchical classification of the unigenes is shown in **Fig. 8-B** and **Fig. 8-C**. It can be seen from the above that the main components of *P. bournei* EOs are sesquiterpenes. Therefore, 185 unigene in terpenoid backbone biosynthesis (pathway ID: map00900) pathway, 36 unigene in sesquiterpenoid and triterpenoid biosynthesis (pathway ID: map00909) pathway and 11 unigenes in monoterpenoid biosynthesis (pathway ID: map00902) pathway would play an important role in the synthesis of *P. bournei* EOs.

Go functional annotation of DEGs

GO annotation was carried out on DEGs in twigs and leaves of *P. bournei* after fertilization. Significant differences were shown in the number of annotated DEGs in GO level 1 classification: cellular component (CC), molecular function (MF) and biological process (BP) (**Table 3**). DEGs in twigs were mainly involved in CC and MF after fertilization, while DEGs in leaves were assigned to CC, MF and BP, with the percentage of >50%.

According to GO level 2 classification of the DEGs (**Fig. 9**) the number of up-regulated and downregulated genes (p-ajust < 0.05) involved in the processes of 'binding', 'catalytic activity', 'cellular process', 'metallic process', 'cell', 'cellular part' and 'membrane' in twigs and leaves of *P. bournei* after the compost treatment were significantly higher than that after the compound fertilizer treatment. In other words, the effect of compost on the leaves was stronger than that on the twigs. For *P. bournei* leaves the number of up-regulated genes was more than that of down regulated genes after the compost treatment, while the number of down-regulated genes was more than that of up-regulated genes after the compound fertilizer treatment; for *P. bournei* twigs, the number of down-regulated genes involved in the processes of 'cellular process' and' molecular process' was more than that of up-regulated genes; the number of down regulated genes involved in 'cellular activity' and 'cellular part' and 'membrane' was more than that of up-regulated genes after the compost treatment; no differences were displayed in the number of up-regulated and down-regulated genes involved in ‘binding’ and ‘cell’. The number of down-regulated genes involved in the processes was smaller than that of up-regulated genes after the compound fertilizer. Number of DEGs participating in metabolic process was listed as follows: LCF_ Down (108), LCF_ Up (33), LOF_ Down (746), LOF_ Up (691), TCF_ Down (86), TCF_ Up (118), TOF-Down (271), TOF-Up (242).

Table 3 GO level 1 classification of DEGs in leaves and twigs of *Phoebe bournei* after fertilization

Groups	CC		BP		MF	
	DEGs number	Percentage (%)	DEGs number	Percentage (%)	DEGs number	Percentage (%)
NF_twig_vs_CF_twig_G	2093	66.78	1546	17.73	1589	50.7
NF_twig_vs_OF_twig_G	4022	26.85	3157	10.27	3393	41.82
NF_leaf_vs_CF_leaf_G	2064	74.27	1660	59.73	1451	52.21
NF_leaf_vs_OF_leaf_G	10296	62.57	8673	52.71	8107	49.27

Identifition of DEGs in KEGG pathways

The main components of EOs from *P. bournei* are sesquiterpenes and few monoterpenes (**Fig. 2**). The metabolic pathways related to their synthesis are terpenoid backbone biosynthesis (TBB), glycolysis / gluconeogenesis (GG), pentose phosphate pathway (PPP), sesquiterpenoid and triterpenoid biosynthesis (STB) and monoterpenoid biosynthesis (MB). Among them TBB can be regarded as the core metabolic pathway (**Fig. 10**). Fertilization has different effects on these metabolic pathways (**Table 4**). In the TBB pathway, 18 and 4 DEGs were identified in the leaves and twigs by the compost treatment, and 4 DEGs in the leaves and twigs after the compound fertilizer application. In the GG pathway, 96 and 26 DEGs were obtained in the leaves and twigs treated with the compost, and 17 and 23 DEGs in the leaves and twigs after the compound fertilizer application. In the PPP pathway, 55 and 7 DEGs were found in the leaves and twigs after the compost treatment, and 12 and 5 DEGs in the leaves and twigs by the compound fertilizer treatment. In these three metabolic pathways the number of DEGs in leaves and twigs by the compost treatment was significantly higher than that of the compound fertilizer treatment. In STB pathway only one DEG was obtained in the leaves and twigs by the compost treatment. In the MB pathway one and two DEGs were also identified in the leaves and twigs treated only with the compost. In general, the percentage of DEGs in twigs and leaves treated with the compost was relatively high in the five pathways (**Table 4**).

Table 4 The number of DEGs involved in sesquiterpenes biosynthesis pathway after fertilization.

Groups	Pathways									
	TBB		STB		MB		GG		PPP	
	DEGs	percentage/%	DEGs	percentage/%	DEGs	percentage/%	DEGs	percentage/%	DEGs	percentage/%
NF_leaf_vs_OF_leaf	18	9.73	1	2.78	1	9.09	96	6.34	55	9.89
NF_leaf_vs_CF_leaf	4	2.16	0	0.00	0	0.00	17	0.11	12	2.16
NF_twig_vs_OF_twig	4	2.16	1	2.78	2	18.18	26	0.09	7	1.26
NF_twig_vs_CF_twig	4	2.16	0	0.00	0	0.00	23	0.05	5	0.90

The number of significantly up- or down-regulated unigenes was different in the five pathways (**Table 5**). After the compost treatment up-regulated and down-regulated unigenes in GG and PP pathway were found, and the number of down-regulated unigenes was more than that of up-regulated unigenes. In TBB pathway the number of up-regulated unigenes was the same as that of down-regulated unigenes. A up-regulated unigene and a down-regulated unigene were identified in STB and MB pathways, respectively. The number of down-regulated genes was higher than that of up-regulated genes in leaves treated with the compound fertilizer. The number of up- or down- regulated unigenes in the leaves treated with the compound fertilizer was significantly smaller than that in the leaves with the compost.

