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Shoot Culture of *Ocimum sp.* and Its Phytochemical Profile

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The success of the *in vitro* culture technique is influenced by many factors, including the type of explants, culture medium and exogenous plant growth regulators (PGR). These factors greatly influence the speed and effectiveness of plant regeneration as well as the profile of the phytochemical compounds produced. Therefore, this study aims to determine the effect of the PGR combination on the growth response of in vitro node explants as well as the phytochemical profiles of *Ocimum sp.* Node explants obtained from 2 weeks old in vitro seedlings were cultured on MS medium with the addition of PGR, namely BAP and Kinetin (0.2 and 5 mg/l) combined with NAA (0.1 and 0.2 mg/l) and synthetic cytokinins alone namely Thidiazuron (TDZ) (1, 3, and 5 mg/l). The growth response of the explants and the potential for regeneration were observed for 8 weeks of culture. Effects of adding activated charcoal (AC) to root media were observed on growth of plantlets aged 2 weeks. Phytochemical profile of *In vitro* shoot was analyzed using GC-MS and LC-MS to be compared with its profile in *in vitro* callus tissue. The results showed that MS medium with the addition of a combination of cytokinin and auxin was able to induce shoot regeneration in node explants of *Ocimum sp. in vitro*. The Kinetin/NAA combination produced better shoot height growth, while the BAP/NAA combination produced a higher leaves number. Thidiazuron at all concentrations was able to induce shoots that were more likely to form rosettes. The addition of AC to the rooting medium did not have a positive effect on the response of shoot and plantlet growth.

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Chromatographic screening showed different profiles of secondary compounds in the callus and shoot tissues of *Ocimum sp.* in vitro. Callus composed of actively dividing cells do not produce some of the secondary compounds as produced by in vitro shoots. This shows the difference in the potential of cells or tissues in synthesizing secondary metabolites.

Keywords: Cytokinin; GC-MS/LC-MS; node tissues; secondary metabolites; thydiazuron.

1. INTRODUCTION

Basil is a medicinal plant that is a member of the Lamiaceae family, which has more than 150 species [1]. Basil plants belong to Ocimum species as a member of Lamiaceae family are aromatic or ornamental plants which contain many essential oils and various secondary metabolites such as anthocyanins, flavonoids, and rosmarinic acid [2]. For pharmaceutical purposes, the utilization of Lamiaceae species has major constraints, due to variations at the individual level caused by heterogeneous genetics and biochemistry since hybridization and polyploidy between species occur frequently in this genus [3]. In addition, propagation by seed has problems due to poor viability and low germination frequency. In vitro culture technique is an alternative method to be able to produce offspring that are true-to-type and could improve secondary metabolite products. In vitro systems have been widely implemented in the medicinal plants micropropagation. The production of secondary metabolites which naturally depend on the growth phase of plants and only in small amounts makes tissue culture techniques an alternative solution to these problems.

In vitro micropropagation is an effective and relatively fast way of propagation of species that require high progeny uniformity. Plant cell, tissue and organ culture technology has been proven to be an alternative for all plant systems in producing natural products. Therefore, large-scale propagation of aromatic and medicinal plants using in vitro techniques is gaining increasing interest [4,5]. The success of the in vitro culture technique is influenced by many factors, such as the type of explants, culture medium and exogenous growth regulators (PGR). These factors greatly influence the effectiveness of plant regeneration and the phytochemical compounds produced both quantitatively and qualitatively. In vitro studies in the genus Ocimum have been reported using a variety of explants, such cotyledonary nodes, the first true leaves, hypocotyls, cotyledons [6,7,8], and shoot bud [9]. *In vitro* system can produce different profiles of secondary metabolites when compared to the original plant [10]. Therefore, the goal of this study is to determine the combination of growth regulators effect on the shoot induction of *Ocimum sp.* node explants in vitro and its phytochemical profiles.

2. MATERIALS AND METHODS

2.1 Seed Germination and Explants Preparation

Basil seeds (*Ocimum sp.*) were washed with running tap water for 1 hour and sterilized with 10% commercial bleach solution for 15 min. Subsequently, seeds were rinsed with sterile distilled water for 5 minutes three times. The sterilized seeds were germinated in water medium solidified with agar without the addition of either macro and micronutrients or PGR. Two weeks old seedling derived from seed germination were ready to be used as a source of explants.

2.2 Shoot Induction Media

Murashige and Skoog (MS) basal salt medium were used supplemented with 3% sucrose and solidified with 1.1% (w/v) agar. The pH was adjusted to 5.8 by adding NaOH or HCl 0.1 N prior to sterilization using autoclave at 121◦C, 1.5 atm for 15 minutes. A combination of cytokinins (BAP/ kinetin 2 and 5 mg/l) and auxin NAA (0, 0.1 and 0.2 mg/l) as well as TDZ alone in several concentrations (1, 3, and 5 mg/l) was added to observe their effect on regeneration and multiplication of shoot. All treatments consisted of six combinations of BAP-NAA and 3 concentrations of single TDZ.

