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Effects of Carbon and Nitrogen Sources on the Essential Oil Production and Its Composition in Callus Culture of *Mentha piperita* L.

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탄소와 질소원이 박하정유와 정유성분에 미치는 영향

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ABSTRACT : The effects of carbohydrate sources(sucrose, glucose) and ratio of ammonia to nitrate on essential oil production and its com- positions in the callus culture of *Mentha piperita* L. were studied.

An ammonia : nitrate ratio of 1:2 was more effective for essential oil production regardless of the media used ; Lin-Staba(LS) and modify Murashige-Skoog(MS) medium.

Menthol biosynthesis was enhanced when ratio of ammonia to nitrate was 1:3 in the LS medium while the ratio was 1:2 in the MS medium.

Lower sucrose concentration(20g /l) was much better than higher sucrose concentration(30g /l) for both oil and menthol biosynthesis in the LS medium but higher sucrose concentration(30g /l) was more effective for those in the MS medium. When sucrose was replaced with glucose, menthol biosynthesis was sharply decreased or absent regardless of media used.

Plant cell culture techniques have been widely applied for in vitro production of secondary metabolites. Kireeva et al(1978) reported that menthol and menthone biosynthesis were inhibited and mainly their precursors (piperitone and pulegone) were accumlated in the callus cells as compared with that of intact plant. These results have been confirmed again by Chae and Park(1991).

The medium components which generally affect the secondary metabolite production are concentrations of nitrogen and sugars as well as growth regulators. Bricout and Paupardin (1975) stated that essential oil in the callus cells was synthesized partially at the expense of glucose present in the medium. Wang and Staba(1963) found that sucrose was hydrolyzed to dextrose and fructose in suspension culture of peppermint callus.

We have previously examined the effects of auxin, media and light on callus formation (Park and Chae, 1991) and have reported that essential oil and its composition were varied with growth regulators in the callus culture of *Mentha piperita*(Jin and Chae, 1991). In this paper, the effects of carbohydrate sources and

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relative concentration of nitrate to ammonia on essential oil yield and its composition in callus culture of *Mentha piperita* L. were examined.

MATERIALS AND METHODS

Park and Chae(1990) previously reported on the callus initiation and growth. Induced calli were subcultured for 3 weeks on Lin-Staba (LS) medium containing 2,4-D(2mg/l) for proliferation before final culture on the LS and modify Murashige-Skoog(MS basal medium+600mg/l casein hydrolysate) medium with different nitrogen and carbohydrate sources. The relative concentration of nitrate and ammonia in the LS and MS media were presented in table 1. And different carbohydrate sources and their amounts in the LS and MS media were shown in table 2.

Rapid solvent extraction method(McCarthy et al, 1980) was modified for essential oil extraction from callus cells. Calli were freeze dried and soaked for 24hrs in solution mix with 2 volumes of pentane and one volume of dichloromenthane before application of charcoal to purify and anhydrous sodium sulfate for dehydrolyzation. Oil content was determined by the weight method.

Oil composition was analyzed by gas chromatography(Hewlett-Packard 5890A) with supelcowax 10 fused silica capillary column and flame-ionization detector. N_2 was used as carrier gas, and programmed temperature was 100-200°C(5°C/min). Injecor and detector temperature were 230°C and 250°C, respectively.

Table 1. Total amount of nitrogen and ratio of ammonia to nitrate nitrogen in Lin-Staba and MS-modify media.

Source	Lin-Staba medium			MS-modify medium		
	L1	L2	L3	M1	M2	M3
NH_4^+	8.99	17.98	8.99	20.61	41.22	10.30
NO_3^-	18.39	27.38	27.79	39.40	60.02	29.10
NH_4^+/NO_3^-	1/2	1/1.5	1/3	1/2	1/1.5	1/3

Table 2. Concentration of sucrose and glucose in Lin-Staba and MS-modify media (unit : g/l).