For the twigs treated with the compost, only 3 and 2 up-regulated unigenes were enriched in TBB and MB pathways, and only one down-regulated unigene was identified in STB pathway. The number of down-regulated unigenes in GG and PPP pathways was larger than that of up-regulated unigenes. The number of up-regulated and down-regulated unigenes in most of the pathways in the twigs treated with the compost was significantly smaller than that in the leaves with the compost. For the twigs treated with the compound fertilizer, the up-regulated and down-regulated unigenes were involved in TBB, GG and PPP. In comparison with the compost, the effects of the compound fertilizer on *P. bournei* twigs were relatively weak.

In summary, the effect of the compost on *P. bournei* was much stronger than that of the compound fertilizer, especially on *P. bournei* leaves. The significantly up/down regulated unigenes might have contributed to the differences in sesquiterpene metabolism before and after the fertilization treatments.

Table 5 The number of up / down regulated unigenes in the five pathways (q-value < 0.05).

Groups	Pathways								
	TBB		STB		MB		GG		PPP
	Up/Down	percentage/%	Up/Down	percentage/%	Up/Down	percentage/%	Up/Down	percentage/%	Up/Down
NF_leaf_vs_OF_leaf	5/5	27.78/27.78	1/0	5.56/0	0/1	0/5.56	41/48	42.71/50	20/26
NF_leaf_vs_CF_leaf	0/2	0/50	-	-	-	-	2/4	11.76/23.53	1/7
NF_twig_vs_OF_twig	3/0	75/0	0/1	0/25	2/0	50/0	10/15	38.46/57.69	2/5
NF_twig_vs_CF_twig	1/0	25/0	-	-	-	-	17/3	73.91/13.04	2/2

Identification of genes involved in biosynthesis of sesquiterpenoids.

TBB contains two metabolic pathways: mevalonate (MVA) pathway and 2C-methyl-D-erythritol 4-phosphate (MEP) pathway (**Fig.10**). MVA pathway is mainly responsible for the biosynthesis of sesquiterpenes and triterpenes, while MEP pathway is mainly responsible for the biosynthesis of monoterpenes [21]. The main components of *P. bournei* EOs are squiterpenes, and therefore, TBB is the core pathway of EO metabolite synthesis. 3-hydroxy-3-methyl-glutaryl-CoA (HMG CoA) and 1-deoxy-d-xylulose-5phosphate (DXP) are two key substrates for the synthesis of sesquiterpenes and monoterpenes. DXP is produced by 1-deoxy-D-xylulose-5-phosphate synthase (DXS) catalyzing glycraldehyde 3-phosphate (G3p) and pyruvate. DXS has been proved to be an important rate-limiting enzyme in MEP pathway [21,36]. DXS should be an important regulatory site for the sesquiterpenes biosynthesis. This indicated that DXS would play an important role in the anabolism pathway of *P. bournei* EO. The expression of genes regulating DXS in different branches varied with development, tissue type and environmental conditions. After the treatment of the compost significant changes are found in the expression of DXS genes in leaves and twigs. Three unigenes about DXS in the leaves were changed: one up-regulated unigene (TRINITY_DN56563_c0_g1) and two down-regulated unigenes (TRINITY_DN73974_c0_g2, TRINITY_DN60255_c0_g7); the unigene (TRINITY_DN70707_c0_g6) in the twigs may be a DXS gene. However, one down-regulated DXS unigene (TRINITY_DN73974_c0_g2) was found only in leaves after the compound fertilizer treatment. If an enzyme is regulated by two or more genes, these unigenes may belong to a multigene family or part of a larger gene [37]. The expression of DXS genes is usually divided into three categories (DXS1, DXS2, DXS3), while most belongs to DXS2 regulating plant secondary metabolite biosynthesis [36,38]. According to Swissprot Description (**Table 6**), only TRINITY_DN60255_c0_g7 is the unigene regulating DXS2, which is closely related to the metabolism of *P. bournei* EO. The expression of the unigene was not significantly changed in all twigs, but obviously decreased in leaves treated with the compost (**Fig. 11**). One unigene (TRINITY_DN73974_c0_g2), not being DXS2 gene, was enriched only in leaves after the compound fertilizer treatment.

A key step in MVA pathway is HMG-CoA synthesis by Hydroxymethylglutaryl-CoA synthase (HMGS) catalyzing Acetyl-CoA and Acetoacetyl-CoA [39]. HMGs is the first committed enzyme in the MVA pathway. The change of HMGS expression level has an important impact on the production of secondary metabolites [40]. The expression of two unigenes (TRINITY_DN79706_c2_g1, TRINITY_DN84575_c6_g2) enriched in *P. bournei* regulating HMGS was both up-regulated in

twigs and leaves after the compost treatment and twigs after the compound fertilizer treatment (**Fig.11 and Table 6**). Here, the expression of TRINITY_DN79706_c2_g1 in leaves was similar to that in twigs after the compost treatment (**Fig. 11**).

Another important step in MVA pathway is mevalonate synthesis by 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) catalyzing HMG-CoA. HMGR is also an important rate-limiting enzyme in MVA pathway, and the overexpression of HMGR can promote the synthesis of sesquiterpenes in plants [41]. HMGR is usually divided into HMGR1, HMGR2 and HMGR3 [42]. The overexpression of HMGR1 and HMGR3 can also have a positive effect on the synthesis of secondary metabolites [42-43]. The expression level of HMGR1 gene (TRINITY_DN83281_c2_g2) enriched in all the twigs was not significantly changed, but clearly decreased in the leaves by the compost treatment. This indicated that the compost treatment would reduce the EO yield of *P. bournei* leaves. Similarly, the expression of HMGR3 gene (TRINITY_DN76855_c1_g3) was clearly decreased in leaves treated with the organic and compound fertilizer, except in all the twigs **(Fig.11**). Both the two fertilization treatments, especially the compost treatment, to some extent, had a negative effect on the yield and the constituent of *P. bournei* EOs.