2.3 Shoot Regeneration and Multiplication

The first nodes of seedling were used as explants and placed vertically on shoot induction medium. Each of nine PGR treatment were repeated in six bottles and each bottle contained

two explants. All cultures were kept in incubation room under continuous illumination from fluorescent white light (light intensity about 600 lux) at 24±2°C, with relative humidity of 60-65%. The leaves number were counted, and shoot height were measured every week for four weeks.

2.4 *In vitro* Rooting

Single shoots were separated from the shoots clumps and then transferred to the rooting medium (MS medium without hormones) with or without the addition of 0.1% (w/v) AC (activated charcoal). Shoot and root development was observed two weeks after subculture.

2.5 Phytochemical Analysis using GC-MS and LC-MS

Phytochemical analysis using GC-MS and LC-MS based on [11] with modification. Phytochemical screening and identification of phytochemical compounds were carried out on in vitro shoots and callus of Ocimum. Shoot samples were derived from MS hormone free medium while callus tissues were derived from MS + 2.4-D 1 mg/l + BAP 0.5 mg/l. The screening stage includes sample extraction using 95% methanol with a ratio of 1:5, followed by dilution of the extract with 95% methanol solvent to a concentration below 100 ppm. After being homogenized with a vortex and centrifuged at 8000 rpm for 10 minutes the resulting supernatant was used for the protein precipitation and purification stages with Solid Phase Extraction (SPE). The purified solution was then filtered with a 0.45 µm cellulose acetate filter membrane and the solution was ready to be analyzed by LC-MS. The Shimadzu LC-MS – 8040 LC-MS was used for this study. The filtration was carried out using polytetrafluoroethylene (PTFE) membrane filter (0.45 *µ*m size). One microliter (1 *µ*L) of the filtrate was introduced (injected) into the liquid chromatographical system. This was separated on Shimadzu Shim Pack FC-ODS (2 mm x 150 mm, 3 µm) column. Running was undertaken at a flow rate of 0.5 mL/min. The column temperature was fixed at 35°C with isocratic mobile phase mode. Scan range from *0,6 sec/scan (mz: 10-1000)* was used to acquire mass spectra.

The GC system (Shimadzu GC-MS QP 2010 SE) with ZB – AAA (10 mL x 0,25 mmLD (Phenomenex Inc) capillary column was used for this study. Helium (99.99%) gas was used to maintain flow rate of 0.6 mL/min at a constant column. GC-MS spectral lines was detected using the ionization energy method, in a 0.2 second scan time with a ranging fragment from 40 to 600 m/*z*, with one microliter (1 *µ*L) injection quantity (split ratio 10:1). The temperature was maintained at 250°C. The column oven temperature was at 50°C running for 3 minutes and 10°C temperature increase per minutes up to 280°C, with a final temperature of about 300°C for 10 minutes. The compound profiles including peak number, RT (retention time) per minute, curve area, composition and the structure and analysis were compared with the standard library of phenolic compounds.

2.6 Data Analysis

Quantitative data on shoot and root growth were analyzed using the One way-ANOVA method and Tukey's test as a follow-up on MS Excel 365 with a significance value of $p < 0.05$ while descriptive growth data were analyzed using relevant references. Phytochemical profiles were analyzed descriptively based on the types of compounds and their appearance on the chromatogram.

3. RESULTS AND DISCUSSION

3.1 Shoot Induction

The node explants showed a positive response in shoot induction on all media with various combinations of cytokinin:auxin. In general, shoot induction begins with the emergence of lateral shoots in the leaf axils 3-7 days after (Fig. 1A). Furthermore, adventitious shoots emerge from the node tissues in varying numbers (Fig. 1B). After four weeks of culture, every combination of cytokinin:auxin showed different effect on shoot growth (Fig. 2). In general, the kinetin/NAA combination produced higher shoots (0.30 – 2.14 cm) than those produced by the BAP/NAA combination (0.03 – 0.55 cm). On the other hand, the kinetin/NAA combination produced fewer leaves (1.27 – 9.07) than those produced by the BAP/NAA combination (2.93 – 17.87). According to [12] auxin and cytokinins are needed synergistically to control shoot growth. High leaf number was produced in the medium with the addition of 2 or 5 mg/l BAP combined with 0.1 mg/l NAA.

