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ELICITATION, BIOCONVERSION AND QUANTIFICATION OF BERBERINE FROM *CISSAMPELOS PARIERA* CALLUS CULTURES

Vikash Shad [1] and M. A. Deepa [*2]

Research Centre[1], Bharathiar University, Coimbatore – 641046 Tamil Nadu, India
Kristu Jayanti College [2], K Narayanapura, Bangalore – 560077, Karnataka, India

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Correspondence to Author:
M. A. Deepa

Associate Professor,
Departmet of Life Sciences, Kristu Jayanti College, K. Narayanapura, Nothanur (Post), Bangalore - 560077, India

E-mail: deepa.ma@kristujayanti.com

ABSTRACT: *Cissampelos pariera,* an important medicinal plant was subjected to elicitation and bioconversion of precursors for the formation of Berberine, an important medicinal component. *In vitro* cultures of *Cissampelos pariera,* was successfully established using stem and leaf explants. MS Media fortified with IAA (2 mg/l) alone and MS media enriched with IAA (2mg/l) showed profuse callusing from leaf and stem explants respectively. Elicitation with yeast resulted in the highest percentage of berberine yield from leaf callus (0.055%). Elicitation with *Bacillus subtilis* showed the presence of 0.0294% berberine in leaf callus. Elicitation with *Aspergillus niger* showed the highest yield of 0.0019% from stem callus. Addition of precursor components Tryptophan and Phenylalanine did not increased the berberine from leaf callus. But addition of precursor components Tryptophan and Phenylalanine showed the bioconversion to 0.0019% and 0.0022% of berberine in stem callus. The study reports a standardized protocol for the efficient conversion of precursors to breberine and also to elicit the production using elicitors.

INTRODUCTION: Tissue culture mediated production or enrichment of phytochemicals is one of the promising techniques used in the recent years. Plants are able to synthesis complex compounds that may be difficult to synthesize in a chemical laboratory[1]. But most of the time the extraction of these chemicals from plants leads to complete destruction of plants from the environment. This leads to the depletion of species. Thus plant cell cultures are found to be an attractive alternative to meet the demands, without destroying the plant species.

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The callus induced from the respective plant parts are also reported to produce the medicinally important compounds in many plants. Eliciting the production of compounds by subjecting the callus to disease causing microorganisms is applied in many plants to increase the production of secondary metabolites. Many organisms and the chemicals were used and proved as promising elicitors for various plant systems.

This triggers the defense mechanism in the plant and induces the production of secondary metabolites in larger quantities. These biological elicitors are mostly used in the form of heat killed cell extracts or filtrates of fungi, bacteria etc. More over using cells as bioreactors and bioconversion of precursors into the required final compounds are also commonly applied in many economically important plant species. *Cissampelos pareira* also known as Patha, velvet leaf, ice vine, patindu and

abuta belongs to the family Menispermaceae. It is also called Laghu patha in the Ayurvedic medicine and "Malaith thaangki" in Siddha medicine. It is distributed throughout India. In Himachal Pradesh, common in Chamba, Kangra, Sirmour, Una, Bilaspur, Ahju forest, Jawar forest at 1000 m, Solan and most other places upto 1500m elevation. It is also found in Bangalore, Tamil Nadu and most of the places of Kerala (Chauhan N.S 834, 876, 1224, 1370, 1923). The plant is common in orchards, hedges, parks and gardens on the moist soils distributed throughout tropical and sub-tropical India and on the hilly tracts along water courses.

It is a sub-erect or climbing herb used which reaches 3 to 6 m along the ground or into the crowns of the trees. The stem is woody, flexible, and slender (to 1 cm) and twines for support. Alternate leaves are usually softly pubescent on both the surfaces. The leaves are about 1 to 4 inches in length having a diameter of about 1-3 inch. It has veins that are 5-7 in numbers. Venation is palmate in widely oval or nearly around 4-10 cm blades. Petiole is 3-7 cm long. The root system consists of flexible, light brown lateral roots with sinkers and moderately abundant fine root system.