Farnesyl pyrophosphate synthase (FPPS) is an important branch-point enzyme, whose activity can change the direction of isoprene metabolism in the TBB pathway and plays an important role in isoprene metabolism [44-46]. The expression of FPPS gene (TRINITY_DN61719_c0_g1) was down-regulated in *P. bournei* leaves and twigs by the fertilization treatment, but the down-regulation of the expression was the most significant in the leaves by the compost treatment (**Fig.11**). The change in the expression of FPPS gene could be a main reason that the EOs of *P. bournei* twigs and leaves were varied in the main components. Geranylgeranyl diphosphate synthase (GGPPS) is also an important branch enzyme in terpenoid biosynthesis [40]. Contrary to FPPS, the expression of GGPPS gene (TRINITY_DN67962_c1_g3) was up-regulated in all the twigs and leaves, particularly in the leaves by the compost treatment. Therefore, changes in the expression of FPPS and GGPPS genes may be an important factor for the change of species or content of the components in *P. bournei* EOs.

The terminal products geranyl diphosphate (GPP) and farnesyl diphosphate (FPP), produced by Acetyl-CoA, Acetoacetyl-CoA, Pyruvate and D-Glyceraldehyde-3P going through MVA and MEP processes, are further catalysed by terpene syntheses (TPS) to form sesquiterpenes and monoterpenes (**Fig. 10**). Here, the expression of four unigenes (TRINITY_DN64565_c0_g4, TRINITY_DN83483_c1_g2, TRINITY_DN64925_c1_g1 and TRINITY_DN71731_c0_g2) about TPS was significantly changed in *P. bournei*. However, these sesquiterpenes (Germacrene D, (E, E)-farnesyl-P, terpineol and (+)-Neomenthol) produced by the regulation of the four genes were not the main components of *P. bournei* EOs.

In addition to TBB pathway, and the synthesis of volatile components and the yield in *P. bournei* EOs is also related to GG and PPP. The first four substrates (Acetyl-CoA, Acetoacetyl-CoA, Pyruvate and D-Glyceraldehyde-3P) in the TBB process are produced by the metabolism of GG and PPP (**Fig. 10**). After fertilization the expression of many genes regulating the two pathways was also affected to a great extent. From the above KEGG enrichment results, except for the twigs treated with the compound fertilizer, the number of down-regulated genes was higher than that of the up-regulated genes in other samples. These genes to some extent indirectly affected the yield and composition of *P. bournei* EOs.

Table 6 The information of DEGs in map00900, map00909 and map00902

Gene ID	Swissprot Description	Log2FC	Pvalue	Pajust	Up/ Down	Group
TRINITY_DN79706_c2_g1	Hydroxymethylglutaryl-CoA synthase	1.39516492	7.82E-03	4.90E-02	up	NF_leaf_vs_OF_leaf
		1.707138136	3.68E-04	7.63E-03	up	NF_twig_vs_OF_twig
TRINITY_DN84575_c6_g2	Hydroxymethylglutaryl-CoA synthase	2.150896458	1.68E-03	2.41E-02	up	NF_twig_vs_OF_twig
		2.493661561	2.26E-04	1.19E-02	up	NF_twig_vs_CF_twig
TRINITY_DN56563_c0_g1	1-deoxy-D-xylulose-5-phosphate synthase 1 (DXS1)	1.06898745	4.73E-04	5.48E-03	up	NF_leaf_vs_OF_leaf
TRINITY_DN73974_c0_g2	1-deoxy-D-xylulose-5-phosphate synthase 1 (DXS1)	-5.5884809	7.98E-05	1.26E-03	down	NF_leaf_vs_CF_leaf
		-4.21307726	5.45E-06	1.31E-03	down	NF_leaf_vs_OF_leaf
TRINITY_DN60255_c0_g7	1-deoxy-D-xylulose 5-phosphate synthase 2 (DXS2)	-1.557267	2.84E-03	2.25E-02	down	NF_leaf_vs_OF_leaf
TRINITY_DN70707_c0_g6	Probable 1-deoxy-D-xylulose-5-phosphate synthase, chloroplastic	1.923531945	8.85E-07	5.23E-05	up	NF_twig_vs_OF_twig
TRINITY_DN83281_c2_g2	3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 (HMGR1)	-1.92527724	9.50E-07	2.87E-05	down	NF_leaf_vs_OF_leaf
TRINITY_DN76855_c1_g3	3-hydroxy-3-methylglutaryl-coenzyme A reductase 3 (HMGR3)	-1.57011729	9.19E-06	2.04E-04	down	NF_leaf_vs_OF_leaf
		-1.34758953	1.21E-03	4.35E-02	down	NF_leaf_vs_CF_leaf
TRINITY_DN61719_c0_g1	Farnesyl pyrophosphate synthase 2 (FPPS2)	-1.57943298	6.17E-05	1.02E-03	down	NF_leaf_vs_OF_leaf
TRINITY_DN64139_c1_g1	Probable phytol kinase 3	1.818575961	8.01E-08	3.31E-06	up	NF_leaf_vs_OF_leaf
TRINITY_DN75454_c1_g3	3 beta-hydroxysteroid dehydrogenase/Delta 5-->4-isomerase	1.019357385	2.79E-03	2.22E-02	up	NF_leaf_vs_OF_leaf
TRINITY_DN67962_c1_g3	Geranylgeranyl pyrophosphate synthase, chloroplastic (GGPPS)	1.132667099	1.63E-03	1.47E-02	up	NF_leaf_vs_OF_leaf
TRINITY_DN64565_c0_g4	TPSGD_VITVI/ Terpene synthase family (Pfam)	-2.39226398	3.13E-03	3.79E-02	down	NF_twig_vs_OF_twig
TRINITY_DN83483_c1_g2	Geraniol synthase, chloroplastic	1.966248415	4.55E-04	8.97E-03	up	NF_twig_vs_OF_twig
TRINITY_DN64925_c1_g1	Alpha-terpineol synthase, chloroplastic	-6.04327435	6.47E-06	1.52E-04	down	NF_leaf_vs_OF_leaf
TRINITY_DN71731_c0_g2	Salutaridine reductase	1.419760693	1.80E-04	4.35E-03	up	NF_twig_vs_OF_twig