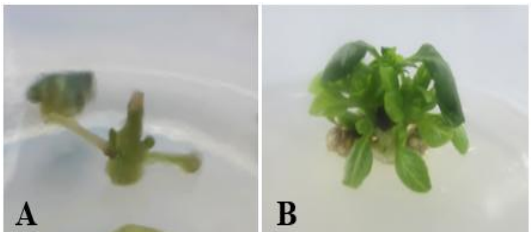

Fig. 1. Development of node explants and shoot regeneration. A. lateral shoots in the leaf axils, B. Multiple shoots derived from node explants

The concentration of TDZ tested was able to produce leaves but the number was less than the use of BAP or kinetin. Four weeks after culture the number of leaves produced at all TDZ concentrations tended to be the same between 10.7 to 14.4 (Fig. 3). Shoots derived from TDZ treatment were generally clustered to form a rosette (Fig. 4A). Therefore, shoot height on medium with the addition of TDZ could not be measured properly when compared to shoots on media with the addition of a combination of cytokinins and auxin (Fig. 4B). Thidiazuron also showed poor response in shoot formation of *O. sanctum* when compared with zeatin and BA [13]. However, TDZ could induce rapid micropropagation of *O. basilicum* using various types of explants except root [14]. An efficient method for micropropagation of basil on MS medium supplemented with TDZ was established using epicotyl, hypocotyl and shoot tip explants cultured [15].

3.2 Root Induction

Four weeks after culture the regenerated shoot was able to form roots without subculture to rooting medium. The quality and quantity of roots formed on shooting medium were varied. In general, medium supplemented with BAP and NAA showed poor respons in root formation (Fig. 5). Meanwhile the combination of Kinetin and NAA appears to be most suitable for producing a high number of roots. This is contrary to the effect of the combination of these growth regulators on shoot height (Fig. 2). The addition of kinetin alone produces more roots than the addition of BAP alone. Kinetin is a synthetic cytokinin which generally has a stronger effect than its natural compounds.

In this study the addition of AC to the medium tends to reduce the growth of *Ocimum sp.* in vitro. Shoot and leaf number in the medium without AC were always higher than in the medium with the AC (Fig. 6). While the addition of AC slightly increased shoot height. In *O. sanctum* micropropagation, the percentage of roots, roots number, and root length were positively affected by the addition of AC when compared to control media without AC [9].

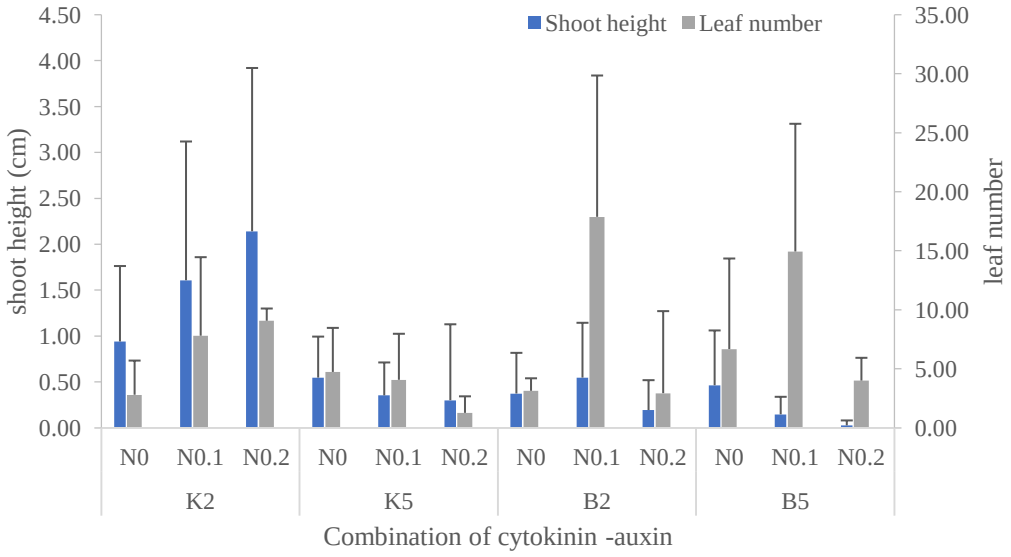

Fig. 2. Shoot regeneration derived from node explants of *Ocimum sp.* four weeks after culture. Notes: B: BAP; K: Kinetin, N: NAA

