한국생물공학회지 제8권 제4호
Korean J. Biotechnol. Bioeng.
Vol. 8. No. 4. 390-396(1993)

Effects of Various Elicitors on the Production of Berberine in Plant Cell Suspension Cultures of *Thalictrum rugosum*

Jeong-Hwan Yun, In-Suk Park and Dong-Il Kim
Department of Biological Engineering, Inha University
Incheon 402-751, Korea

Thalictrum rugosum 세포배양에 의한 berberine 생산에 미치는 여러 가지 elicitor의 영향

윤 정 환 · 박 인 석 · 김 동 일
인하대학교 공과대학 생물공학과

ABSTRACT

Effects of various elictitors were investigated to enhance the production of berberine in plant cell suspension cultures *of Thalictrum rugosum*. Treatments of yeast elicitor, 15 different types of abiotic elicitors, 16 kinds of fungal elicitors from three species of fungi were performed. Cell growth and berberine production were examined and compared for both normal and elicitor treated cultures. No distinguished increases in berberine yield by the addition of elicitors could be attained.

INTRODUCTION

The production of commercially important secondary metabolites via plant cell suspension cultures attracts an increasing attention in these days. To increase the productivity, various techniques have been studied including selection of overproducing cell lines, optimization of process variables, development of process strategies, and bioreactor design for scale-up. On top of that, the utilization of either biotic or abiotic elicitors is thought to be another factor in increasing productivity. Increased synthesis, i. e. elicitation, of secondary products in response to stress of various types appears to be the general response of cultured cells(1). It is possible to use an elicitor to promote the timely expression of secondary metabolites in culture. Microbial challenge of cells and whole plants leads to the production of phytoalexins and the pathways used by the plants to produce these compounds are modified by the transient expression of increased level of key enzymes. Treatment of cultured parsley cells with elicitor preparation from cell walls of various phytophathogenic fungi caused the accumulation of furanocoumarins and structurally related phenylpropanoid derivatives in the culture fluid, and large but transient increases in a number of enzyme activities in the cells (2, 3, 4, 5). Abiotic elicitors such as ultraviolet irradiation, salts of heavy metals, surfactants, carbohydrates, and metabolic inhibitors have also been used.

DiCosmo and Misawa (1) have summarized the diverse sources of biotic and abiotic elicitors as well as the phytochemical products seen in plant cell culture challenge with these elicitors.

Most work on elicitors has been for the production of phytoalexins which are low molecular weight, antimicrobial compounds that are both synthesized by and accumulated in plants in response to microbial infection, mechanical or chemical damage (6). However, the secondary metabolites of commercial interest are usually not phytoalexins. Elicitors do not always elicit the biosynthesis of most secondary metabolites, but they show the possibility of induction and removal of repression. The productions of berberine, a protoberberine alkaloid and sanguinarine, a benzophenanthridine alkaloid, were reported to be susceptible to being elicited (7, 8, 9). Interestingly, these alkaloids were classified as prohibitins which are pre-infectional plant metabolites (10) and are produced constitutively. The induction systems using elicitors or stress may be a significant process variable to be considered.

In this study, various kinds of elicitors were selected and their effects on the production of berberine, an isoquinoline alkaloid, in plant cell suspension cultures of *Thalictrum rugosum* were investigated for the purpose of enhancing productivity.

MATERIALS AND METHODS

The cell suspension culture of *Thalictrum rugosum* was kindly provided by Dr. Henrik Pedersen(Rutgers University, NJ, USA) and has been maintained in Murashige and Skoog(MS) medium prepared from MS salt mixture (Gibco laboratories) with the addition of 2 μM 2, 4-dichlorophenoxyacetic acid (2, 4-D), vitamin stock solution and 30 g/L of sucrose as carbon source. The pH was adjusted to 6.0 with 1 N KOH. The suspension cultures were grown in 125ml Erlenmeyer flasks with 50ml of medium on a rotary shaker at 150rpm, 25℃. Subcultures have been done weekly by 1:3 dilution. For the determination of dry cell weight, cell suspensions were filtered, washed with distilled water, and dried at 60℃ to constant weight.