Discussion

In this study, it is found that the yield of EO from *P. bournei* leaves is decreased after the compost treatment. No significant changes are found in the component categories of EOs from *P. bournei* leaves and twigs, and sesquiterpenes are still the main constituents after the application of compost and compound fertilizer. But both the compost and the compound fertilizer had certain effects on the main components of EOs obtained from *P. bournei* leaves and twigs. Firstly, the compost treatment had a positive effect on (+) - calarene, which is the primary component of EO from *P. bournei* leaves, while the compound fertilizer had a negative effect on it. However, the compound fertilizer had a positive effect on germacrene B, another main component. Secondly, the effect of the compost treatment is not significant on most of the main components in the twig EO, while the effect of the compound fertilizer treatment is more obvious on some main components of the twig EO. Felice Senatore et al. confirmed that composting with high organic content can change the composition of essential oil and help to improve some main components, while fertilization treatment has no significant effect on the yield of essential oil [47]. Sandra Kleine and Caroline Müller also elaborated that the content of terpenoids in *Tanacetum vulgare* leaves changes with the change of fertilization conditions [48].

In order to investigate the changes of the main components of EOs from *P. bournei*, we performed the transcriptome sequencing for *P. bournei* leaves and twigs. Many DEGs are annotated from the twigs and leaves of *P. bournei* after the fertilization treatmenst and the number of DEGs in twigs and leaves after

the compost treatment is significantly higher than that of twigs and leaves after the compound fertilizer treatment. According to the analysis of GO function annotation the total number of DEGs significantly down-regulated is more than that of DEGs up-regulated in leaves, while the total number of DEGs significantly up-regulated is more than that of DEGs down-regulated in twigs.

The main components of *P. bournei* EOs are sesquiterpenes. In the TBB pathway of sesquiterpenes synthesis the significant up-regulation or down-regulation of DEGs' expression plays a key role in the yield and composition of *P. bournei* EOs. Here, the expression of several genes regulating two key speed limiting enzymes (DXS and HMGR) and two important branch-point enzymes (FPPS and GGPPS) in TBB pathway are noteworthily down-regulated, and the expression of the genes regulating HMGS, another key enzyme, is significantly up-regulated. Among them only one gene (TRINITY_DN76855_c1_g3) regulating HMGS1 has been recorded in NCBI. The significant up / down regulation of the gene expression levels should be an important factor in the changes of the yield and the main components of *P. bournei* EO, and the number of down-regulated genes accounts for a large proportion, leading to the decrease of EO yield and the changes of the components of *P. bournei* EOs. In addition, the fertilization treatment also affected the biosynthesis pathway of GG and PPP, which provide the initial substrates for TBB synthesis.

The number of down-regulated genes in GG and PPP pathways is more than that of up-regulated genes in other samples except the twigs after the compound fertilizer treatment. Therefore, the down-regulation of the gene expression levels can reduce the primary substrates of sesquiterpenes and monoterpenes, which may indirectly affect the yield and composition of *P. bournei* EOs.

In general, both the two fertilization treatments had certain effects on the components and the yield of *P. bournei* EOs: the compost treatment increased the relative content of the primary components in the essential oil from *P. bournei* leaves, while the compound fertilizer treatment reduced its content; the two fertilization treatments had no significant effects on most of the main components of the essential oil from *P. bournei* twigs.

The two types of fertilizers used in this experiment are common fertilizers and complex mixtures. It is still unclear that the specific elements in the two fertilizers are responsible for the effects. Therefore, the experiment of using different single elements to treat *P. bournei* needs to be performed in the future work. On the other hand, the composition of the essential oil determines its bioactivity. The biological activity of the essential oil from *P. bournei* after the fertilization treatment merits further investigation.

Conclusions

The EO yield of *P. bournei* leaves after the compost treatment was decreased, but the relative content of the primary component (+) - calarene was increased in the essential oil; the compound fertilizer had no significant effects on the yield of the EO from *P. bournei* twigs, but had certain effects on the main components of the essential oils. After the fertilization treatments the expression levels of genes regulating DXS2, HMGR, FPPS and GGPPS were significantly down-regulated, indicating that the genes may be the important factors resulting in the changes in the yield and main components of the essential oils.

Methods

Fertilization method

The test samples were *P. bournei* trees from *P. bournei* plantation planted by the project staff with the seeds purchased from Jindong Forest Farm in Yongzhou city, Hunan Province, China, not the wild *P. bournei* trees, and they were treated with compost and compound fertilizer once a year, respectively. Leaves and twigs were collected from the *P. bournei* plantation in July of 2020.

Distillation Of Essential Oil

Fresh leaves / twigs (1000 g) were mixed with and 3000 g water and homogenized with for 2 min, then distilled for 5 h. The yield of the essential oil (%) was calculated as follows: mass of essential oil / sample dry weight × 100%. The essential oil (10 μL) was diluted with 1 mL absolute ethanol, then dehydrated with anhydrous sodium sulfate at room temperature (about 25 ℃) for 2 h, centrifuged, and the upper diluent was used for detecting the components of the essential oil via GC/MS.

Component Analysis By Gc/ms

The detection method of the essential oil from *P. bournei* was adjusted according to some reported methods [49–50]. GC detection conditions were the following: initial temperature was 50 ℃, increased to 260 ℃ by 5 ℃/min and the sample was injected into a capillary column (30 m × 250 μm × 0.25 μm) at split ratio of 10:1. The information of MS program was input: scanning range was 30-550AMU (m/z), with an ionizing voltage of 70 eV and an ionization current of 150 μA of electron ionization (EI). The flow velocity of helium was 1.2 mL/min. The ion source temperature was 230 °C, and the quadropole temperature was 200 °C.

Rna Sequencing, Data Processing, And Gene Annotation

Total RNA from each sample was isolated from frozen tissues (leaves and twigs) of *P. bournei* using the using TRIzol® Reagent according to the manufacturer's instructions (Invitrogen, Carlsbard, CA, USA) and genomic DNA was removed using DNase I (TaKara). The integrity and purity of the total RNA

quality was determined by 2100 Bioanalyser (Agilent Technologies, Inc., Santa Clara CA, USA) and the quantity was measured using ND-2000 (NanoDrop Thermo Scientific, Wilmington, DE, USA).