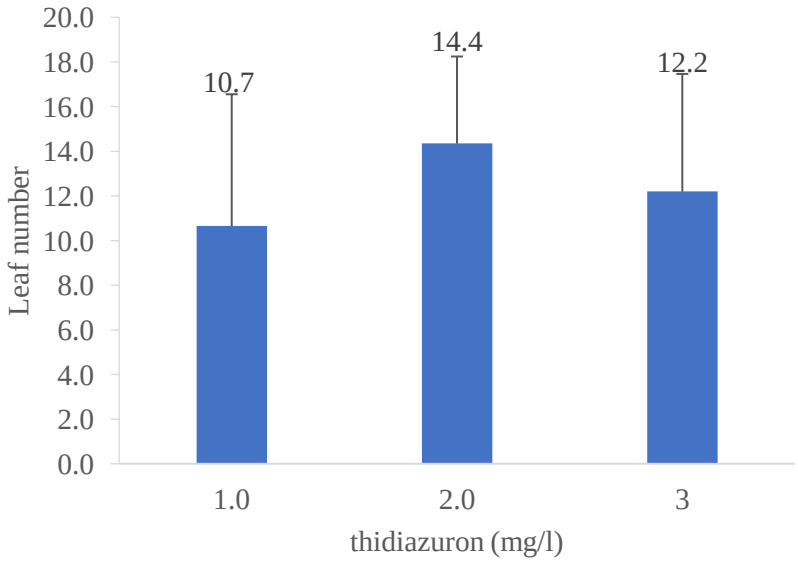

Fig. 3. The leaf number of *Ocimum sp.* in vitro in media containing TDZ

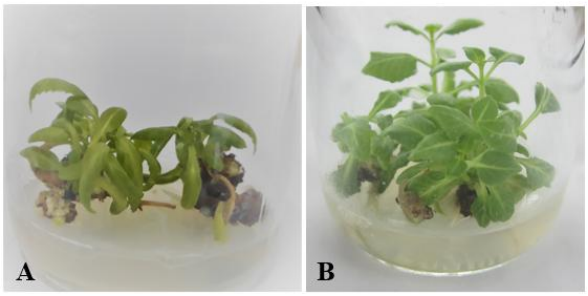

Fig. 4. Shoot growth of *Ocimum sp.* in vitro. A. MS medium + TDZ alone, B. MS medium + combined cytokinin-auxin

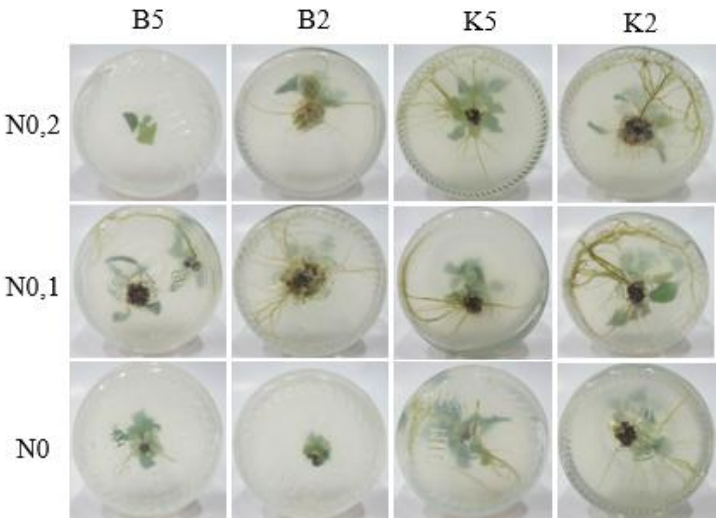

Fig. 5. Rooting in shoot induction medium with various combinations of auxin-cytokinin (mg/l) (N = NAA, B = BAP, K= Kinetin)

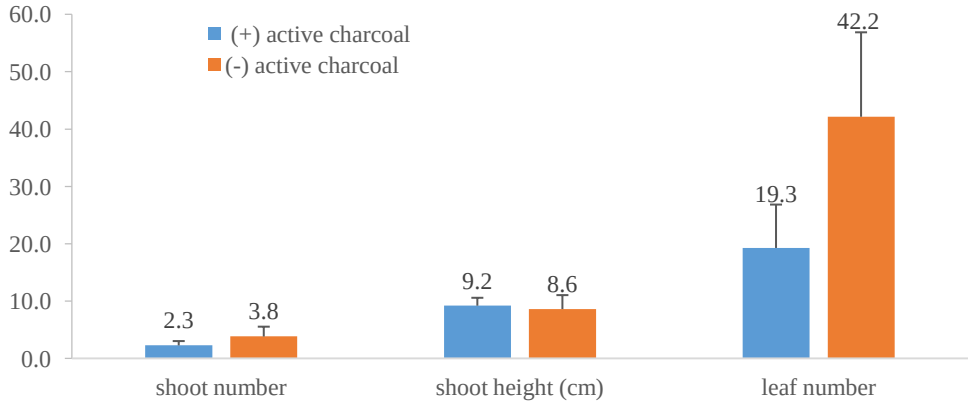

Fig. 6. Effect of AC (activated charcoal) in hormone free MS medium on shoot growth of *Ocimum sp.*

