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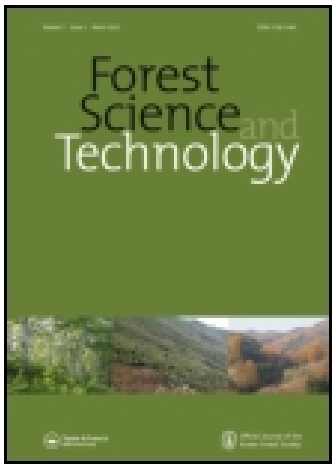

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Selection of High Berberine Yielding *Phellodendron insulare* Nak. Lines and the Antimicrobial Activity of Their Extracts

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High berberine yielding *Phellodendron insulare* Nak. lines were selected by aggregate cloning method and the antimicrobial activity of their extracts was assessed. The berberine producing cork tree lines were selected by adopting a colorimetric method. In all 300 high berberine producing lines were selected with a colorimetric reagent containing 5M HCl and H_2O_2 and established from dissociated cell aggregates. The crude extracts from these lines showed antibacterial activities against tested *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimulium*, and *Listeria monocytogenes*. The cork tree extracts were found to be inhibitory to these test organisms. Further the antimicrobial activity of plant extracts was on par with the berberine isolated from the extracts from native cork trees. These results have potential for developing alternative plant products as antimicrobial substances for application in agriculture and food industry.

Key words : *Berberine, cell line selection, antimicrobial activity*

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INTRODUCTION

The current use of microbial derived antibiotics have proved harmful to some humans, also are met with the problem of drug resistance by microorganisms. Hence, alternative and safe antimicrobial substances, especially of plant origin need to be developed. They posses relatively low antimicrobial property compared to available antibiotics. The availability of less number of useful antimicrobial substances dictates investigations on the look for new potential plant substances for therapeutic applications.

There are many reports concerning substances with antimicrobial properties from higher plants. The antimicrobial properties of individual part extracts from plants like phenol (Nagabhushan and Bhide, 1992; Moon *et al.*, 1997), alkaloids (Verpoorte *et al.*, 1978), and volatile oil (Yousef and Tawil, 1980) have been reported.

Cork tree belongs to the family *Rutaceae* and its 10 species are distributed in the Far East regions, such as Korea, China, and Japan. Among them cork tree is found in Korea with restricted distribution in Ulleung island. This tree is frequently planted as an ornamental or a roadside tree. Its wood, root, and bark extracts serve as dye applications for wool and leather. The crude extracts of barks is well known source of antimicrobial substances. Hong *et al.* (1988) have reported that cork tree bark extract was very effective in inhibition of Japanese apple canker caused due to *Valsa ceratosperma*. These results indicate that such extracts can find possible bio-pesticide functions for pathogen control. In a previous study, we developed formulation containing *P. amurense* extracts and berberine derivatives for effective prevention of plant powdery mildew, anthracnose of pepper, damping off of cucumber, and gray mold of strawberry (Data not shown -add reference if available).

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The crude plant extracts and especially berberine and its derivatives show a wide range of strong antimicrobial activity against several food related pathogens. However, the availability of *P. amurense* (cork tree) is restricted due to shortage of natural resources. One way to generate *P. amurense* biomass on a large scale is to use plant cell and tissue cultures. However, this approach has, in general, has met with limited success due to low yields (Ebel, 1986; Cusido *et al.*, 1999), but such limitations can be overcome by optimization of the culture conditions and the selection of high-yielding cell lines (Kang *et al.*, 2004).

The production of useful secondary metabolites by cell cultures of *P. amurense* has been reported (Park *et al.*, 1992; Park *et al.*, 1992; Choi *et al.*, 1996). The *P. amurense* immobilized cells produced about 70% more berberine than free cells and ca-alginate entrapped cells yielded 50-70%. The cell cultures could be *in vitro* manipulated by altering several factors such as continuous light culture, DMSO or XAD-2 resin addition which in general stimulated the more berberine release from cells. Choi *et al.* (1996) concluded that optimization of nutrient components can improve the yields of berberine and palmatine. This study establishes efficient selection method for selection of berberine rich cork tree lines. Here we also report the antimicrobial potential of cork tree cell extracts.

