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식물세포 배양 및 융합을 통한 유용물질 개발(I)

김길웅* · 박용구* · 곽성희*

Development of Useful Secondary Product Through Plant Cell Culture(I)

Kim, K.U.*, Y.G. Park* and S.H. Kwak*

ABSTRACT

Water extracts from *Polygonum aviculare* and *Salix koreansis* markedly inhibited the germination of lettuce and rice seeds, indicating the presence of biologically active substances. The biochemical substances such as salicylic and + vanillic acid, tannic acid + gallic acid, p-coumaric acid, p-cressol, sinapic acid and catechol etc. belonging to phenolic compounds were detected in the cultured cells, suggesting that the secondary metabolites can be synthesized in plant cell and tissue culture. In addition, fatty acid like linolenic acid and organic acid such as oxalic acid were presented in the highest amount, 3.7 mg/g and 14.288 mg/g, respectively, which seem to be related to exhibiting phytotoxicity of *P. aviculare*. Petroleum ether extract exhibited another potential relating to inhibitory effect which needs further investigation. Calli from two plant sources were easily introduced by uses of 1.0 mg/l of 2.4-D and 0.1 to 0.2 mg/l of BAP in MS basal medium which can be implemented for a large scale production through cell culture.

Key words : Biochemical substances, phenolic compounds, secondary metabolites.

INTRODUCTION

The plant kingdom has great potential as a source of compounds. It is known that 1,500 novel products are isolated from plants each year and approximately 300 of those compounds were biologically active.[1] In addition, the potential of plant cell culture has been suggested as a method of generating many novel compounds and many researchers are already analysing plants which have been traditionally used as a herbal residues or in tribal rituals. Further, it has been known that relative amount and number of secondary metabolites often differ significantly when plants are transferred into tissue and cell suspension culture. Generally secondary metabolites are stored within the plant cells. If certain amount of compounds may be released into culture medium, it may be possible to increase

* 경북대학교 농과대학 (Dept. of Agronomy, Kyungpook National Univ., Taegu 702-701, Korea)
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secondary metabolite productivity by treatment of plant growth regulators. Indeed, many novel metabolites have been isolated from plant cell and tissue culture, e.g. rutacultin from *Ruta graveolens*, hindokiol and furrigiol from *Thuja occidentalis*[3] and shikonin from *Lithospermum erythrorhizon*[8] which represent the first commercial production of plant biochemicals using plant cell and tissue culture. Thus it seems to be of great importance to search for useful biochemical from wild plant sources and to increase product formation using plant cell and tissue culture.

The main purpose of the works was to identify the presence of biochemically active substances in plant species such as *Polygonum aviculare* and *Salix koreansis*, and to reproduce them through plant cell and tissue culture.

MATERIALS AND METHODS

Water extracts and germination test.

Aqueous extracts of two samples such as *P. aviculare* and *S. koreansis* were made from dry leaves of them harvested at autumn. one hundred ml of water was added to each of 20 g dry material and kept at 25℃ for 24 h. The extracts was diluted to 1, 2, 5 and 10%. Two selected species such as *Lactuca sativa* and *Oryza sativa* were used for assaying the presence of germination inhibiting substances. Twenty seeds of two species were seeded on the filter paper in petri dishes with three replications. Ten ml of each concentrate was added to each petri dish. Experiment was conducted at the growth chamber (temp. 25℃, light intensity 3,000 lux, photoperiod 16 h). Germination rates were observed at 6 and 10 days after incubation of *P. aviculare* and *S. koreansis*.

Isolation and identification of phenolic compounds.