Isolation of Yeast Elicitors

Elicitor was isolated from yeast extract (Difco) by ethanol precipitation as described by Hahn and Albersheim (11). A typical isolation of elicitor was initiated by dissolving 200g of yeast extract in 1l of deionized water. Ethanol was added to 80%(v/v). The precipitate was allowed to settle for 4 days at 6℃ and the supernatant solution was decanted and discarded. The precipitate was dissolved in 1l of deionized water and ethanol precipitation was repeated. The precipitate formed again was dissolved in 800 ml of deionized water and dialyzed against deionized water at 6℃. The precipitate formed inside the dialysis bag was removed by filtration through a 1.2μm Millipore filter. The dialyzed fraction was made 60% (v/v) with respect to ethanol. The precipitate which formed was removed by centrifugation at 21,500g for 15min. The supernatant solution was decanted and evaporated under reduced pressure at 40℃ to a volume of 350ml. The yeast elicitor obtained was dispensed into tubes, autoclaved, and stored in the refrigerator.

Preparation of abiotic elicitors

The chemicals known to be elicitors were selected from various literatures. The concentrations were determined according to the literatures and stock solutions for each chemical were made 50x to allow for the addition of 1ml into a culture flask containing 50ml of cell suspension. The stock solutions were autoclaved before the application, except for heat labile chemicals which were filter sterilized using a 0.22 μm Millipore membrane filter.

Strains, media, and culture conditions for the preparation of fungal elicitors

Among the diverse range of pathogens known to elicit phytoalexin production, three strains were purchased from the American Type Culture

Collection(ATCC, Rockville, MD, USA). The strains are *Phythophthora megasperma* f. sp. *glycinea* (ATCC 28001), *Colletotrichum lindemuthianum* (ATCC 11225), and *Verticillium dahliae* (ATCC 7611).

The asparagine medium (12) used for the culture of *P. megasperma* consisted of the following, in the amounts specified per liter : sucrose, 15 g; asparagine, 2.0g; $MgSO_4 \cdot 7H_2O$, 0.2g; $FeSO_4 \cdot 7H_2O$, 1mg; $CaCl_2 \cdot 2H_2O$, 10mg; thiamine-HCl, 1mg; K_2HPO_4, 1.04g; KH_2PO_4, 1.90 g; β-sitosterol, 20mg; $ZnSO_4 \cdot 7H_2O$, 1mg; $CuSO_4 \cdot 5H_2O$, $NaMoO_4 \cdot 2H_2O$, and $MnCl_2 \cdot 2H_2O$, each 0.02mg; and $CaCO_3$, 3g. The culture conditions for *P. megasperma* have been described by Ayers *et al.* (13). Large cultures were grown in 2.8 L Fernbach flasks containing 50 ml of asparagine medium. The liquid cultures were inoculated with mycelial agar fragment from the periphery of mycelia growing on an agar slant. On the 2nd and 4th day after inoculation, the liquid cultures were vigorously swirled by hand for a while to aid the spread of the mycelia on the surface of the media. Cultures were incubated at 25℃ without further agitation until the mycelia covered the surface of the liquid.

The strain of *C. lindemuthianum* was cultured in the same way as shown above in the medium described by Anderson-Prouty and Albersheim (14), contained in 900ml of water, 15g of sucrose, 1g of KH_2PO_4, 0.25g of $MgSO_4$, 0.027g of $FeCl_3$, and 100ml of casein hydrolysate extract. The medium for growth and the culture conditions for *V. dahliae* were exactly the same as those of *P. megasperma*. All disposal of cultures was carried out by autoclaving first and then discarding.

Isolation of elicitors from fungal cultures

The same procedure, originally described for *P. megasperma* (15), was used for all three fungal cultures. One hundred g batches of mycelia were harvested by suction filtration on a coarse sintered-glass funnel from liquid cultures of each pathogen. The mycelia were washed on a coarse sintered-glass funnel with 4l each of distilled water, of 10mM potassium phosphate, pH 7.2, and of 500mM potassium phosphate, pH7.2. The mycelia were then homogenized in a Waring Blendor in 250ml of 500 mM buffer. The homogenized mycelia were washed 8 times with 500 ml aliquots of 500mM buffer by suction filtration through a 37 μm screen supported on a coarse sintered-glass funnel. The remaining cell membrane and wall material was washed with 8l of deionized water and 2l of chloroform-methanol (1:1, v/v). The mycelial walls were then washed with 1l of acetone and were air-dried. Mycelial walls were suspended in water(10ml/g of walls), and the suspension was autoclaved for 3hr. The solubilized elicitor was separated from the wall residue by centrifugation followed by passage through a 0.22 μm Millipore filter. The heat-solubilized wall fraction was concentrated to 1% of its original volume by rotary evaporation at 40 ℃ under reduced pressure and dialyzed against deionized water. The heat-solubilized, nondialyzable material obtained from purified walls of each of the three strains were termed as 'wall-released elicitor'.