MATERIALS AND METHODS

Plant material

Cork tree was collected from Peak Sunginbong, Ulleung, Korea. The cambial tissues measuring 5 cm in length were detached from a mature plant. Each tissue portion was further segmented in to 2-3 cm, surface sterilized with ethanol 70% (v/v), for 2 min and treated with NaClO 5% (v/v) for 15 min. The tissue was finally rinsed 4 times with sterile distilled water to wash off the excess reagents.

Selection of berberine producing cell lines

Primary callus cultures were established from cambial tissues of cork tree. The liquid culture medium (LS medium) consisted of 1.0 mg/L 2, 4-D and 0.1 mg/L BAP, and 3% sucrose (Lismaier and Skoog, 1965). Cell cultures were grown in the same medium in dark at 25°C and sub-cultured after every 4 weeks. Suspension cultures were initiated by transferring callus to liquid LS medium.

Selection of high berberine producing cork tree cell line by cell aggregate method (Park and Choi, 1992). Suspension cells were dissociated and then filtered in screen mesh (# 300). Passed cells were gathered, and cultured on LS solid medium supplemented with 1.0 mg/L 2, 4-D and 0.1 mg/L BAP, and 3% sucrose. Microcalli were transferred on LS solid medium for 4 weeks. The cultures are maintained at 25±2°C and dark condition.

To examine for berberine producing cell lines, plant cells (0.2 g F.W.) were transferred to flask containing 1.0 mL of ether. The contents were subjected to sonication for 1 h and carefully evaporated to dryness on a water bath. The residue obtained was dissolved in 200 µL methanol, and 50 µL of extract solution was transferred into wells of the 96-well plate. To each extract containing well, 150 µL of was colorimetric reagent (mixture of 5M HCl and H_2O_2 (1:1, v/v)) was added and placed at 25°C for 10 min. After the reaction, a 10 µl of the supernatant from each well was dropped onto a filter paper for visual determination of color. Selected cell lines were also subjected HPLC analysis to quantify berberine.

Cultivation of selected cell lines

The selected cell lines were cultured on LS medium consisting of 1.0 mg/L IAA, 80 mM nitrate, 8.98 mM phosphate and 7% sucrose (Choi *et al.* 1996). All cultures were maintained 4 weeks in the dark condition at 25°C on a gyratory shaker.

Extraction and HPLC analysis of berberine

Samples for berberine analysis were collected by taking 0.5 g of cells (fresh weight) and analyzed by HPLC. Dried cells were extracted with 10 ml methanol by sonication for 1 hour on water bath maintained at 50°C. Berberine was quantitatively determined employing Gilson HPLC system equipped with a UV detector. Separation was accomplished using µ-Bondapak C18 (300×3.9 mm) column; solvent 5 mM 1-octansulfonic acid: acetonitrile (30:70); flow rate 0.5 mL/min; wavelength 254 nm. The estimates were obtained by constructing a berberine calibration curve.

Antimicrobial assay against test microorganisms

Bioassay of plant extracts was carried out by disc-diffusion method (Zaika *et al.*, 1978). Test microbial strains were procured from Genetic Resources Center, Korean Research Institute of Bioscience and Biotechnology (KRIBB). *Escherichia coli* (KCTC 1682), *Staphylococcus aureus*

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(KCTC 1621), *Salmonella typhimulium* (KCTC 1925), and *Listeria monocytogenes* (KCTC 3569) were utilized as test organisms. The stock bacterial cultures were inoculated to liquid LB medium, cultured overnight (8 h). The overnight grown bacterial cultures were mixed with autoclaved molten LB agar medium cooled to 50°C. The resulting mixture (20 mL) was poured into a petri dish (90 cm× 20 mm) to prepare a solid agar plate, and stored at 4°C until required.