Source	Lin-Staba medium			MS-modify medium		
	L1	L4	L5	M1	M4	M5
Sucrose	20	30	-	30	20	-
Glucose	-	-	20	-	-	20

RESULTS

Callus growth and oil content in the LS and MS media with different ratio of ammonia to nitrate were shown in table 3. In general, oil content was higher in the LS than in the MS medium. The ratio of ammonia to nitrate with 1:2(L1 and M1 in table 1) showed the highest oil content regardless of the media used. However, the end products(menthone and menthol) were higher in both L3($NH_4^+ : NO_3^- =$ 1:3) and M3($NH_4^+ : NO_3^- = 1:2$) medium(table 4).

As increased sucrose content from 20g/l

Table 3. Effect of the ratio of ammonia to nitrate nitrogen in Lin-Staba and MS-modify media on growth and essential oil production in *Mentha piperita* callus cultures.

Media	Fresh wt.(mg)	Dry wt.(mg)	Oil wt.(mg)	Oil content(%)*
L1	35,189	1,329	22.0	1.66
L2	45,074	1,998	30.0	1.50
L3	48,307	2,176	34.0	1.56
M1	30,001	1,979	12.6	0.64
M2	38,189	2,276	3.4	0.15
M3	62,458	2,788	13.7	0.49

* Oil content was calculated on the dry weight basis.

Table 4. Effect of the ratio of ammonia to nitrate nitrogen in Lin-Staba and MS-modify media on the composition of essential oil in *Mentha piperita* callus cultures.

Media	Composition of essential oil(%)			
	Pulegone	Menthylacetate	Menthone	Menthol
L1	4.37	6.67	2.48	2.17
L2	1.04	–	0.42	0.35
L3	1.32	–	2.25	7.31
M1	1.88	5.60	3.77	19.31
M2	1.34	–	–	1.07
M3	0.94	–	0.15	0.23

Table 5. Effect of the concentration of sucrose and glucose in Lin-Staba and MS-modify media on growth and essential oil production in *Mentha piperita* callus cultures.

Media	Fresh wt.(mg)	Dry wt.(mg)	Oil wt.(mg)	Oil content(%)*
L1	35,189	1,329	22.0	1.66
L4	68,502	3,698	11.0	0.30
L5	49,567	2,027	45.0	2.22
M1	30,001	1,979	12.6	0.64
M4	38,290	1,723	3.8	0.22
M5	32,148	1,553	4.9	0.32

* Oil content was calculated on dry weight basis.

Table 6. Effect of the concentration of sucrose and glucose in Lin-Staba and MS-modify media on the composition of essential oil in *Mentha piperita* callus cultures.

Media	Composition of essential oil(%)			
	Pulegone	Menthylacetate	Menthone	Menthol
L1	4.37	6.67	2.48	2.17
L4	1.04	10.39	0.10	–
L5	1.49	4.94	1.49	–
M1	1.88	5.60	3.77	19.31
M4	1.86	–	–	0.58
M5	0.22	–	–	0.15

(L1) to 30g/1(L4), callus growth was enhanced but oil content was dropped from 1.66% to 0.3% in the LS medium(Table 5). However, higher concentration of sucrose (30g/1=M4) increased oil weight and oil content in the MS medium(Table 5). When sucrose was replaced with glucose in the LS medium(L5), oil weight and oil content were increased than in the L1 medium while there was no difference in the MS medium(M5 vs M4) as shown in table 5.

Menthylacetate was the main component and no menthol was biosynthesized as increased sucrose ontent(L4) in the LS medium (Table 6). On the contrary, higher sucrose content(M1) brought in higher accumulation of menthone and menthol in the MS medium (Table 6). The LS medium containing glucose instead of sucrose(L5) increased oil content (Table 5) but menthol was not produced (Table 6), and both oil contents(Table 5) and end products(Table 6) were much lower in the MS medium.

DISCUSSION

The result that we had previously recognized higher oil content in the LS medium rather than MS medium in the callus culture of *Mentha piperita* L. was recomfirmed again in this study(Table 3 and 5). However, increased oil content was not accompanied with higher accumulation of end products(Table 4 and 6).