Besides the 'wall-released elicitor', elicitors from culture filtrates were prepared. The liquid media obtained by the filtration to remove mycelia were concentrated 100times by rotary evaporation under reduced pressure at 40℃ and dialyzed against deionized H_2O. Dialysis divided the filtrate into 'small extracellular elicitor's and large extracellular elicitor' based on the ability to pass through or to be retained by the dialysis membrane, respectively. In addition, 'autoclaved fungal elicitor' was also collected by washing the harvested mycelia and autoclaving the mycelia resuspended in water.

Determination of carbohydrate concentration in various biotic elicitors

For the determination of carbohydrate concentration in fungal or yeast elicitors, the orcinol-sulphuric acid procedure was used as described (16). To 1ml of adequately diluted standard

sugar solution and samples (water as blank), 8.5ml of orcinol-sulphuric acid(7.5 volume of H_2SO_4-water(3:2, v/v)added, after cooling, to 1 volume of 1.6%(w/v)orcinol in water) was added. After heating at 80℃ for 45min, the tubes were cooled and the absorbance was read at 505nm. Glucose was used as the standard. For the treatment of biotic elicitors, stock solution was added to make 100 μg carbohydrate per ml of plant cell culture.

Alkaloid analysis

The sample for intracellular berberine analysis were collected by taking 0.5g of cells by fresh weight. To each sample, 20 ml of HPLC grade methanol was added. The suspension was sonicated at 125 W for 10 min and filtered to be used for HPLC analysis. The filtrates obtained during the cell mass measurement were collected for the analysis of extracellular berberine in the medium. A filtered sample(20 μl) was injected into the HPLC system with a UV detector (Young-In HPLC 9500 System). The mobile phase contained 1 mM tetrabutylammonium phosphate in water adjusted to pH 2 with phosphoric acid(60%) and acetonitrile(40%). The flow rate was 2ml/min and the measuring UV wavelength was 265nm.

RESULTS AND DISCUSSION

Time course responses after addition of yeast elicitor

The utilization of elicitors may increase productivity and it is possible to promote timely expression of secondary metabolites if we use elicitors. Recently, Funk *et al.* (8) reported the yeast elicitor preparation is effective in inducing glyceollin isomer synthesis in cells of *Glycine max* and enhancing berberine biosynthesis up to four-fold in cells of *Thalictrum rugosum*. A success in using yeast elicitors to elicit quaternary benzophenanthridine alkaloids in *Eschscholzia* cell cultures was also reported(9). A practical advantage of yeast elicitor over other biotic elicitors of fungal origin is that it has been isolated from a microorganism approved for human consumption(8).

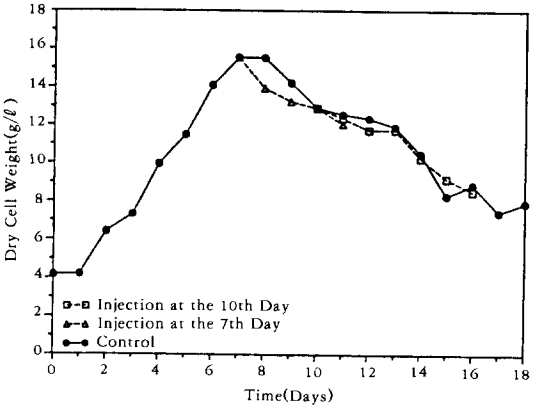

Fig. 1. Effect of yeast elicitor on the cell growth.