Paper discs (8 mm ψ) containing various concentrations of different cell extracts, were prepared and placed on the agar plate for antimicrobial assay. Crude extracts were membrane filter sterilized (0.2 μm, Gelman). After 24 hr of incubation at 37°C, the inhibitory zone diameters were measured by using autocaliper (CD-15B, Mitutoyo Corp., Japan).

Statistical analysis

The experiments were repeated for a minimum of three times. Each numeral value represents the mean and standard deviation (SD) by analysis of variance (ANOVA).

RESULTS AND DISCUSSIONS

Selelction of berberine rich cell lines

All cell lines of *cork tree* do not show berberine production in cell cultures except on elicitation.

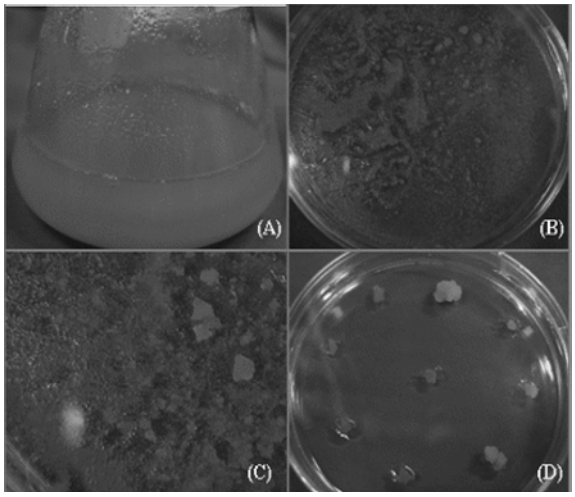

Figure 1. Selection of high berberine producing cork tree cell line by cell aggregate method. A: Suspension cell cultures on LS liquid medium within 2 weeks, B: Dissociation and plating of suspended cells on LS solid medium (after 1 week of plating), C: Micro-calli formation on LS solid medium (after 3 weeks of plating), D: Microcalli were transferred on LS solid medium for 4 weeks (after 7 weeks of plating).

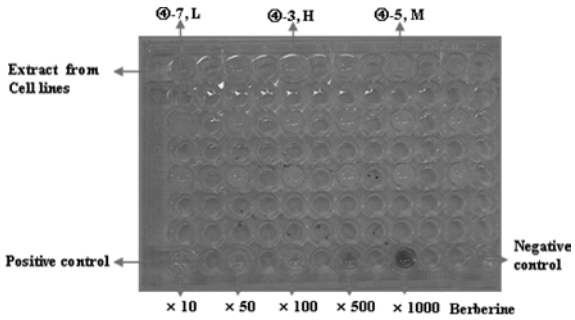

Figure 2. Cell line selection by colorimetric method. H: high berberine; M: medium berberine; L: low berberine producing cell lines; Positive control: 5M HCl + H_2O_2 + authentic berberine; Negative control: reagent mixture + Methanol.

We attempted here to select high berberine producing cell line by cell aggregate cloning method (Figure 1). Callus derived suspended cells (Figure 1A) were plated on LS solid medium (Figure 1B). The cell clumps obtained showed further growth (Figure 1C) as microcallus, and finally calli were formed after 7 weeks of plating (Figure 1D). In this manner, 300 cell lines were obtained by adopting cell aggregate cloning method.

Cell lines obtained exhibited varied morphological features like color and growth pattern among cell lines. The concentration of berberine was established by colorimetric reaction (Figure 2) and this property was adopted for the selection of berberine rich cell lines. In all 300 cell lines biosynthetically different in production of berberine were selected. Almost all cell line extracts on treatment with colorimetric reagent showed weak reaction. Authentic berberine showed dark yellow color, and was considered as a positive control. The color intensities linearly correlated with increasing amounts of berberine (Figure 2). The berberine amounts were determined simultaneously by HPLC analysis (Figure 3).

A visual screening method for the selection of cell lines with varying amounts of berberine was established. HCl and H_2O_2 solution reacts or couples with berberine to form dark yellow colored complex. Berberine is an alkaloid, widely distributed in numerous plants families. It is considered as a derivative of di-isoquinoline and a strong quaternary base. In this study, the intensity of color may attributed to high acidification. Ananichev *et al.* (1966) have reported that the reaction of berberine with hydrogen peroxide is very sensitive. However, the exact mechanism of this reaction is yet to be understood.