It is highly rich in alkaloids namely as curine, curine-4-Methyl-Ether, 4"-0-Methyl curine, alkaloids, Berberine, Cissamine, Cissampareine, Cyclanoline, Cycleanine, D-Quercitol, Hayatidine, Hayatine, Hayatinine, Isochondodendrine, Menismine, Perierine, Quercitol, Saponin, Tetrandrine and Tetrandrine-N-2'-oxide[2]. But Berberine is reported as the major alkaloid from the *Cissampelos pareira.* Berberine is proved for the following medicinal activity.

Berberine an alkaloid extracted from this plant shows both Anti-adipogenic and anti-inflamatory effects on 3T3-L1 adipocytes and the anti-adipogenic effect is due to the down regulation of the adipogenic enzymes and transcription factors[3]. It shows activity against gastrointestinal peptides elevation and mucous secretion in hyperthyroid diarrheic rats. Antidiarrheal effects of Berberine may be due in partially to the reduction of the number of globlet cells and the amount of mucous secretion through re-balancing gastrointestinal peptides[4]. It induced antiproliferative activities in human promonocytic U937 cells. 75µg/ml^{-1} induces significant changes in mitrochondrial membrane in U937 cells. It induces apoptosis of U937 cells through the mitrochondrial/ caspase-dependent pathway[5]. It protects against endothelial injury and enhances the endothelium- dependent vasodialation which is mediated through activation of AMPK signaling cascade. Berberine and its derivatives may be useful for the treatment or prevention of endothelial dysfunction associated with diabetes and cardiovascular disease[6.] It is a novel natural hypolipidemic agent.

It exerts anticancer activities both in vitro and in vivo through different mechanisms. It shows inhibitory effects on the proliferation and reproduction of certain tumorigenic microorganisms and viruses such as *Heliobacter pylori* and Hepatitis B virus[7.] 0.176 %of berberine extracted from the roots of *Cissampelos pareira* showed to possess in-vitro antioxidant activity and immunomodulatory activity in mic[8]. In the current study, an attempt was made to induce callus from leaf and stem explants of *Cissempelos pariera* and to enhance the production of Berberine using elicitors and precursors.

MATERIALS AND METHODS:

Cissampelos pareira (L.) (Menispermaceae) was collected from herbal garden of FRLHT, Bangalore and planted in the green house for further use. The plant material was identified and authenticated taxonomically at the Life Science Department of Kristu Jayanti College, Bangalore. A voucher specimen of the collected sample was deposited in the department herbarium for future reference.

Leaf explants were collected from the mother plants and washed thoroughly with running tap water. The explants were treated with teepol for 3 min to remove surface dust and dirt and washed 4-5 times in distilled water. Then the explants were surface sterilized with 10% Sodium hypochlorite for 5 min and a thorough wash in sterile distilled water for 7-8 times. The explants were trimmed and inoculated on MS medium fortified with NAA (2mg/l) for inducing callus. The cultures were incubated at 25±2°C.

Elicitation: The induced callus was sub-cultured to MS media supplement with IAA (2mg/l) for Leaf

and NAA (2mg/L) for stem and various elicitor organisms to increase the berberine content.

***Saccharomyces cerevisiae*:**
Yeast granules dissolved in double sterilized water was added to MS medium supplemented with respective growth hormones at specific concentrations

***Bacillus subtilis*:**
The bacteria *Bacillus subtilis* grown enriched in the Nutrient broth and after 48 hours of incubation were heat killed and added into the MS medium containing growth regulators in specific concentrations.

***Aspergillus niger*:**
The fungus *Aspergillus niger* was cultured on the PDA (Potato Dextrose Agar) for 48 hours, heat killed and then transferred onto the MS medium containing growth hormones in specific concentration.

Bioconversion:
Two precursors viz. Tryptophan and Phenylalanine at concentrations ranging from 5 mg -25 mg/l was weighed and added directly to the MS media supplemented with IAA (2mg/l) for Leaf and NAA (2mg/L) for stem.

HPLC Estimation of Berberine:
The callus obtained from all the above experiments were dried and extracted with ethanol. The extract was evaporated and the percentage yield of extract. The residue was redissolved in 5 ml of ethanol and subjected to HLC analysis. Isocratic elution through C18 reverse phase column with solvents water/acetontrile/trifluroacetic acid (60:40:0.1) was used. Flow rate was 1.0 ml min^{-1}. The compound was identified as berberine HCl by comparing the Rf values, retention time and peak of the sample and the standard berberine HCl purchased from Sigma Chemical Co. Inc, USA.

