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Full Length Research Paper

Rosmarinic acid biosynthesis in callus and cell cultures of *Agastache rugosa* Kuntze

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Callus and cell suspension cultures of *Agastache rugosa* were established to investigate the production of rosmarinic acid in vitro. For callus induction 2,4-D treatment was more effective than that of NAA. The best callus induction rate (92%) and callus growth *A. rugosa* were obtained in MS medium containing 2 mg/l 2, 4-D. In cell suspension culture of *A. rugosa*, the maximum growth (7.7 g/l) and the highest rosmarinic acid production (11.5 mg/g) were attained in the liquid B5 medium supplemented with 2 mg/l 2,4-D and 0.1 mg/l BAP at 10 days after culture. The present results demonstrate that cell culture of *A. rugosa* might be an alternative approach for the production of rosmarinic acid.

Key words: *Agastache rugosa*, callus, cell suspension, rosmarinic acid

INTRODUCTION

Agastache rugosa Kuntze, belonging to the mint family (Labiatae), is a perennial medicinal plant widely distributed in China, Japan, Korea and Siberia. It is used in Chinese traditional medicine for the treatment of cholera, vomiting, and miasma, and has been reported to have antitumor, antifungal, antiatherogenic and cytotoxic activities (Oh et al., 2005; Shin and Kang, 2003; Hong et al., 2001).

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid (Figure 1). It is isolated as a pure compound for the first time as rosmarinic acid from the plant, *Rosmarinus officinalis* (Scarpati and Oriente, 1958), and commonly found in the species of the Boraginaceae and the subfamily Nepetoideae of the Lamiaceae. Rosmarinic acid has a multitude of biological activities, e.g. antiviral, antibacterial, anti-inflammatory and antioxidant. The presence of rosmarinic acid in medicinal plants, herbs and spices has beneficial and health promoting effects to human being (Ly et al., 2006; Parnham and Kesselring, 1985).

Rosmarinic acid is readily accumulated in undifferentiated plant cell cultures, in some cases it's concentrations are much higher than in the plant itself. The first plant cell cultures accumulating rosmarinic acid were derived from *Coleus blumei* (Razzaque and Ellis, 1997; 1997; Zenk et al., 1977).

Secondary metabolites from plant have been incurporated into a wide range of both commercial and Industrial applications, and in many cases, rigorously controlled plant in vitro culture can generate the same valuable natural products. Plant as well as its cell culture has served as resources for preservatives, natural pigments, flavors, enzymes, cosmetics, and bioactive compounds (Mary AL, 2005). Callus is an unorganized tissue mass growing on solid substrate which forms naturally on plants in response to wounding, infestations, or at graft unions. Cell suspension cultures are suspensions of individual plant cells and small cell clusters grown in liquid medium. Suspension cultures are established by transferring small pieces of callus to liquid medium and then, subsequently placed on a gyratory shaker. Within a few days individual plant cells and microcalli should be detached from the original inoculums and growing in the constantly agitated medium (Mary AL, 2005).

In this study, it is described efficient protocols for the growth and rosmarinic acid productions in callus and cell suspension cultures of *A. rugosa*.

MATERIAL AND METHODS

Seed sterilization and germination

Seeds of *A. rugosa* were surface-sterilized with 70% (v/v) ethanol for 30 s and 2% (v/v) sodium hypochlorite solution for 10 min, then

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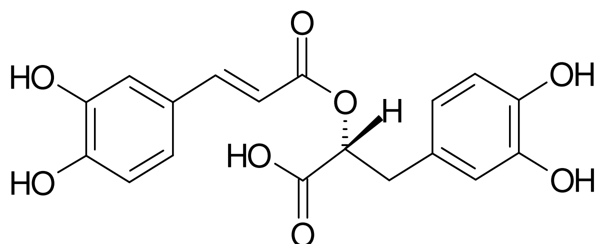

Figure1. Chemical structure of rosmarinic acid.