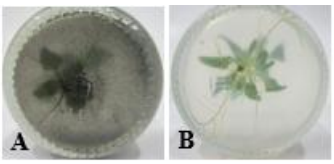

Fig. 7. Root induction of Ocimum regenerated shoot. (A) medium containing AC and (B) in medium without the addition of AC

According to [16] the addition of AC in the culture medium resulted in 100% rooted explants of five Agave clones. In aromatic rice the callus induction and plant regeneration frequencies in tissue culture medium could be enhanced by adding optimum concentration of AC combined with nanocarbon [17]. This suggests that AC appears to affect nutrient absorption in the culture medium. However, in this study AC did not show a significant effect on the root's growth (Fig. 7).

Activated charcoal (AC) is a porous carbonized substance with a large inner surface area [18] therefore they can adsorbe many substances [19]. Although AC can adsorp unwanted substances/inhibiotors [20] the addition of AC into tissue culture media is often associated with its role in increasing cell growth and development. The addition of AC simulated the dark condition in soil [21]. Analysis of the main metabolic pathways in wheat seedling revealed that AC stimulates the expression of nine genes in the phenylpropanoid biosynthetic pathway that promote cell differentiation and seedling growth [18]. However, AC addition did not show significant difference in in vitro root length of *Solanum sessiliflorumto* [22]. Even the addition of AC to the culture media has an unfavorable effect, namely a drastic reduction in the concentration of PGR and other organic supplements. On AC-free media resulted in a higher number of regeneration of plantlet shoots than media with low AC concentrations, while the number of shoots would decrease if the media was added with high AC concentrations [21]. It is possible that the addition of high concentrations of AC may induce nutrient deficiencies in culture media which related to high adsorption capacity and affected th explant growth [19].

3.3 Phytochemical Profiles of *Ocimum sp. In vitro*

The results of phytochemical analysis of *Ocimum sp.* using GC-MS method separated 78 volatile polar compounds with retention times ranging from 1.176 to 36.591 min (Fig. 8A-B). Meanwhile, analysis using LC-MS method detected 97 compounds with retention times ranging from 1.475 to 58.098 min (Fig. 8C-D). The first peak was β-ocimene with molecular weight of 136.2380 (Fig. 9A), while the last peak was ocimumoside B with molecular weight of 893.2060 (Fig. 9B). The compound with the smallest curve area of 73.9199 was germacrene B (retention time 5.483 min) (Fig. 9C) while the compound with the largest curve area of 1554.2209 was gallic acid (retention time 3.042 min) (Fig. 9D).

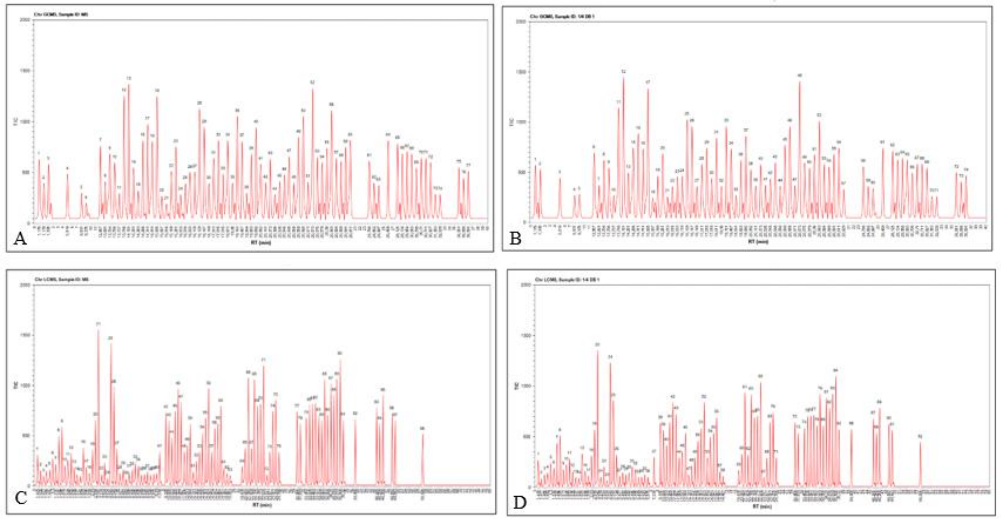

Fig. 8. Chromatography profile of *Ocimum sp.* in vitro. A-B. GC-MS analysis, B-C. LC-MS analysis, A,C Shoot tissues, B,D callus tissues