RESULTS AND DISCUSSION:
The current work was carried out with the aim of the increasing the production of berberine in leaf and stem explants. The MS Medium supplemented with IAA (2mg/l) was found to be the optimum combination for inducing profuse callus from leaf explants. But stem explants showed profuse callusing when cultured on MS medium fortified with NAA (2mg/l). When the medium was supplemented with elicitors (heat killed organism) along with respective hormones, both the explants maintained the callusing ability. The leaf explants responded within 20 days and stem within 28 days. The wet weight of the callus was taken on the 35th day and was left in the hot air oven and after 5 hrs, dry weight was recorded (**Table 1**).

TABLE 1: HPLC QUANTIFICATION OF BERBERINE-ELICITOR MEDIATED ENHANCEMENT

Sl. No.	Explants	Elicitor	Concentration of Berberine
1	Leaf as on control	Control	Nil (Not detected)
2	Leaf	*Aspergillus niger*	Nil (Not detected)
3	Leaf	Yeast	0.055%
4	Leaf	*Bacillus subtilis*	0.0294%
5	Stem	Control	Not detected
6	Stem	*Aspergillus niger*	0.0019%
7	Stem	Yeast	NIL
8	Stem	*Bacillus subtilis*	NIL

Bioconversion of precursors:
Addition of precursor components Tryptophan and Phenylalanine did not increased the berberine content in the leaf callus. Whereas addition of precursor components Tryptophan and Phenylalanine showed the bioconversion of 0.0019% and 0.0022% of berberine from stem callus (**Table 2**).

TABLE 2: HPLC QUANTIFICATION OF BERBERINE-PRECURSOR MEDIATED ENHANCEMENT

Sl. No.	Explant	Elicitor	Concentration of Berberine
1.	Leaf as on control	Control	Nil (Not detected)
2.	Leaf	Tryptophan	Not detected
3.	Leaf	Phenylalanine	Not detected
4.	Stem	Control	Not detected
5.	Stem	Tryptophan	0.0019%
6.	Stem	Phenyl alanine	0.0022%

Several studies on cell cultures derived from a range of plant species have shown that elicitation is an effective means in increasing the production of secondary metabolites. Parameters such as elicitor specificity, concentration and exposure time as well as culture conditions and growth stage of the cells can have a considerable influence on the elicitation process[9]. Elicitation with yeast resulted in the highest percentage of berberine yield from leaf callus (0.055%). Similarly, it was also, reported that yeast when used as elicitor caused a rapid increase in rosamaric acid in *Salvia miltirrhiza*[10].

Highest percentage of Oleanolic acid, in *Calendula officinalis* cell cultures treated with Yeast extract was reported after 72hrs of treatment with 300 mg/l, i.e showing a 5 fold increase over the control[11]. Elicitation with *Bacillus subtilis* showed the presence of 0.0294% berberine in leaf callus. Addition of *A. niger* elicitor, to the *P. rosea* cells enhanced the metabolites content with an increase in the dose up to 1.5% (v/v)[12]. Elicitation with *Aspergillus niger* showed the highest yield of 0.0019% from stem callus. Similarly, it was previously reported that addition of *Aspergillus flavus* mycelial extracts enhanced the production of anthocyanins in *Daucus carota* cell cultures[13]. Whereas, the highest berberine production in *Coptis japonica* cells ie 5% by dry weight was reported earlier[14].

Feeding of L-Phenylalanine caused 3-5 fold increase in 5-methoxypodophyllotoxin in cell suspension cultures of *Linum flavum L.* An exogenous supply of a biosynthetic precursor to the culture media may improve alkaloid accumulation where production is limited by lack of precursor[15]. This is may be reason that in the current study also addition of precursors resulted in increased production of berberine. Thus, elicitation with fungal and bacterial organisms is a promising technique to increase the berberine content in callus cultures of *Cissampelos Pareira.* The process of bioconversion of precursors to berberine was achieved successfully when precursors when added to the culture media.

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