Table 1. Effect of different concentrations of 2, 4-D and NAA on callus induction from leaf explant of *Agastache rugosa* at 6 weeks after incubation on MS solid medium.

Auxins (mg/l)	Callus Induction (%)
Control 0.0	0.0 ± 0.0
2,4-D 0.1	13.1 ± 2.4
0.5	43.7 ± 3.2
1.0	83.5 ± 9.1
2.0	92. 3 ± 7.8
4.0	88.3 ± 7.4
NAA 0.1	0.0 ± 0.0
0.5	5.7 ± 0.8
1.0	31.8 ± 3.7
2.0	57.6 ± 7.6
4.0	56.8 ± 4.9

rinsed three times in sterilized water. Ten seeds were placed on 25 ml of agar-solidified culture medium in Petri dishes (100 x 15 mm). The basal medium consisted of salts and vitamins of MS medium (Murashige and Skoog, 1962) and solidified with 0.7% (w/v) agar. The medium was adjusted to pH 5.8 before adding agar, and then sterilized by autoclaving at 121℃ for 20 min. The seeds were germinated in a growth chamber at 25℃ under standard cool white fluorescent tubes with a flux rate of 35 µmol s-1 m-2 and a 16 h photoperiod.

Callus induction and culture

Leaf explants were cut aseptically at the ends, into sections of approximately 7 x 7 mm in size. Explants were placed on the medium in the Petri dish (100 x 15 mm). For callus induction from explant, the basal medium was supplemented with various concentrations (0.1, 0.5, 1.0, 2.0 and 4.0 mg/l) of 2, 4-D and NAA, and 30 g/l sucrose. After 3 to 4 weeks, calli were subcultured onto medium consisting of MS salts and vitamins, and containing 2 mg/l 2,4-D.

Cell suspension culture

Callus was transferred to 30 ml of MS liquid medium, containing 3% (w/v) sucrose, in 100 ml flasks. Suspension cultures were maintained at 25℃ on a gyratory shaker (120 rev/min) in a growth chamber under standard cool white fluorescent tubes with a flux rate of 35 µmol s^{-1} m^{-2} and a 16 h photoperiod. Growth rates were determined by measuring the dry weight of cultured cells at 10 days after culture. The addition to the culture medium with 2 mg/l 2,4-D of various concentrations of the cytokinins (BAP and kinetin) was tested to promote the growth of cell cultures. All experiments were repeated three times.

HPLC analysis of rosmarinic acid

Harvested cell cultures of *A. rugosa* (1 g) were frozen in liquid N2, ground to a fine powder using a mortar and pestle, and extracted twice with methanol (10 ml) for 24 h at 25°C. Extracts were reduced to dryness under vacuum dried, and dissolved in methanol. The extracts were analyzed by high performance liquid chromatography (HPLC) on a C_{18} reverse phase column (4.6 x 250 mm; Ultrasphere, Beckman-Coulter) at room temperature. The solvent gradient used in this study was formed through with an initial proportion of mix of 70% solvent A (3% acetic acid in water) and to 30% solvent B (methanol). After 50 min, the solvent gradient had reached 100% solvent B. The flow rate of the solvent was kept constant held at 1.0 ml/min. Samples (20 µl) were detected at wave lengths of 280 nm. We identified the rosmarinic acid in solution A by matching the retention times and spectral characteristics to those from single HPLC run of a known rosmarinic acid standard.

RESULTS AND DISCUSSION

Callus induction and growth of *A. rugosa*

Basal MS medium was supplemented with different concentrations of 2,4-D and NAA for the induction of callus from *A. rugosa*. Callus induction did not appear during the culture period in the control, while there was a little extension growth of the leaf explants. At the initial stage (1 to 2 weeks after incubation) of callus induction medium with 2, 4-D and NAA, the leaf explant swelled and friable callus formation was observed at 3 to 4 weeks after incubation. Six weeks after incubation 2,4-D treatment was more effective than NAA for callus induction and the best callus induction rate (92.3%) was obtained in MS medium containing 2 mg/l 2,4-D. There was no significant difference in the percentage of explants producing callus (83.5 – 92.3%) among the 1 - 4 mg/l 2,4-D treatments. In NAA treatment, 2 mg/l NAA showed the highest callus induction rate (57.6%) and no callus was formed during the whole culture period at the lowest concentration (0.1 mg/l) (Table 1).