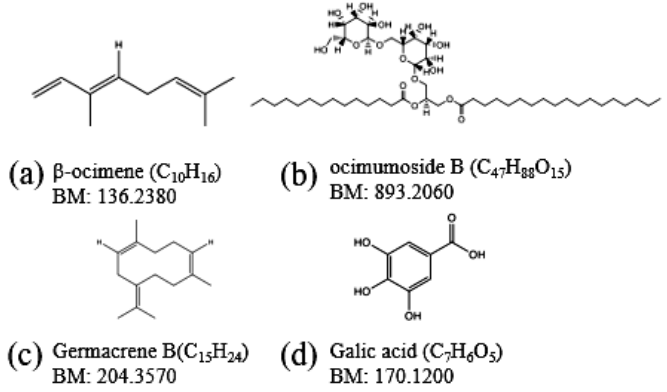

Fig. 9. Phytochemical types in *In vitro Ocimum sp.* (a). the first retention time, (b). the last retention time, (c) the smallest curve area, (d) the largest curve area

Table 1. Volatile non-polar compounds in shoot and callus in vitro of Ocimum

No	Compounds	Shoot	Callus	No	Compounds	Shoot	Callus
1	methyl formate	+	+	40	citronellyl formate	+	+
2	**1,3-pentadiene****	**+**	**-**	41	geranyl acetate	+	+
3	methyl butyrate	+	+	42	lavandulyl acetate	+	+
4	1-octen-3-ol	+	+	43	citronellyl acetate	+	+
5	2-phenylethyl alcohol	+	+	44	**α-calcorene****	**+**	**-**
6	p-cymene	+	+	45	(E,E)-α-farnesene	+	+
7	β-phellandrene	+	+	46	α-bourbonene	+	+
8	α-terpinolene	+	+	47	α-cadinene	+	+
9	α-pinene	+	+	48	α-cubebene	+	+
10	β-pinene	+	+	49	α-humulene	+	+
11	β-myrcene	+	+	50	α-muurolene	+	+
12	limonene	+	+	51	γ-muurolene	+	+
13	β-ocimene	+	+	52	α-ylangene	+	+
14	α-terpinene	+	+	53	germacrene A	+	+
15	**p-menthadiene****	**+**	**-**	54	β-bisabolene	+	+
16	γ-terpinene	+	+	55	germacrene B	+	+
17	3-hexenyl acetate	+	+	56	germacrene D	+	+
18	(2R)-nonan-2-ol	+	+	57	β-caryophyllene	+	+
19	thymol	+	+	58	β-chamigrene	+	+
20	4'-ethylacetophenone	+	+	59	β-elemene	+	+
21	**4'-methoxyacetophenone****	**+**	**-**	60	bicyclo-germacrene	**+**	**+**
22	dehydro-1,8-cineole	+	+	61	**longifolene***	-	+
23	geranial	+	+	62	allo-aromadendrene	+	+
24	sabinene	+	+	63	β-selinene	+	+
25	1,4-cineole	+	+	64	γ-cadinene	+	+
26	cis-rose oxide	+	+	65	geranyl propionate	+	+
27	linalool	+	+	66	elemol	+	+
28	citronellal	+	+	67	farnesol	+	+
29	geraniol	+	+	68	α-copaene	+	+
30	isoborneol	+	+	69	spathulenol	+	+
31	citronellol	+	+	70	10-epi-γ-eudesmol	+	+
32	isomenthol	+	+	71	β-eudesmol	+	+
33	neoisomenthol	+	+	72	11-selina-4-α-ol	+	+
34	eugenol	+	+	73	nerolidol	+	+
35	safrole	+	+	74	T-cadinol	+	+
36	1-octen-3-yl acetate	+	+	75	selina-4,11-diene	+	+
37	methyleugenol	+	+	76	geranyl butyrate	+	+
38	coniferylalcohol	+	+	77	geranyl isobutyrate	+	+
39	geranyl formate	+	+	78	citronellyl butyrate	+	+

*Note: '+' compounds are detected as peaks according to the retention time; '-' compounds are not detected as peaks according to the retention time; * compounds are only detected in callus; ** compounds are only detected in shoots*