Transcriptome assembly was performed using the Trinity method [51]. The functions of *P. bournei* genes were annotated on the basis of the information from the following databases: National Center for Biotechnology Information (NCBI) non-redundant protein sequences (Nr) database, NCBI non-redundant nucleotide sequences (Nt) database, Gene Ontology (GO) database, protein family (Pfam) database, Clusters of Orthologous Groups of proteins (KOG/COG) database, manually annotated and reviewed SwissProt protein sequence database, Kyoto Encyclopedia of Genes and Genomes (KEGG) datebase, and Orthology (KO) database.

Gene expression levels were calculated using fragments per kilobase per million reads (FPKM) method in RSEM software [52]. The analysis of differential expression genes (DEGs) was performed using the DESeq R package (1.10.1), and the genes with an adjusted P-value < 0.05 identified by DESeq were considered as differentially expressed.

Statistical analysis

All experiments were repeated at least three times. The probability value of $p < 0.05$ was considered as significant difference.

Abbreviations

EO
essential oil; DEGs:differential expression genes; TPS:terpene syntheses; HMGR:3-hydroxy-3-methylglutaryl-coenzyme A reductase; GPP:geranyl diphosphate; FPP:farnesyl diphosphate; DXP:1-deoxy-d-xylulose-5phosphate; DXS:1-deoxy-D-xylulose-5-phosphate synthase; FPPS:Farnesyl pyrophosphate synthase; GGPPS:Geranylgeranyl diphosphate synthase.

Declarations

-Ethics approval and consent to participate

Not applicable

-Consent to publish

All authors have approved the manuscript and agree with submission to “BMC PLANT BIOLOGY”.

-Availability of data and materials

We will provide the file in an editable file format (docx), and we are willing to share our manuscripts and related data.

-Competing interests

The authors have no conflicts of interest to declare.

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- Authors' Contributions

LL is responsible for data analysis and article writing; GX H is the manager of the project and provides financial support; Professor DQ Z directs the writing of the thesis.

We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the manuscript and agree with submission to BMC PLANT BIOLOGY.

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Figures

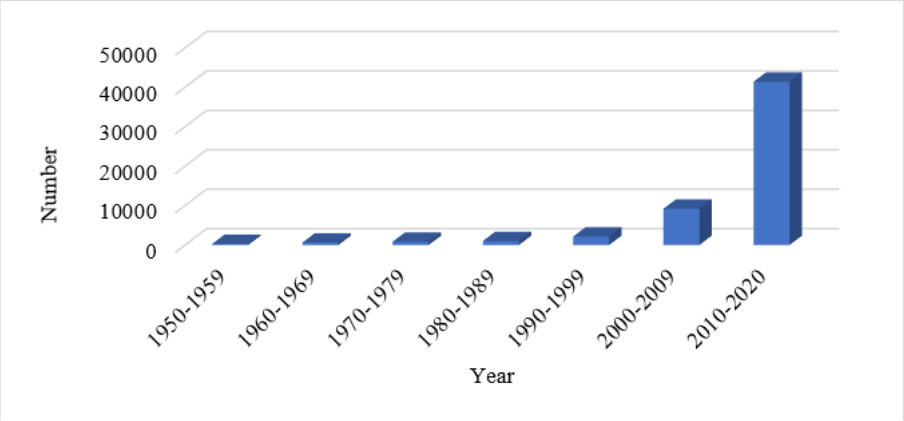

Figure 1

Number of publications regarding PEOs found in the Web of Science from the 20th century to the present (21st century).

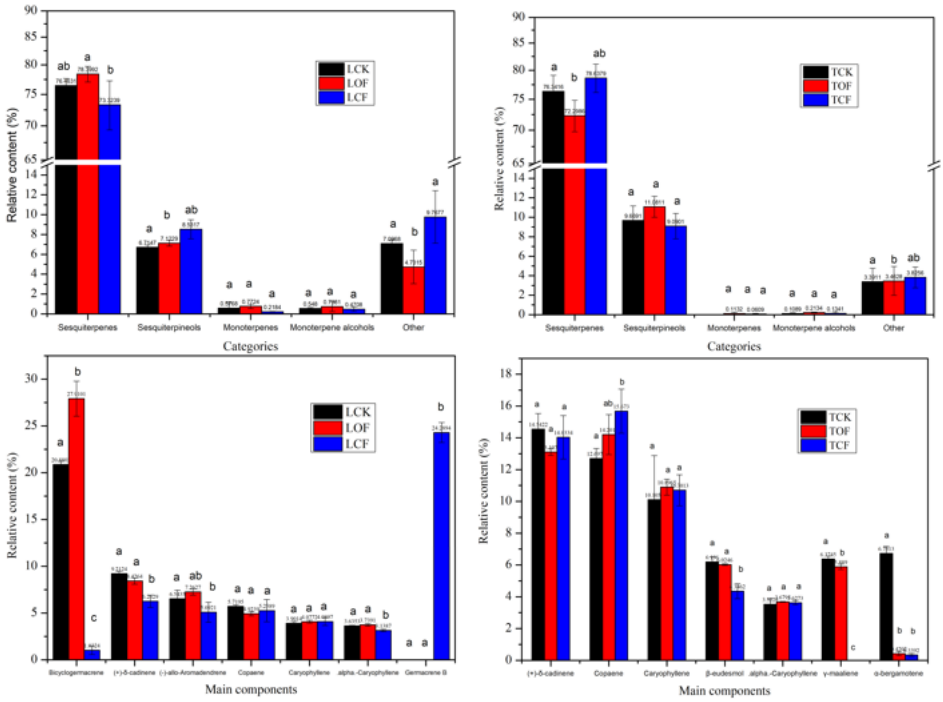

Figure 2

Note: 1) LCK — leaf control group; LOF — leaves treated with compost; LCF — leaves treated with compound fertilizer; TCK — twig control group; TOF — twigs treated with compost; TCF — twigs treated with compound fertilizer, the same below.