The friable callus was obtained from leaf explants of *A. rugosa* cultured on solid MS medium with 2, 4-D treatment and were subcultured in the dark at 25℃ on agar-solidified MS medium with 2 mg/l 2, 4-D for maintenance of *A. rugosa* callus cultures (Figure 2A).

In plant tissue culture, several auxin type herbicides such as chlorophenoxy acids (e.g., 2, 4-D, 2,4,5-T, and MCPA), benzoic acids (e.g., dicamba), and pyridines (e.g., picloram) and synthetic auxin (NAA) have been applied to callus induction. For callus induction of *A. rugosa*, treatment with 2, 4-D was more effective than NAA. Amoo and Ayisire (2005) published similar result with callus induction from cotyledon explants of *Parkia biglobosa* (Jacq.) Benth. Unlike in NAA, successful callus

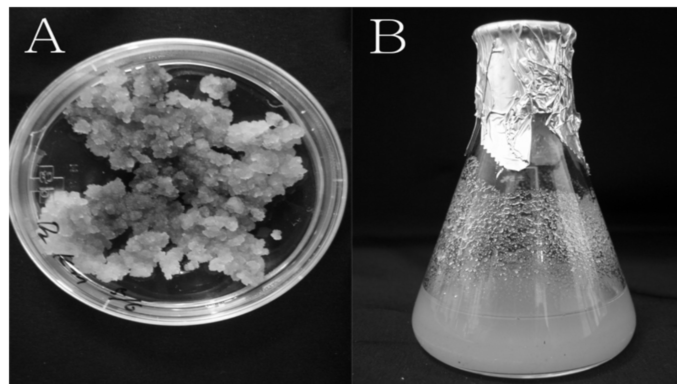

Figure 2. Callus and cell suspension culture of the *Agastache rugosa*. A) Callus cultured on solid MS medium with 2 mg/l 2,4-D treatment in the dark at 25℃ for 4 weeks. B) Suspension cells cultured in 30 ml of MS liquid medium containing 2 mg/l 2, 4-D for 10 days.

Table 2. Effect of different concentrations of 2,4-D on growth and rosmarinic acid (RA) production in suspension culture of *Agastache rugosa* at 10 days after culture in MS liquid medium.

2,4-D (mg/l)	Dry weight (g/l)	RA/ D.W. [a] (mg/g)
0.1	1.6 ± 0.1	8.5 ± 0.8
0.5	3.9 ± 0.5	8.7 ± 0.7
1	5.4 ± 0.7	9.3 ± 0.9
2	5.9 ± 0.7	9.5 ± 1.0
4	5.2 ± 0.6	9.4 ± 0.8

[a] = Dry weight
Data are means ± SE

induction was observed in all concentrations of 2,4-D.The fact that callus was induced by 2,4-D but not by NAA suggests that cotyledon explants of *P. biglobosa* are auxin specific. Rita and Floh (1995) also reported similar observation with the leaf explants of Cuphea ericoides. Zafar et al. (1995) also reported callus induction from cotyledon, hypocotyl and root explants of *Medicago littoralis* in the presence of 2,4-D alone and when it was replaced with NAA, the explants either died or showed poor callus formation. Harvey and Grasham (1969), while working on 12 species of conifers also reported species specificity for IAA, NAA and 2,4-D in their effectiveness for callus induction (Table 1/ Figure 2).