Generally, the concentration of the plant sec-

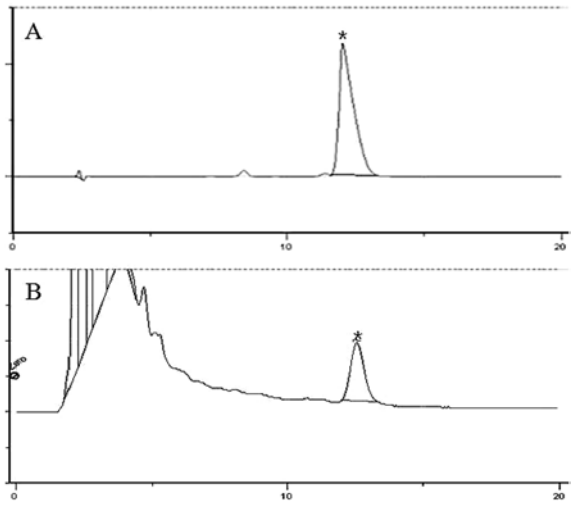

Figure 3. HPLC analysis of berberine chlorides and methanol extracts from PC 4-3 cell line. A; Berberine chloride standard (R.T.=12.04), B; Methanol extracts from PC *4-3* cell line (R.T.=12.07). *R.T.* means retention time in HPLC.

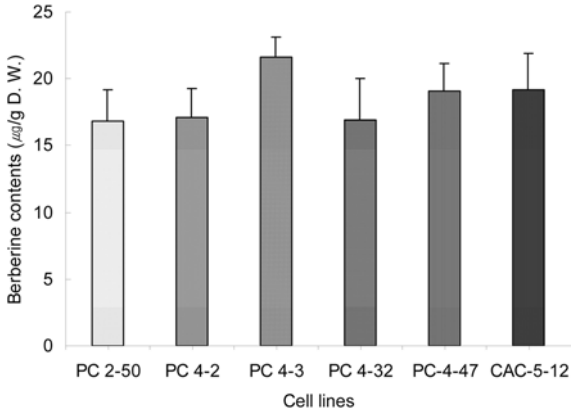

Figure 4. The berberine contents on selected cell lines selection through cell aggregate selection methods. The berberine contents in cell lines were obtained by HPLC analysis. The error bar show standard deviation from the mean. PC: cork tree cell lines.

ondary metabolites has been determined either with HPLC or GC analysis (Figure 3). However, these analytical methods are cumbersome due to the involved complicated procedures, time, high cost and labor. Thus these results demonstrate that colorimetric method is efficient for screening and selection of cork tree with high berberine or its derivatives.

Berberine production by selected cell line cultivation

Among the selected cell lines, six of them were high berberine producing, of which PC4-3 line was considered as a reliable callus line with high berberine yield and rapid cell growth (Figure 4). *The alkaloid contents of six lines were more or less fluctuated from 15 to 23 μg per g dry weight.*

For production of bereberine, selected lines were cultured on slightly modified with respect to LS basal medium. Berberine was detected from the extracts of cultured cells, but the amount varied depending upon cell line and culture conditions (Data not shown). However, cell lines which did not show positive reaction with color reagent were considered as berberine non-producers. The di-isoquinoline extracted from the plant extracts was tentatively identified as berberine based on HPLC retention time, UV spectra, and EI mass spectrometry. The Mass Spectrum (MS) analysis of purified berberine from the cultured cells was identical to the MS of authentic sample. The Mass spectrum of compound showed relative intensities for molecular ion peaks at: 337 (M^+, 2), 336 (18), 270 (12), 252 (100), 235 (47) 217 (23.5), 142 (29), 119 (41.2), and 105 (6) attributing to the fragmentations of isoquinoline alkaloid, berberine (data not shown).