Table 2. Non-volatile compounds in shoot and callus *In vitro* of Ocimum

No	The compounds	Shoot	Callus	No	The compounds	Shoot	Callus
1	β-ocimene	+	+	50	thymoquinol 2-O-B-glucopyranoside	+	+
2	**α-terpinene***	**+**	**-**	51	cirsillineol	+	+
3	α-pinene	+	+	52	eupatorin	+	+
4	limonene	+	+	53	nevadensin	+	+
5	α-terpinolene	+	+	54	gardenin B	+	+
6	β-myrcene	+	+	55	5,6,4'-trihyfroxy-7,3'- dimethoxyflavone	+	+
7	β-phellandrene	+	+	56	chlorogenic acid	+	+
8	cinnamic acid	+	+	57	gardenin D	+	+
9	estragole	+	+	58	gardenin E	+	+
10	thymol	+	+	59	labiatenic acid	+	+
11	1,8-cineole	+	+	60	thymonin	+	+
12	geraniol	+	+	61	squalene	+	+
13	borneol	+	+	62	gardenin C	+	+
14	linalool	+	+	63	**gardenin A***	**+**	**-**
15	p-coumaryl alcohol	+	+	64	α-amyrin	+	+
16	citronellal	+	+	65	apigenin-7-O-glucoside	+	+
17	4-methoxycinnamaldehyde	+	+	66	cosmoslin	+	+
18	p-coumaric acid	+	+	67	kampferol-3-O-rhamnoside	+	+
19	safrole	+	+	68	acacetin-7-glucoside	+	+
20	eugenol	+	+	69	acacetin-7-galactoside	+	+
21	gallic acid	+	+	70	quercetin-3-O-rhamnoside	+	+
22	coniferaldehyde	+	+	71	luteolin-7-glucoside	+	+
23	methyleugenol	+	+	72	betullinic acid	+	+
24	coniferylalcohol	+	+	73	hirsutrin	+	+
25	caffeic acid	+	+	74	quercetin-3-glucoside	+	+
26	ferulic acid	+	+	75	chicoric acid	+	+
27	menthyl acetate	+	+	76	kampferol-3-(2''-acetylrhamnoside)	+	+
28	germacrene A	+	+	77	luteolin-7-O-(6''-malonylglucoside)	+	+
29	α-cubebene	+	+	78	quercetin-3-O-malonylglucoside	+	+
30	**germacrene B***	**+**	**-**	79	apigenin-7-(6''-p-coumarylglucoside)	+	+
31	α-copaene	+	+	80	luteolin-7-aplosyl (1-2) glucoside	+	+
32	β-caryophyllene	+	+	81	naringin	+	+
33	β-chamigrene	+	+	82	apigenin-7-[rhamnosyl (1-2) galacturoside]	+	+
34	α-farnesene	+	+	83	acacetin-7-rutinoside	+	+
35	β-selinene	+	+	84	kaempferol-7-rhamnoside-4'-glucoside	+	+
36	germacrene D	+	+	85	luteolin-3'-methyl ether-7-apiosyl (1-2) glucoside	+	+
37	α-zingiberene	+	+	86	apigenin-7-glucoside-4'-trans-caffeate	+	+
38	**β-cadinene***	**+**	**-**	87	kampferol-3-(5''-feruloylaloside)	+	+
39	ocimarin	+	+	88	luteolin-7-O-rutinoside	+	+
40	**nerolidol**	**+**	**-**	89	kampferol-3-(6''-caffeoyglucoside)	+	+
41	sinapic acid	+	+	90	quercetin-3-(3''-p-coumarylglucoside)	+	+

No	The compounds	Shoot	Callus	No	The compounds	Shoot	Callus
42	apigenin	+	+	91	tulsinol E	+	+
43	acacetin	+	+	92	apigenin-7-rhamnoside-4'-rutinoside	+	+
44	genkwanin	+	+	93	kaempferol-3-glucoside-2''-rhamnoside-7-rhamnoside	+	+
45	luteolin	+	+	94	apigenin-7-rutinoside-4'-trans-caffeate	+	+
46	kaempferol	+	+	95	quercetin-3,7,4'-triglucoside	+	+
47	quercetin	+	+	96	ocimumoside A	+	+
48	ladanein	+	+	97	ocimumoside B	+	+
49	salvigenin	+	+				

Note: '+' compounds are detected as peaks according to the retention time; '-' compounds are not detected as peaks according to the retention time

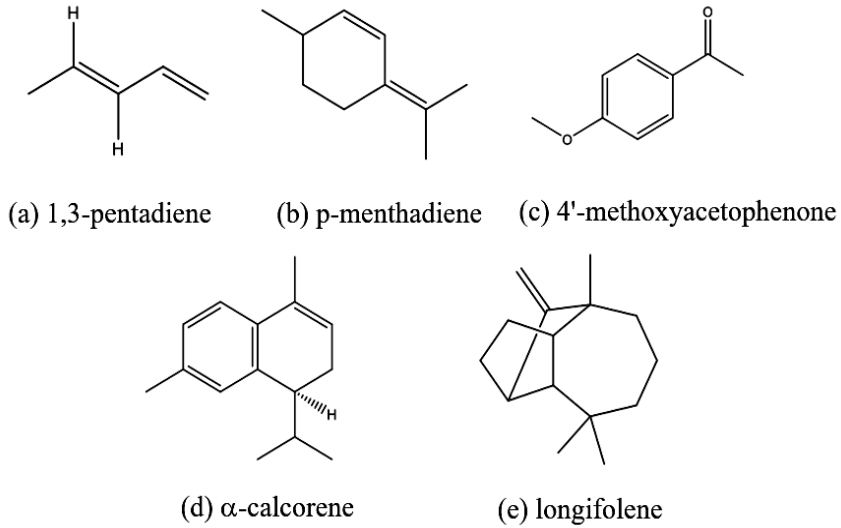

Fig. 10. Specific volatile non-polar compounds detected in shoot and callus in vitro of *Ocimum sp.* (a-d). the compounds only detected in shoot, (e) the compound olny detected in callus