Cell suspension culture and rosmarinic acid production of *A. rugosa*

The friable callus of *A. rugosa* were transferred to 30 ml of MS liquid medium containing 30 g/l sucrose and 2 mg/l 2,4-D in 100 ml flasks. Suspension cell cultures were maintained at 25℃ on a gyratory shaker (120 rev/min) in a growth chamber under standard cool white fluorescent tubes with a flux rate of 35 µmol s^{-1} m^{-2} and a 16-h photoperiod. At the time of first week after transferring the callus to liquid media some smaller pieces fell off from the bigger callus clumps. After several subculture of suspension cells of *A. rugosa*, white and friable callus were easily broken apart and dispersed into small cell aggregates (Figure 2B).

To study the effects of different concentrations of 2,4-D on cell growth and rosmarinic acid biosynthesis in suspension cell culture of *A. rugosa*, suspension cells were subcultured to MS liquid medium supplemented with various concentrations of 2,4-D (Table 2). Our results revealed that the addition of 2,4-D exhibited a positive effect on *A. rugosa* suspension cell growth and rosmarinic acid biosynthesis and overall growth at concentrations between 1 and 4 mg/l. The addition of 2 mg/l 2,4-D which was selected as the mean of the optimal concentration for suspension cell growth and rosmarinic acid production in *A. rugosa* (Table 2).

In general, treatment of low levels of cytokinins in suspension cell culture medium improved cell growth. To improve cell growth and rosmarinic acid biosynthesis in cell suspension culture of *A. rugosa*, different concentrations (0.01, 0.1, 0.5, and 1.0 mg/l) of cytokinins, BAP and kinetin were added in MS liquid medium with 2 mg/l 2,4-D. Our results revealed that the concentration between 0.01 - 0.5 mg/l BAP and kinetin increased suspension cell growth but that of 1 mg/l both BAP and kinetin inhibited. BAP was relatively more effective than kinetin for suspension cell growth at 10 days after culture. The addition of the two individual cytokines (BAP and kinetin) in MS liquid medium with 2 mg/l 2,4-D increased rosmarinic acid biosynthesis at all tested concentrations. In cell suspension the combination of 0.1 mg/l BAP and 2mg/l 2,4-D performed the highest growth (7.0 g/l) and rosmarinic acid yield(10.7 mg/g D.W.). The presence of 2,4-D alone was less effective than a combination of 2,4-D and cytokinins for rosmarinic acid production and cell growth except for BAP 1 mg/l and kinetin treatment (Table 3). These results suggest that low concentrations of a cytokinin (BAP or kinetin) are optimal for rosmarinic acid production and cell growth in suspension cell culture of *A. rugosa* table 3.

Although secondary metabolite biosynthesis in suspension cell cultures is genetically controlled, it may also be influenced by nutritional and environmental factors. To examine the effect of different media on suspension cell growth and rosmarinic acid production, suspension cells of *A. rugosa* were cultured for 10 days in full- and half-strength of B5 and MS basal media supplemented with 2 mg/l 2,4-D and 0.1 mg/l BAP. Cell suspensions grown in B5 medium produced the highest levels of growth (7.7 g/l) and rosmarinic acid production (11.5 mg/g D.W.), as compared to cell suspensions grown in half-strength B5 medium, and half- and full- strength MS medium (Table 4).

Suspension cell cultures are rapidly dividing homoge-

Table 3. Effect of cytokinins on growth and rosmarinic acid (RA) production in suspension culture of *Agastache rugosa* at 10 days after culture in MS liquid medium containing 2 mg/l 2,4-D.