The relative concentration of ammonia to nitrate had important role on both oil content and end products biosynthesis in the cultured cells. Relatively higher oil content was obtained in the ammonia : nitrate ratio of 1:2 in the LS medium while menthone and menthol were accumulated more in the ammonia : nitrate ratio of 1:3(Table 3 and 5). However, both higher oil content and end product accumulation were observed in the ammonia : nitrate ratio of 1:2 in the MS medium(Table 3 and 5). Ikeda et al(1977) reported that the balanced relationship between ammonia and nitrate was more important than the amount of total nitrogen on ubiquinone production in tobacco cell suspension culture. Similar result was observed by Yamakawa et al(1983) for anthocyanin production by *Vitis* suspension culture and solasodine formation in suspension culture of *Solanum*(Nigra et al 1990). Hagimori et al (1982) found that digitoxin production was enhanced in the ratio of ammonia to nitrate was 1:2 while digitoxin was decreased rapidly when only either one of nitrogen sources was provided. The results of the L3 and M1 medium in table 4 suggested that each medium has its own proper ratio between ammonia and nitrate for end product formation.

The effect of increasing sucrose concentration on oil content and final product formation was varied with media used(Table 5 and 6). Sucrose concentration of 20g /l in the LS medium was appeared as proper level for both oil and end product biosynthesis. However, the positive effect of increased sucrose (30g /l) in the MS medium on both oil and end product biosynthesis in this study was in agreement with that of reports by Yamamoto et al(1989) who observed that accumulation of anthocyanin was enhanced by increased sucrose concentration in suspension culture of *Euphorbia millii*. Similar result was reported (Hagimori et al, 1982) for the digitoxin formation in *Digitalis purpea* liquid culture. Therefore, different level of sucrose concentration with different media must be considered in secondary metabolite production through in vitro culture of cells.

Glucose was appeared to be inferier to sucrose for menthone and menthol accumulation regardless of the media used in this paper. Replacement of sucrose with glucose brought in decrease of diosgenin production of *Dioscorea deltoidea* culture(Tal et al, 1982) and digioxinformation of *Digitalis purpuea* culture (Hagimori et al, 1982). However, there were some reports indicated no difference between sucrose and glucose in the production of shikonin(Mizukami et al, 1977), ubiquinone (Ikeda et al, 1976) and anthocyanin(Matsumoto et al, 1973).

Yamakawa et al(1983) indicated that the ratio of nitrogen to carbohydrate was important for anthocyanin formation in grape cell culture. Higher anthocyanin was biosynthesized in the ratio of sucrose to nitrogen was 2.5 : 1. In this experiment, the calculated ratio of sucrose to nitrogen in the L3 and M1 medium(Table 1) which showed higher accumulation of menthone and menthol(Table 4) were 1.59:1 and 1.46:1, respectively. This implied that end products were influenced by the ratio of nitrogen to carbohydrate as well as ammonia to nitrate in peppermint callus cultrue.

摘　　要

본 연구에서는 탄소원, 질소원의 비율, inositol 및 비타민이 박하정유 생산과 멘톨생합성에 미치는 영향을 조사한 결과 암모니아태질소와 초산태질소의 비율이 1:2일 때 사용한 배지에 관계없이 정유생산이 많았다. 멘톨 생합성이 높았던 경우는 LS배지에서는 암모니아태와 질산태질소의 비율이

1 : 3이었고 MS배지에서는 1 : 2이었다. Sucrose 대신 glucose를 사용하였을 때는 사용한 배지에 관계없이 정유생산과 멘톨 생합성이 모두 감소하였다. Inositol의 농도를 증가하였을 경우 세포의 생장량은 증가하였으나 멘톨 함량은 감소하였다. 비타민 농도를 기본배지의 두배로 하였을 때 LS배지에서는 정유함량이 증가하였으나 MS배지에서는 감소하였다. 멘톨 함량은 모두 감소하였다.

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