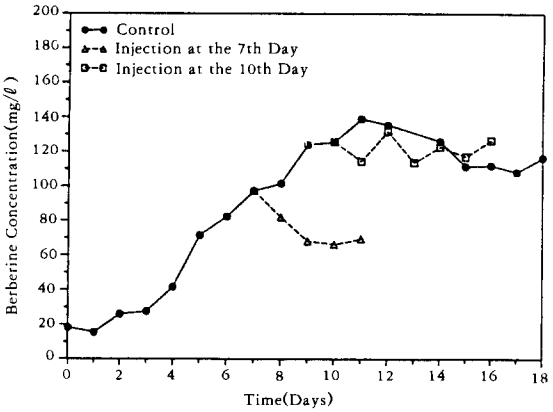

Fig. 2. Effect of yeast elicitor on the production of total berberine.

Since the same *Thalictrum* cell was known to be elicited by yeast elicitor (the cell line used in this study was originally developed by Funk's group), a similar experiment was performed as a confirmation. The results are shown in Fig. 1 and Fig. 2. The growth was inhibited by addition of yeast elicitor as expected. However, the berberine production result was the opposite to that reported by Funk *et al.* (8). In place of increasing productivity, yeast elicitor had a negative effect or berberine biosynthesis. Earlier injection on the 7th day was worse. Injection at the late growth phase, which is usually known as the best time to maximize product formation, reduced berberine production continuously. Four days after the

treatment, berberine level was only 50% of the control culture. In order to be certain, different lots of yeast elicitor were prepared and tested, but the results were the same.

It is not easy to explain these poor results. Probably, the instability of the culture may be the only reason why the cultures do not respond to elicitation any more. Funk *et al.* expressed their four-fold increase on the basis of specific productivity even though cell mass decreased sharply (8). In terms of volumetric berberine yield, this increase might be much lower. Compared to considerable increase by elicitor treatment in other well-elicited systems, the value for berberine elicitation was not so significant.

Examination of abiotic elicitors

Abiotic elicitors induce the formation of some secondary metabolites. These include salts of heavy metals, surfactants, carbohydrates, activated carbon, antibiotics, metabolic inhibitors, and ultraviolet irradiation. A distinct difference in the primary mechanism of action of biotic and abiotic elicitors was shown by Yoshikawa(17). According to him, the unique response of soybean cotyledon to abiotic elicitors is significant because it offers insight into the frequently observed accumulation of plant phytoalexin in response to stress that is a change in the normal physiological state of the plant cell environment. Many results have been published on the utilization of abiotic elicitors and part of them were summarized by DiCosmo and Misawa(1). Addition of vanadyl sulfate to cell suspension cultures of *Catharanthus roseus* was found to increase ajmalicine, catharanthine, and tryptamine levels (18, 19). Phaseollin and pisatin accumulated in bean and pea cotyledons respectively after treatment with mercuric chloride(20). When sweet potato was injured through some poisonous agent such as mercuric chloride, biochemical alterations were almost the same as seen in sweet potato infected by *Ceratostomella fimbriata* (21).

In shikonin production, a small amount of agar powder induced shikonin derivatives syntheis (22). sometimes, and abnormal metabolite was produced when the medium was supplemented with activated carbon (23). Chitosan also induced phytoalexin production in both tissue and cell suspension cultures (24, 25). Schwochau and Hadwiger (26) reported the stimulation of pisatin production in *Pisum sativum* by actinomycin D and other compounds including heavy metals and cycloheximide. Triton surfactants induced accumulation of phytoalexins in cotyledons of the French bean(27).

From the examples mentioned above, we can see that abiotic elicitors induce certain secondary products by either host-defense mechanism or acting as stress. Among those known abiotic elicitors, 15 chemicals were chosen and tested the possibility of inducing berberine production. The concentrations applied were searched in the references and injected at the late growth phase.

The effects of various abiotic elicitors on cell growth and berberine production are demonstrated in Table 1. As can be anticipated, some toxic chemicals such as heavy metal salts and metabolic inhibitors suppressed cell growh and killed the cells. Among 15 chemicals examined, cupric sulfate, cAMP or glucan stimulated berberine production notably while others did not show much effect. The heavy metal salts generally enhance the specific content of berberine due to cell mass decrease. On the basis of dry cell weight, and increase in specific content of berberine after treatment with cupric sulfate was as high as 60%. The more interesting fact is the change of distribution of products. On account of severe stress and concurrent cell lysis, more than 80% of berberine was found extracellularly. Therefore, treatment with cupric sulfate provides two advantages if applied when cell density is high : the stimulation of berberine production in a short time and the release of berberine into the medium. Stimulation of berberine production in *T. rugosum* suspension cultures in response to addition of cupric sulfate were discussed in detail

Table 1. Effects of various abiotic elicitors on cell growth and berberine production.