The heterogeneous nature of cell suspension cultures is another possible cause for the large line-to-line variation in metabolite production. Cell suspension culture normally consists of cells with diverse morphology and aggregation states. Individual cells in these physiological states respond differently to the environment. The difference in the distribution of these cells among cell lines often leads to variability in secondary metabolite production (Hall and yeoman, 1987). Compounds that are present in the intact plant may be not present in cell culture. On the contrary compounds that are not present in the intact plant may be present in cell culture. Our results indicate that berberine non-producing cell line can be separated at the level of cell line selection. Yamada *et al.* (1983) have reported a similar result using homogeneous stable strains of high vitamin B_6 yielding cell lines. They obtained four times more secondary product than that of normal cell lines of *Cytisus scoparius*. Using the cell aggregate selection method, we obtained six berberine producing cell lines. However, further experiments on serial selection are still warranted for this system. Results described in this study may help to overcome the obstacles of low and unstable productivity involved in plant secondary metabolite production. This study can serve as a model system in the study of production of useful secondary metabolites from

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Table 1. Comparison of antimicrobial activities of extract from PC4-3 cell cultures.

	Escherichia coli	*Staphylococcus aureus*	*Salmonella typhimrium*	Listeria monocytogenes
Positive control				
Berberine chloride 2.5 mg	2.8	2.5	2.5	4.0
Negative control				
Methanol 100 μL	-	-	-	-
Cork tree cell extract				
2.5 mg	1.0	1.1	1.0	1.4
5.0 mg	2.3	2.6	1.9	2.8
10.0 mg	3.8	4.8	2.4	6.0
Plant extract				
bark	1.0	2.5	1.0	3.0
leaf	1.3	1.0	1.3	2.3
stem	1.3	1.0	1.0	2.5

–: no response

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tree species which are in general recalcitrant to *in vitro* manipulation. This system for selecting high berberine yielding cell lines appears to be readily adaptable for the commercially attractive biosynthesis of secondary metabolites of other tree species.

Antimicrobial activity of cell extracts

Cork tree cell extracts with various concentration of berberine inhibited the growth of various microbes tested (Table 1). Berberine chloride inhibited all four test microorganisms and this treatment served as the positive control. The extracts from cork tree showed varied and potent inhibitory response. Antimicrobial activity of plant extract was different based on plant part and test microbials. On the other hand, antimicrobial activities of methanol extract prepared from cell cultures were similar to the one from wild cork tree (Table 1). The antimicrobial activity was dependent of concentration of the in cell extract employed. On the other hand, antimicrobial activity of cell extract of cork tree was similar to cork tree extracts at the same concentrations.

Antimicrobial activity of cell extract was less compared to berberine. The antimicrobial activities demonstrated by cell extracts followed the order: *Listeria monocytogenes* > *Staphylococcus aureus* > *Escherichia coli* > *Salmonella typhimulium*. The highest antimicrobial activity, which was observed for cultured cell extracts, was interesting to note. Antimicrobial activity of selected *R insulare* or PC4-3 was increasing with concentration of cell extracts. Generally, the wild plant extracts had higher biological activity, and all selected cell cultures showed antimicrobial activity. Hence, screening for new biologically active compounds from plant cell cultures is feasible. However, study for the antimicrobial activity through plant cell cultivation very few have been reported. The antimicrobial activity from cell cultures of cork tree has also not been elucidated so far. Furmanowa *et al.* (2002) have reported that antimicrobial activity in the extracts of *Polyscias filicifolia* biomass from bioreactor and callus. Sokmen *et al.* (1999) also reported the establishment of that twenty-four callus, and eleven cell suspension cultures from Turkish medicinal plants whose extracts contain *in vitro* antimicrobial activities. The antimicrobial activity of leaf and callus extracts of *Rauvolfia tetraphylla* and *Physalis minima* is shown to be inhibitory for selected pathogenic fungi and bacteria (Shariff *et al.*, 2006). Thus it is indeed possible to observe more biological activities from extracts of plant cell cultures. Thus based on our results we conclude that the cell extracts from lines of cork tree posses strong antimicrobial activity compared to the extracts from wild plant. Such findings facilitate the isolation of natural antimicrobial substances from nature for medicinal, agricultural and food industry applications.

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