Extraction of phenolic compounds was made by the methods of Kuwastuka and Shindo.[11] 15 g of each dry samples was mixed with 1 liter of metanolic sodium hydroxide (methanol: 0.1N NaOH=7:3) for 48 h, respectively. Crude extracts were filtered and the filterate was adjusted to pH 7.0 with HCl, concentrated to about 300 ml at 40-45℃. Aqueous phase was acidified to pH 2 with HCl and again extracted with either. Then extracts were completely dried. Finally 0.3 ml of TMS(trimethylsilylacetamide, 25% solution in acetonitrile) was added to dried residues, and it was allowed to stand for 3 minutes in water bath at below 60℃ and 2 μl was injected into gas chromatography. Further, fractionations such as free, soluble and insoluble-bound were done by Krygier's method.[10]

Identification by gas chromatography.

The analysis of various extracts was performed on a Pye Unicam gas chromatograph equipped with a flame ionization detector, and glass column, 1.5 m×4 mm (inner diameter), packed with chromosorb W(100-120 mesh) coated with 5% silicon SE 30. The flow rate of carrier gas (nitrogen) was 30 ml/min. The temperatures of injector and detector were maintained at 270℃ and 280℃, respectively. The column temperature was programmed at the rate of 5℃/min from 130℃ to 250℃.

Isolation and identification of fatty and organic acids, total alkaloid and ptroleum ether extract.

Fatty and organic acids were extracted by the method of Court and Hendel.[5] One hundred ml of MeOH containing glutaric acid (ISTD) 50 mg and 7.2 ml H_2SO_4 was added to 10 g of dry sample of *P. aviculare* and then shaked for 24 h.

Crude extracts were filtered with Toyo 5B, and the filtrated was again extracted with 100 ml of chloroform four times and then injected into the gas chromatograph. A Pye Unicam gas chromatograph was used for analysis with glass column, 2.7 m × 4 mm(inner diameter), packed with chromosorb W (100-120 mesh) coated with 5% Silar 10℃. The column temperature was programmed at the rate of 8℃/min from 90℃ to 230℃. The other conditions in the gas chromatogrphy were the same as determination of phenolic substances in the above. The total alkaloid content was determined by Cundiff and Markunas.[6]

Callus induction and chemical substance.

The calli were induced from newly developed leaf of *P. aviculare* and from newly developed shoot of *S. koreansis*. MS basal medium was used with various rates of 2,4-D(0.1, 0.5, 1.0 mg/l) and BAP(0.1, 0.5, 1.0 mg/l) for selection of optimum conditions. Fresh weight of callus was evaluated at 30 days after incubation. Chemical substances such as phenolic compounds in callus of *P. aviculare* were detected with gas chromatography in the same method as mentioned in the above.

RESULTS AND DISCUSSION

Water extracts and inhibitory effect.

Water extracts from *P. aviculare* and *S. koreansis* markedly inhibited the germination of rice and lettuce seeds as the concentration increased from 1% or 2% to 10%. No germination of lettuce (*Lactuca sativa*) seeds was observed in the concentration of 5 to 10% of *P. aviculare* extract and only 3% in rice(*Oryza sativa*) seeds by 10% concentration of *S. koreansis* extracts, indicating that biologically active substances are presented in these two plants(Table 1). Rice[14] reported that 75 weed species exhibited allelopathic effects.

Identification of chemical substances.

The major phenolic acids analyzed by GLC in the leaf sample of *P. aviculare* were tannic acid + gallic acid, salicylic acid + vanillic acid, sinapic acid, p-coumaric acid, p-cresol, catechol etc. (Table 2). The composition of phenolic acids determined varied from each fraction, eg. 11 phenolic compounds in free fraction and 4 from soluble fraction. However, salicylic acid, + vanillic acid and tannic acid + gallic acid were unique compounds which were detected in the all fractions, indicating the most commonly occurred phenolic compounds in *P. aviculare*. There were some similarity in the phenolic compounds detected in tissue cultured cells with those of *P. aviculare* plants although composition and quantity of them somewhat differed, indicating that the secondary metabolites can be directly synthesized in cultured cell(Fig. 1).

These results suggest that plant cell cultures are valuable for biosynthesis of biologically important substances although they do not necessarily

Table 1. Percent germination of two species as affected by aqueous extract from the leave of *Polygonum aviculare* and *Salix koreansis*.