Cytokinins (mg/l)		Dry weight (g/l)	RA/D.W.[a] (mg/g)
Control	**0**	5.9 ± 0.7	9.5 ± 0.8
BAP	0.01	6.1 ± 0.5	9.7 ± 0.9
	0.1	7.0 ± 0.9	10.7 ± 0.9
	0.5	6.6 ± 0.8	10.5 ± 1.2
	1	4.6 ± 0.5	10.4 ± 1.3
Kinetin	0.01	5.9 ± 0.7	9.6 ± 0.8
	0.1	6.6 ± 0.7	10.3 ± 1.0
	0.5	6.3 ± 0.6	10.4 ± 1.1
	1	5.2 ± 0.5	10.2 ± 0.9

[a] = Dry weight
Data are means ± SE

Table 4. Effect of media on growth and rosmarinic acid (RA) production in suspension culture of *Agastache rugosa* at 10 days after culture in different liquid medium containing 2 mg/l 2, 4-D and 0.1mg/l BAP.

Media	Dry weight (g/l)	RA/D.W.[a] (mg/g)
1/2 MS	5.3 ± 0.4	9.4 ± 0.8
MS	6.9 ± 0.8	10.7 ± 0.9
1/2 B5	6.4 ± 0.5	9.8 ± 0.8
B5	7.7 ± 0.9	11.5 ± 1.1

[a] = Dry weight
Data are means ± SE

nous suspensions of cells grown in liquid nutrient media. Cell suspensions are used for generating large amounts of cells for quantitative or qualitative analysis of growth responses and metabolism of natural products, as well as for studies of cell cycle under standard conditions (Namdeo, 2007; Verpoorte et al., 2002; May and Leaver, 1993) (Table 4).

The establishment of suspension cell culture for the production of secondary metabolites using 2,4-D single treatment has also been reported in different plant species: diosgenin from *Dioscorea deltoidea* (Heble and Staba, 1980), β-Carboline alkaloids from *Peganum harmala* (Sasse et al., 1982), betacyanin from *Phytolacca Americana* (Sakuta et al., 1987), diterpenoids from *Torreya nucifera* var. radicans (Orihara et al., 2002).

However, we found the presence of 2,4-D alone was less effective than a combination of 2,4-D and cytokinins for rosmarinic acid production and cell growth. Other studies also reported similar observation that a combination of 2, 4-D and cytokinins is better than 2,4-D single treatment for secondary metabolite production and cell growth in several plants: anthraquinones from *Cassia acutifolia* (Nazif et al., 2000), anthraquinones from *Isoplexis isabelliana* (Arrebola et al., 1999), ginsenosides from *Panax notoginseng* (Zhong and Zhu, 1995), Taxol from *Taxus* spp. (Wu et al., 2001), Taxol from *Taxus baccata* (Cusido et al., 1999).

Here, we described in vitro production of rosmarinic acid from callus and cell suspension culture system of *A. rugosa* and it was found that a combination of 2,4-D and cytokinins are suitable for cell growth and rosmarinic acid production from cell cultures of *A. rugosa* which might be commercially accepted in future.