Elicitor	Concentration	DCW(g/ℓ)	Berberine Concentration(mg/ℓ)			%DCW
			Intracellular	Extracellular	Total	
Control	–	12.86	109.75	7.37	117.12	0.91
$CdCl_2$	1mM	11.00	44.03	76.34	120.37	1.09
$CuCl_2$	1mM	11.28	54.78	67.71	122.49	1.09
$CuSO_4$	1mM	9.29	24.38	110.49	134.87	1.45
$HgCl_2$	1mM	9.23	19.86	97.93	117.79	1.28
NaF	1mM	12.96	104.11	6.78	110.89	0.86
Phenylmercuric acetate	1mM	7.04	13.18	81.45	94.63	1.34
Actinomycin D	2×10^{-5}M	12.18	66.48	47.72	113.20	0.93
Cycloheximide	2×10^{-5}M	12.29	42.83	77.46	120.29	0.98
Agar	30μg/ml	13.01	108.58	6.72	115.30	0.89
cAMP	0.1mM	12.87	123.08	10.11	133.19	1.03
Chitosan	30μg/ml	12.30	90.97	20.49	111.46	0.91
2, 4-dinitrophenol	2×10^{-4}M	11.50	65.70	49.31	115.01	1.00
Glucan	30μg/ml	12.68	114.81	23.20	138.01	1.09
β-Glucan	30μg/ml	12.07	87.02	10.52	97.54	0.81
Tween 80	5mg/ml	12.34	81.25	30.02	111.27	0.90

Table 2. Effects of various fungal elicitors on berberine production: A, autoclaved fungal elicitor; B, large extracellular elicitor, C, small extracellular elicitor; D, wall-released elititor.

Elicitor		Berberine Concentration(mg/ℓ)			%DCW
		Intracellular	Extracellular	Total	
Control		71.90	5.54	77.44	0.63
Phytophthora megasperma	A	57.03	23.20	82.23	0.69
	B	81.09	13.73	94.82	0.77
	C	80.80	7.63	88.43	0.71
	D	75.04	10.77	85.81	0.69
Colletotrichum lindemuthianum	A	43.10	28.26	71.36	0.62
	B	38.64	31.95	70.59	0.58
	C	63.71	18.87	82.58	0.68
	D	55.76	11.86	67.62	0.56
Verticillium dahliae	A	22.47	39.49	61.96	0.54
	B	61.80	34.25	96.05	0.79
	C	68.88	8.02	76.90	0.63
	D	35.78	27.35	63.13	0.52

elsewhere(28). Two other chemicals, cAMP and glucan, are more expensive than cupric sulfate despite the similar effect, so these are not suitable for use.

In addition to heavy metal salts, most of the abiotic elicitors tested induced release of berberine to some extent. Some of them caused cell lysis, but some others did not. In the case of Tween 80, it enhanced the release of berberine noticeably without any indication of cell lysis. The stimulation may be dependent on the concentration and on the cell age. Thus, a careful study is necessary for the chemicals which showed a positive effect.

Effects of fungal elicitors

Synthesis of phytoalexins in plant tissues and cells be stimulated by elicitors that are produced by pathogens. Since the elicitors isolated from the mycelial cultures of *Phythophthora megasperma* and *Colletotrichum lindemuthianum* are well known and have been studied in detail, these two fungal cultures were selected. The reason for selecting *Verticillium dahliae* is that it is known as a pathogen for a wide diversity of plants. Various kinds of elicitors were isolated from these fungal cultures and their effects were examined. The results are summarized in Table 2. The elicitors

were injected at the late growth phase and their effect on final cell mass was negligible.

In general, application of *Phythophthora* elicitors enhanced berberine production. 'Large extracellular elicitors from *Phythophthora* enhanced berberine level up to 22%. Among 12 elicitors tested, 'large extracellular elicitors' from *Verticillium* gave the best result with 24% product increase. Even though some elicitor preparations enhanced berberine yield, it is not easy to say that this is the effect originated from elicitors because the application of elicitors generally induces accumulation of phytoalexins remarkably in a very short period. Adjustment of carbohydrate concentration in the elicitor preparation may result in better outcome, but it may not be so significant as in those other elicitable plant cells.