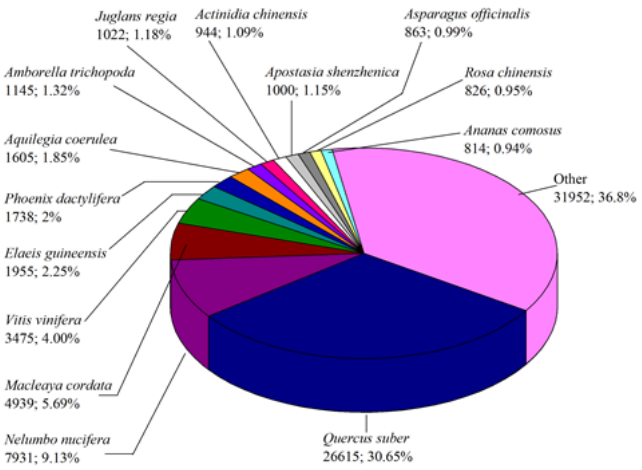

Figure 3

The BLASTX annotation results against NR database.

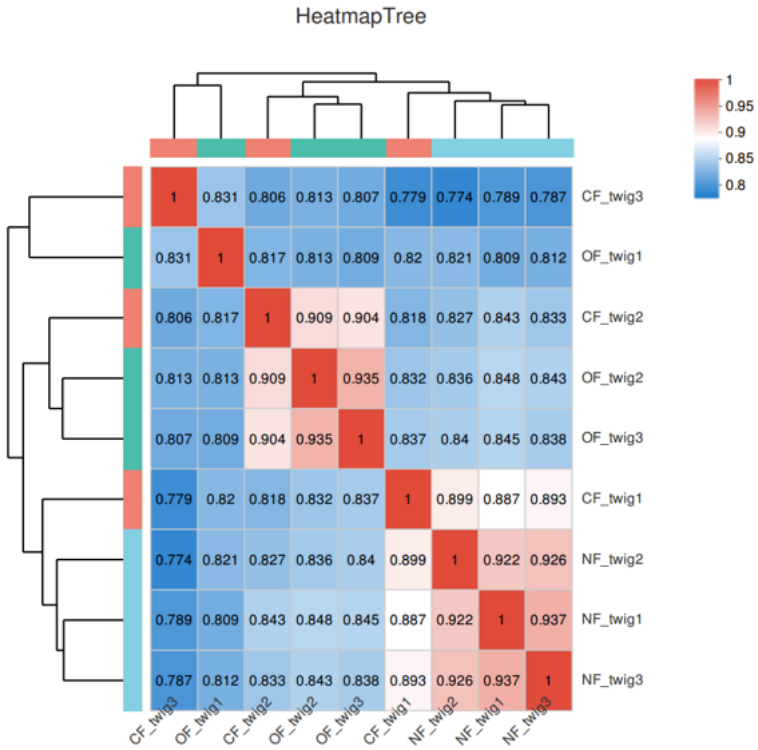

Figure 4

The correlation thermogram of all samples

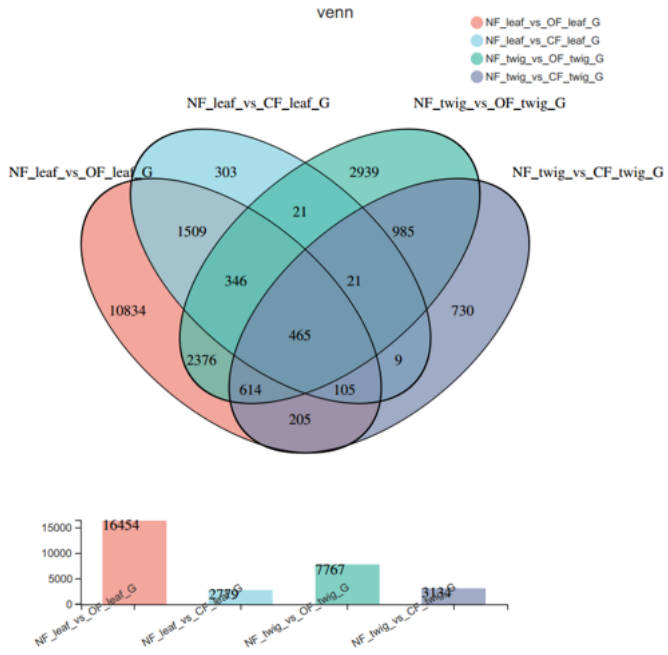

Figure 5

Venn diagram of DEGs after fertilization

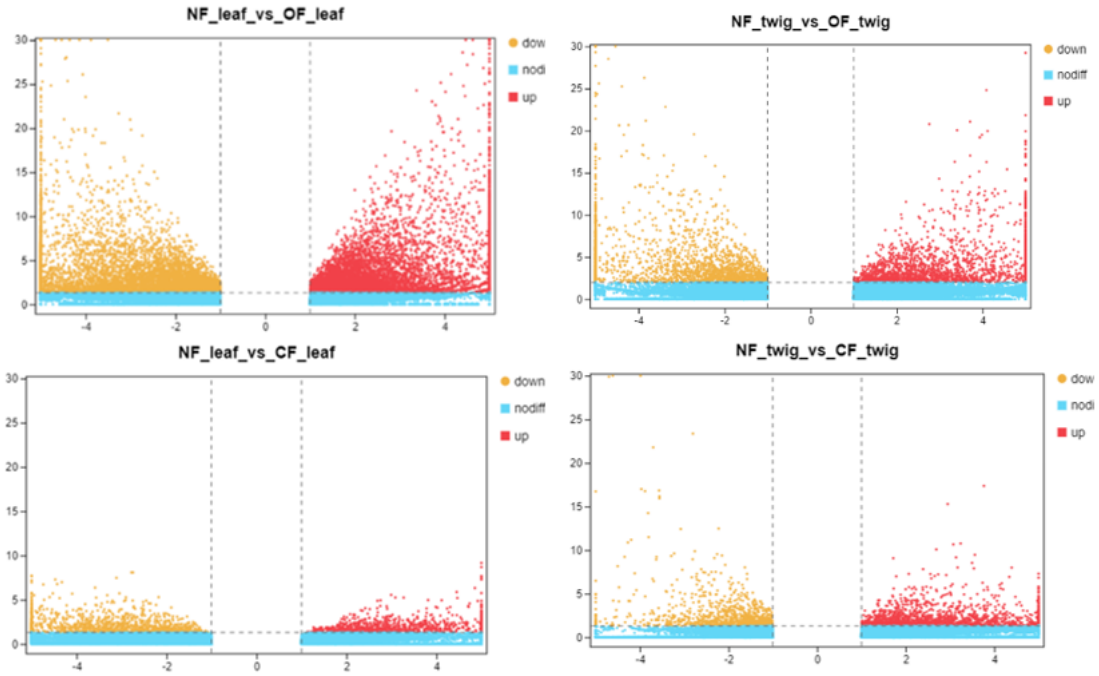

Figure 6

The number of DEGs significantly up-regulated and down regulated after fertilization (p-ajust < 0.05); the red points are significantly