REFERENCES

Amoo SO, Ayisire BE (2005). Induction of callus and somatic embryogenesis from cotyledon explants of *Parkia biglobosa* (Jacq). Benth. Afr. J. Biotechnol. 4: 68-71.
Arrebola ML, Ringbom T, Verpoorte R (1999). Anthraquinones from *Isoplexis isabelliana* cell suspensioncultures. Phytochemistry 52: 1283-1286.
Cusido RM, Palazon J, Navia-Osorio A, Mallol A, Bonfill M, Morales C, Pinol MT (1999). Production of taxol and baccatin III by a selected *Taxus baccata* callus line and its derived cell suspension culture. Plant Sci. 146: 101-107.
Harvey AE, Grasham JL (1969). Growth of the rust fungus *Cronartium ribicola* in tissue cultures of Pinus monticola. Can. J. Bot. 47: 663-666.
Heble MR, Staba E (1980). Steroid metabolism in stationary phase cell suspensions of *Dioscorea deltoidea*. Planta Med. Suppl. 124-128.
Hong JH, Choi JH, Oh SR, Lee HK, Park JH, Lee KY, Kim JJ, Jeong TS, Oh GT (2001). Inhibition of cytokine-induced vascular cell adhesion molecule-1 expression; possible mechanism for anti-atherogenic effect of *Agastache rugosa*. FEBS Lett. 495: 142-147.
Ly TN, Shimoyamada M, Yamauchi R (2006) Isolation and characterization of rosmarinic acid oligomers in *Celastrus hindsii* Benth leaves and their antioxidative activity. Food. Chem. 54:3786-3793.
Mary AL (2005). Valuable secondary products from in vitro culture. CRC Press LLC 285-288.
May MJ, Leaver CJ (1993). Oxidative stimulation of glutathione synthesis of *Arabidopsis thaliana* suspension cultures. Plant Physiol. 103: 621-627.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.
Namdeo AG (2007). Plant cell elicitation for production of secondary metabolites: a review. Pharmacognosy Rev. 1: 69-79.
Nazif NM, Rady MR, Seif E1-Nasr MM (2000). Stimulation of anthraquinone production in suspension cultures of *Cassia acutifolia* by salt stress. Fitoterapia 71: 34-40.
Oh HM, Kang YJ, Kim SH, Lee YS, Park MK, Heo JM, Sun J, Kim HJ, Kang ES, Kim HJ, Seo HG, Lee JH, Yun-Choi HS, Chang KC (2005). *Agastache rugosa* leaf extract inhibits the iNOS expression in ROS 17/2.8 cells activated with TNF-alpha and IL-1beta. Arch. Pharm. Res. 28: 305-310.
Orihara Y, Yang JW, Komiya N, Koge K, Yoshikawa T (2002). Abietane diterpenoid from suspension cultured cells of *Torreya nucifera* var. radicans. Phytochemistry 59: 385-389.
Parnham MJ, Kesselring K (1985). Rosmarinic acid. Drugs of the Future 10: 756-757.
Razzaque A, Ellis BE (1997). Rosmarinic acid production in *Coleus* cell cultures. Planta 137: 287–291.
Rita I, Floh EIS (1995). Tissue culture and micro propagation *of Cuphea ericoides*, a potential source of medium- chain fatty acids. Plant Cell Tissue Organ Cult. 40: 187-189.
Sakuta M, Takagi T, Komamine A (1987). Effects of nitrogen source on betacyanin accumulation and growth in suspension cultures of *Phytolaca americana*. Physiol. Plant. 71: 459-463.
Sasse F, Heckenberg U, Berlin J (1982). Accumulation of b-carboline alkaloids and serotonin by cell cultures of *Peganum harmala* L. Plant.

Physiol. 69: 400-404.
Scarpati ML, Oriente G (1958). Isolamento e costituzione dell'acido rosmarinico (dal rosmarinus off.). Ric. Sci. 28: 2329–2333.
Shin S, Kang CA (2003). Antifungal activity of the essential oil of *Agastache rugosa* Kuntze and its synergism with ketoconazole. Lett. Appl. Microbiol. 36: 111-115.
Verpoorte R, Contin A, Memelink J (2002). Biotechnology for the production of plant secondary metabolites. Phytochem. Rev. 1: 13–25.
Wu J, Wang C, Mei X (2001). Stimulation of taxol production and excretion in *Taxus* spp cell cultures by rare earthchemical lanthanum. J. Biotechnol. 85: 67-73.
Zafar Y, Nenz E, Damiani F, Pupilli F, Arcioni S (1995). Plant regeneration from explant and protoplast derived calluses of *Medicago littoralis*. Plant Cell Tissue and Organ Cult. 41: 41-48
Zenk MH, El-Shagi H, Ulbrich B (1977). Production of rosmarinic acid by cell suspension cultures of Coleus blumei. Naturwissenschaften 64: 585–586.
Zhong JJ, Zhu QX (1995). Effect of initial phosphate concentration on cell growth and ginsenoside saponin production by suspended cultures of *Panax notoginseng*. Appl. Biochem. Biotechnol. 55: 241-246.