Plant species / Conc. / Testing plant(g/100ml)	*Polygonum aviculare*[1]			*Salix koreansis*		
	1	5	10	2	5	10
	% germination					
Oriza sativa	100.0	93.5	95.0	40.0	30.0	3.0
Lactuca sativa	36.50	0	0	90.0	100.0	100.0

[1] Period of extraction : 24 h

Table 2. Constitution of phenolic compound in various fractions identified from *Polygonum aviculare*.[1)]

Phenolic acids	Fraction	*Polygonum aviculare*			
		Free	Soluble	Insoluble	Non
p-cresol		2.4	1.0	5.8	
Catechol		5.8	0.5		
Resorcinol					
Methyl-catechol		5.2		0.5	
p-Cl-benzoic					
Hydroquinone					
Salicylic+					
Vanillic		14.6	45.8	9.2	2.1
Cinnamic					
Porogallol				0.5	
p-OH-benzoic				1.1	
Umbelliferone				0.4	4.0
Protocatechuic		2.5			
Syringic				0.5	
p-coumaric		2.9		41.1	10.3
Tyrosine					
Tannic + Gallic		14.8	17.6	3.6	2.0
Ferulic		6.5			47.9
DL-dopa + Caffeic		1.0			7.3
Sinapic		12.4		1.6	11.9
Chlorogenic		3.31			

[1)] Percent of total GLC analyzed phenolic compounds

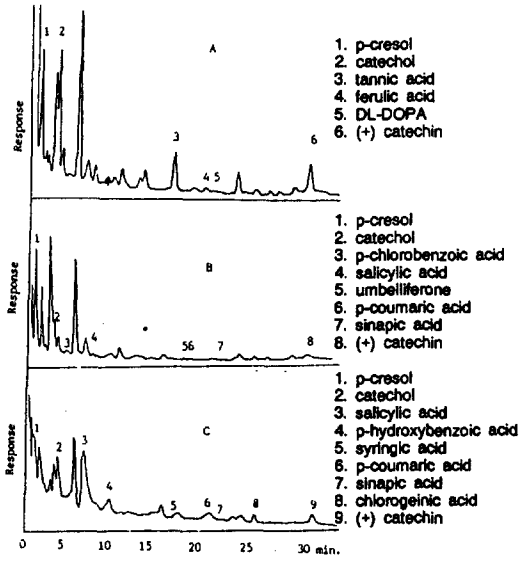

Fig. 1. GC chromatograph of phenolic compounds determined from callus of Polygonum aviculare.(A, free phenolic acids; B, soluble phenolic acids; C, insoluble phenolic acids)

produce the same metabolites as their parent plants.[2)] Reviewed evidences are available on the production of useful secondary plant metabolites by plant cell and tissue culture such as alkaloids, phenols, terpens and others.[2,12)] Six fatty acids such as palmitic, stearic, oleic, linoleic and eicosanoic, three organic acids like oxalic, malic and citric acids were detected by GLC(Table 3) in *P. aviculare* plant. The major fatty acids was linolenic acid presented in the amount of 3.7 mg/g, representing 43% of total fatty acids, which was present in the highest amount. Oxalic acid among organic acids was present in the highest amount representing more than 95% of total organic acids. Thus it is suggested that linolenic and oxalic acids may be two important nonvolatile acids relating to allelopathic effect of *P. aviculare*.

The amount of total alkaloids and petroleum

Table 3. Fatty acids, organic acids, total alkaloid and petroleom ether extracts determined from *Polygonum aviculare* by GLC.