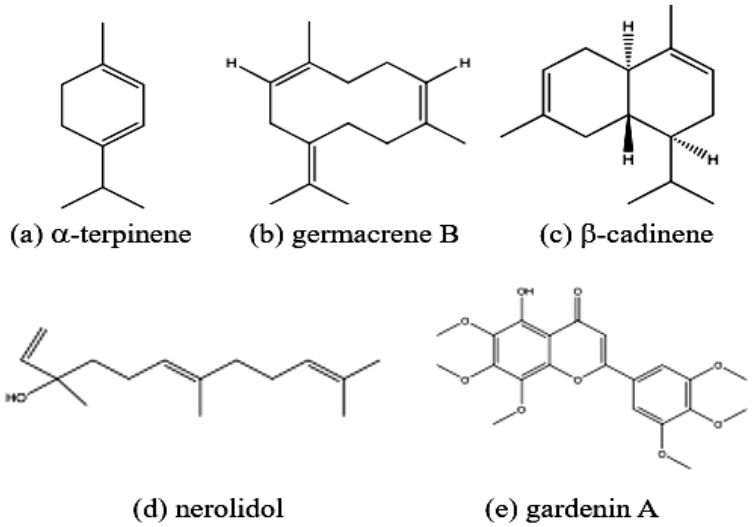

Fig. 11. Specific non-volatile compounds only detected in shoot in vitro of *Ocimum sp.*

The results of analysis using the GC-MS and LC-MS methods showed differences in the phytochemical profiles of Ocimum shoots and callus in vitro. There were 78 volatile non-polar compounds detected by GC-MS (Table 1). Seventy-three of these compounds were produced by both shoot and callus in vitro. However, there are four compounds produced by shoot but not detected in callus, namely 1,3-pentadiene (C_5H_8), p-menthadiene ($C_{10}H_{16}$), 4'-methoxyacetophenone ($C_9H_{10}O_2$), and α-calcorene ($C_{15}H_{20}$). In contrast, the longifolene compound ($C_{15}H_{24}$) only appeared in callus but not in shoot (Fig. 10).

The type of tissues affected the composition of the secondary compounds synthesized in *Ocimum sp.* cultured in medium MS + 0.25 mg/l 2,4-D + 1 mg/l BAP. Non-volatile compounds in Ocimum shoot tissue in vitro detected by HPLC were 97 compounds. However, only 92 compounds were detected in callus tissue (Table 2). Five compounds, namely α-terpinene ($C_{10}H_{16}$), germacrene B ($C_{15}H_{24}$), ocimarin ($C_{12}H_{12}O_4$), nerolidol ($C_{15}H_{26}O$) and gardenin A ($C_{21}H_{22}O_9$) were not detected in callus culture (Fig. 11).

In vitro culture techniques are often used as a strategy to produce secondary metabolites. According to [23] *In vitro* culture can change the pathway of secondary metabolite synthesis by expanding the production of phytochemicals in plants. Metabolite production which may represent a similar metabolite profile to native plants have been reported in types of ogan culture such as shoots or roots [24]. Callus culture also has the potential to produce secondary metabolites that have therapeutic significance [25]. Even callus culture has been reported to be more reliable than collecting plant material from the wild for extracting therapeutic metabolites [26]. On the other hand, PGR are one of the most important factors influencing cell growth, differentiation, and formation of metabolites [27,28]. The composition of in vitro culture and tissue media also determines the accumulation of secondary compounds. In this study, the different phytochemical profiles in shoot and callus tissue were thought to be caused by added exogenous PGR. These results indicate that the production of secondary metabolites in vitro is also influenced by exogenous ZPT which induces specific tissues.

4. CONCLUSION

This study showed that in vitro Ocimum shoots were successfully induced by the addition of a combination of NAA and BAP/Kinetin. The adding of AC had no significant effect on shoot growth or root formation. Regenerated shoots from node explants from in vitro callus tissue had a different phytochemical profile. This proves that PGR not only affects cell growth and development but also the synthesis or production of secondary metabolites.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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