up-regulated genes, the yellow points are significantly down regulated genes, and the blue points are DEGs insignificantly regulated.

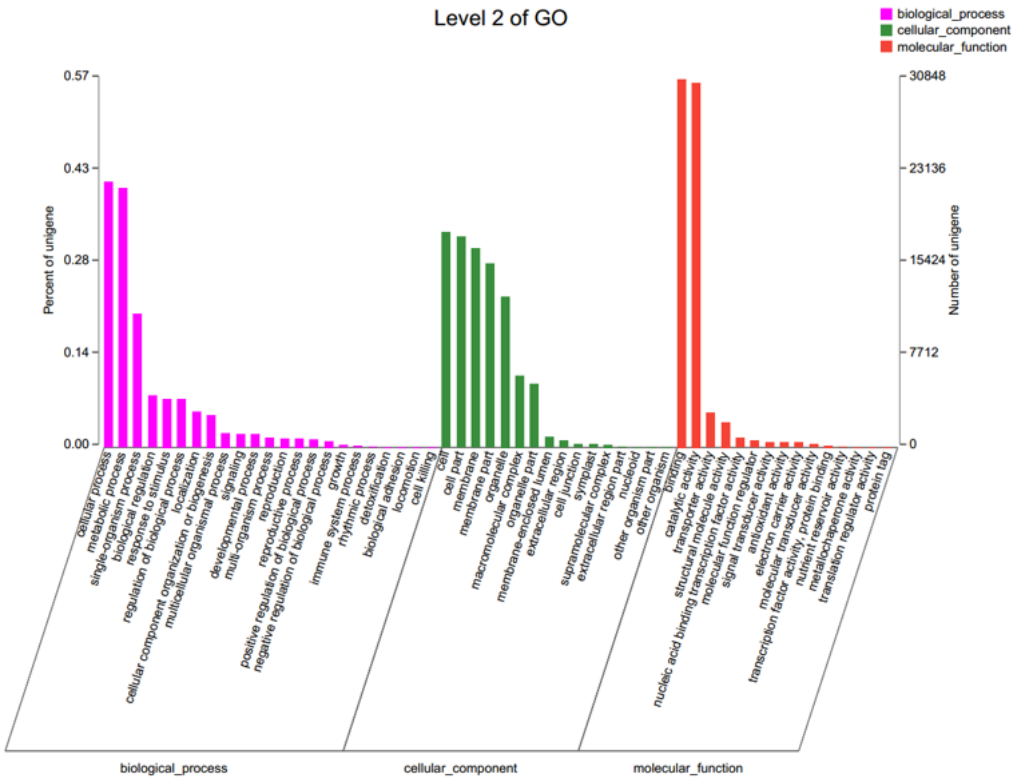

Figure 7

GO functional classification of all assembled unigenes in P. bournei.

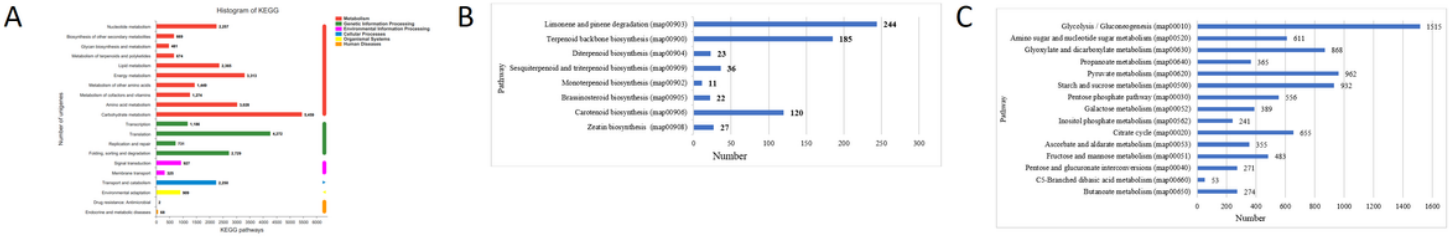

Figure 8

A KEGG classification of assembled unigenes in Phoebe bournei B Pathway classification of metabolism of terpenoids and polyketides C Pathway classification of Carbohydrate metabolism