In conclusion, from series of experiments on the effects of various elicitors, it was found that our *Thalictrum* culture is not inducible markedly by elicitors unlike the published results.

ACKNOWLEDGEMENT

This paper was supported by NON DIRECTED RESEARCH FUND, Korea Research Foundation, 1992. The support is deeply appreciated.

요 약

Thalictrum rugosum 식물세포 현탁배양에 의한 berberine 생산에 미치는 여러 가지 종류의 elicitor 들의 영향에 관해 연구 하였다. 효모 유래의 elicitor, 15가지의 서로 다른 종류의 무생물 종류 elicitor, 3가지 곰팡이로부터 얻은 elicitor 들을 처리하여 세포의 생장 및 berberine 생산에 미치는 영향을 비교 분석해 본 결과, 이들 elicitor들은 *T. rugosum* 에 의한 berberine 생산을 크게 증대시키지 못함을 확인하였다.

REFERENCES

1. F. DiCosmo and M. Misawa(1985), *Trends Biotechnol.*, **3**, 318.
2. E. Kombrink, J. Bollman, E. Schmelzer and H. Hahlbrock (1985), *Plant Physiol.*, **77**, s-51.
3. E. Kombrink and H. Hahlbrock (1985), *Plant Cell Reports*, **4**, 277.
4. K. Tietjen, D. Hunkler and U. Matern (1983), *Eur. J. Biochem.*, **131**, 401.
5. K. Tietjen. D. Hunkler and U. Matern (1983), *Eur. J. Biochem.*, **131**, 409.
6. J. D. Paxton (1981), *Phytopath. Z.*, **101**, 106.
7. C. Funk (1985), *Master's Thesis*, Am Institut für Biotechnologie der *ETH*, Zürich.
8. C. Funk, K. Gügler and P. Brodelius (1987), *Phytochemistry*, **26**, 401.
9. H. Schumacher, H. Gundlach, F. Fiedler and M. H. Zenk (1987), *Plant Cell Reports*, **6**, 410.
10. J. L. Ingham (1981), *Phytopath. Z.*, **78**, 314.
11. M. G. Hahn and P. Albersheim(1978), *Plant Physiol*, **62**, 107.
12. N. T. Keen (1975), *Science*, **187**, 187.
13. A. R. Ayers, E. Jürgen, F. Finelli, N. Berger and P. Albersheim(1976), *Plant Physiol.*, **57**. 751.
14. A. J. Anderson-Prouty and P. Albersheim (1975), *Plant Physiol.*, **56**, 286.
15. A. R. Ayers, J. Ebel, B. Valent and P. Albersheim (1976), *Plant Physiol.*, **57**, 760.
16. C. François, R. D. Marshall and A. Neuberger(1962), *Biochem., J.*, **83**, 335.
17. M. Yoshikawa (1978), *Nature*, **275**, 546.
18. J. I. Smith, N. J. Smart, M. Misawa, W. G. W. Kurz, S. G. Tallevi and F. DiCosmo (1987), *Plant Cell Reports*, **6**, 142.
19. S. Tallevi and F. DiCosmo(1988), *Planta Medica*, **54**, 149.
20. J. A. Hargreaves(1979), *Physiol. Plant Pathol.*, **15**, 279.
21. I. Uritani, M. Uritani and H. Yamada (1960), *Phytopathology*, **50**, 30.
22. H. Fukui, N. Yoshikawa and M. Tabata (1983), *Phytochemistry*, **22**, 2451.
23. L. A. Hadwiger and J. M. Beckman(1980), *Plant Physiol.*, **66**, 205.
25. H. Kohle, D. H. Young and H. Kauss(1984), *Plant Sci. Lett.*, **33**, 221.
26. M. E. Schwochau and L. A. Hadwiger (1968), *Arch Biochem, Biophys.*, **126**, 731.
27. J. A. Hargreaves(1981), *New Phytol.*, **87**. 733.
28. D. -I. Kim(1991), *Biotechnol, Lett.*, **13**, 213.