Plant species	Fatty acids[1]							
	16 : 0	18 : 0	18 : 1	18 : 2	18 : 3	20 : 0	Total	$\Sigma S/\Sigma U$[2]
	mg/g							
P. aviculare	1.90	0.25	1.07	1.36	3.07	0.19	8.47	0.381

Plant species	Organic acids			Total alkaloid	Pet. ether extract
	Oxalic	Malic	Citric		
	mg/g			%	
P. aviculare	14.288	0.506	0.230	0.22	1.65

[1] 16 : 0; Palmitic acid, 18 : 0; Stearic acid, 18 : 1; Oleic acid, 18 : 2; Linoleic acid, 18 : 3; Linoleic acid, 20 : 0; eicosanoic acid

[2] $\Sigma S/\Sigma M$: Total contents of saturated fatty acids/total contents of unsaturated fatty acids.

ether extract were 0.22 and 1.65%, respectively. It is known that petroleum ether extract is related to fat and phenolic compounds. It is required to be verified biological effects of the substances. Total alkaloid was present in relatively low amount which may not play an important role in exhibiting phytotoxic effect of *P. aviculare*.

Callus induction.

The optimum levels of phytohormones for callus induction from *P. aviculare* and *S. koreansis* were 1 mg/l of 2,4-D with 0.1-0.2 mg/l of BAP using MS basal medium(Table 4). Fett-Neto *et al.*[7] and Gibson *et al.*[9] reported that low concentration of NAA and 2,4-D were effective in callus cultures of *Taxus spp.* The similar result was reported by Choi[4] indicating that one mg/l 2,4-D and 1.0 mg/l NAA were slightly effective on cell growth of *Taxus spp.*

The calli were easily inducted from these two different plants under above-mentioned conditions. These results could be utilized in cell suspension culture for a large scale production of useful plant metabolites in a similar way to microbial fermentation in 2nd and 3rd year's experiments. The approaches to increase productivity of the secondary plant metabolite by plant tissue and cell cultures were reviewed by Misawa[12] and Anderson et al.[2] The research along this line looks promising to utilize natural products

Table 4. Effect of 2, 4-D and BAP on callus induction from *Polygonum aviculare* and *Salix koreansis*[1].

Phytohormones		Plant species		
2,4-D (mg/l)	BAP (mg/l)	*Polygonum aviculare*		*Salix koreansis*
		Fresh wt. (mg)		
0.1	0	62.7		50
	0.1	92.7		768
	0.5	195.3		355
	1.0	197.7		177
0.5	0	109.3		233
	0.1	171.2	(264)[2]	729
	0.5	216.2		504
	1.0	214.6		359
1.0	0	106.4		106
	0.1	246.1		772
	0.5	207.5	(312.9)[2]	655
	1.0	226.8		302

[1] MS(Murashig and Skoog) based medium was used.

[2] Fresh weight of *P. aviculare* when BAP applied at the level of 0.2mg/l

摘 要

야생식물인 마디풀과 버드나무의 수용 추출액에는 상치 및 벼종자의 발아를 강하게 억제

하는 물질이 존재하여 이들 물질을 GLC로 검정했다.

마디풀에 페놀물질로는 salicylic + vanillic, tannic − gallic, sinapic, p-coumaric, p-cresol 및 catechol 등의 순으로 함유되어 있었고 이들은 강한 활성을 나타냈다. 배양세포에도 유사한 페놀류가 동정되어 2차대사물질이 세포배양에도 생성됨이 검정되었다. 지방산 가운데는 linolenic 산(3.7 mg/g)과 유기산 가운데는 oxalic산(14.288 mg/g)이 가장 많이 함유되어 있었으며 이들도 생리활성을 지닌 물질로 간주된다. 그 밖에 마디풀에는 석유 에테르 추출물도 1.65% 검정되어 생리활성을 발휘하리라 사료되어 계속 연구중이나 알카로이드 함량은 아주 낮았다. 마디풀과 버드나무로부터 MS기본배지에 2,4-D(1.0 mg/l)과 BAP(0.1-0.2 mg/l)조합 처리하면 캘루스의 유기가 잘 되었으며 대량생산을 위한 세포현탁배양에 이용될 수 있는 기초자료가 된다고 사료된다.

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