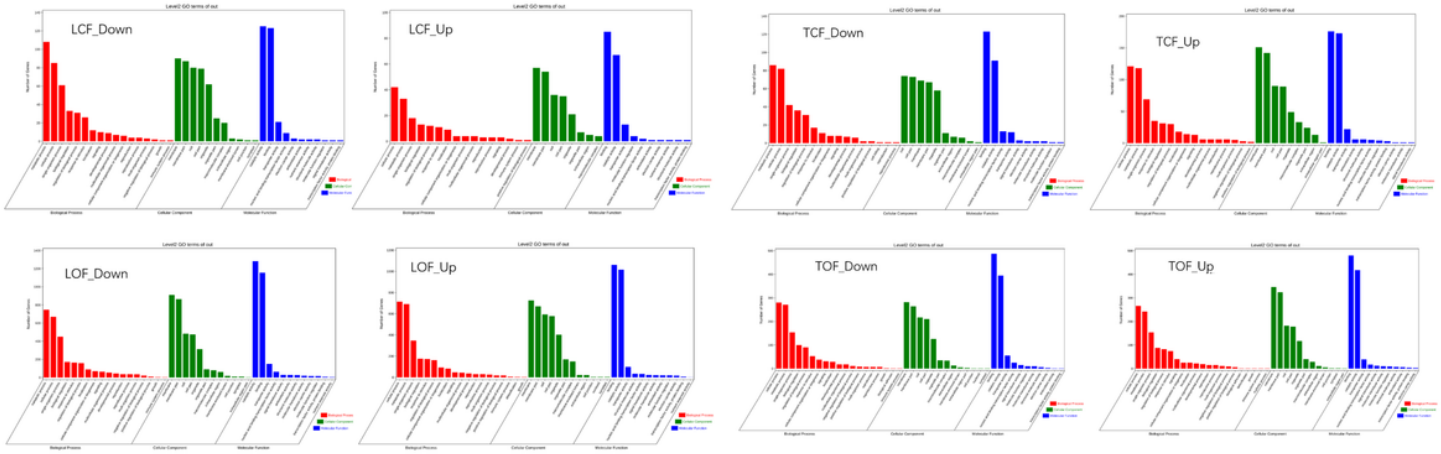

Figure 9

GO level 2 classification of DEGs significantly up-regulated and down-regulated after fertilization.

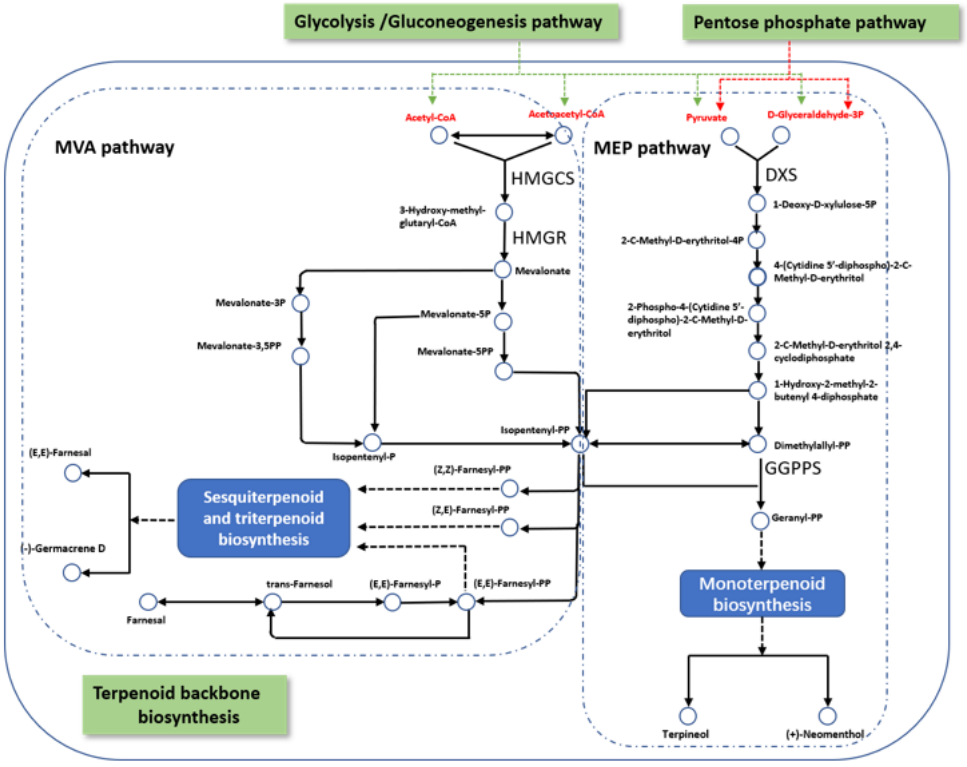

Figure 10

Synthetic pathways of sesquiterpenes and monoterpenes

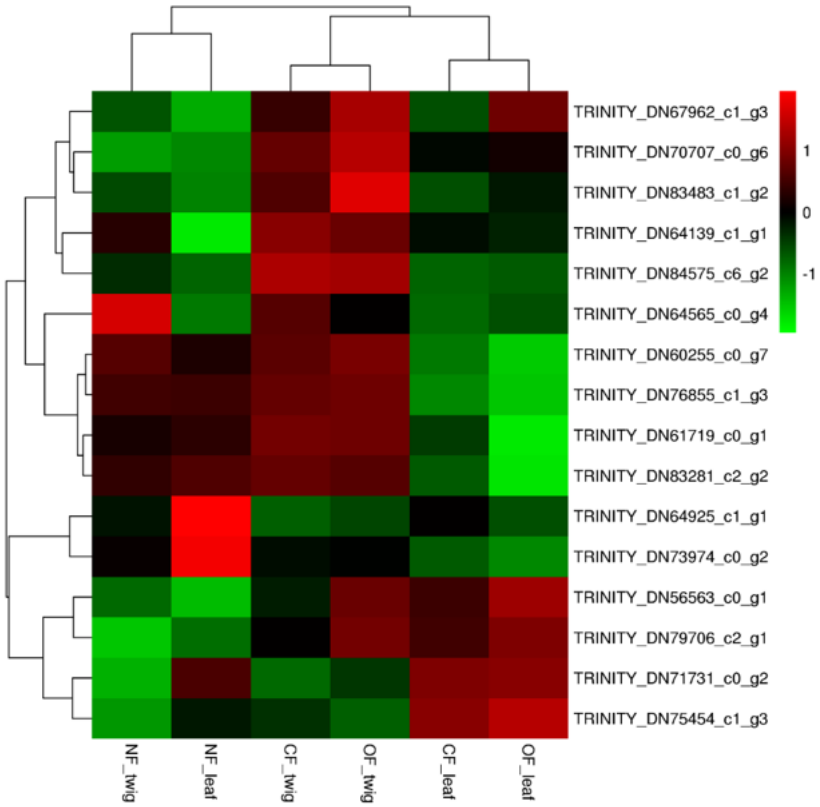

Figure 11

The cluster heat map of DEGs involved in map00900, map00909 and map00902; red means high expression and green means low expression.