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Short Communication

The role of phytohormone on the production of berberine in the calli cultures of an endangered medicinal plant, turmeric (*Coscinium fenestratum* l.)

Talat Khan[1]*, David Krupadanam[2] and Syed Yousuf Anwar[1]

[1]Department of Genetics, Osmania University, Hyderabad-500 007, India.
[2]Department of Chemistry, Osmania University, Hyderabad-500 007, India.

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The present report for the first time describes the results of study aimed at evaluation of the role of phytohormones on the production of berberine from *in vitro* calli cultures. Calli cultures derived from leaf and petiole explants were established in MS medium supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D) plus 6-benzylaminopurine (BAP) and/or kinetin (KN). Berberine an isoquinoline alkaloid, was isolated from 6 - 7 week old calli cultures. Media, phytohormones, and explants used influenced the biomass and berberine content in calli cultures. Berberine with the retention time of 8.49 min and enhanced dry weight (1.788%) from the petiole explant is reported for the first time in this study. The presence of berberine was first checked by preparative thin layer chromatography (TLC) and then confirmed by High Pressure Liquid chromatography (HPLC) and mass spectrometry. Chemical structure was determined through proton NMR and ^{13}C spectra.

Key words: Berberine, calli cultures, *Coscinium fenestratum*, isoquinolene alkaloid, phytohormones.

INTRODUCTION

Herbal remedies have become more popular in the treatment of variety of ailments, and also on account of the increasing costs of personal health maintenance. Indeed, the market and public demand has been so great that there is a great risk that many medicinal plants today, face either extinction or loss of genetic diversity. Reserves of herbs and stocks of medicinal plants in developing countries are diminishing and in danger of extinction as a result of growing trade demands for cheaper healthcare products and new plant-based therapeutic markets in preference to more expensive target-specific drugs and biopharmaceuticals. According to an all India ethno biological survey carried out by the ministry of environment and forests, Government of India, Medicinal plant as a group comprises approximately 8000 species that account for around 50% of higher flowering plant species of India. As the demand for medicinal plant is growing, some of them are increasingly being threatened in the natural habitat. *Coscinium fenestratum* is one among the families having several medicinal values from root to fruit (Narasimhan and Nair, 2004). Because of its medicinal uses in Ayurveda, Unani and Siddha systems of medicine, collection of wild plants from natural habitat has made the species endangered. Therefore, the present study describes an alternate approach of *in vitro* culture for the isolation and characterization of the plant's secondary metabolites (berberine).

*Corresponding author. E-mail: talatkh@yahoo.co.in.

MATERIALS AND METHODS

Calli cultures

Cuttings of the wildly growing plants of *C. fenestratum* were collected from different places of Western Ghat and were established in the green house of Plant Genetics Experimental Farm. Two different explants; leaf and petiole segments were used for *in vitro* studies.

The excised plant parts were thoroughly washed in running tap water for 5 - 8 min and subsequently washed with a mild detergent (Tween-20) for 2 - 3 min. The explants were treated with 0.1% mercuric chloride for 3 - 5 min and rinsed with sterile distilled water 3 times for 3 - 5 min. The surface sterilized explants were cut into small pieces of 0.5 - 1 cm in a sterile petridish (petriplate) and were

Table 1. Role of auxin and cytokinin for callusing response and biomass of different explants of *C. fenestratum*.

Hormone conc. with constant 2.0 mg/l 2,4-D	Petiole Response (%)	Petiole biomass (mg)	Leaf response (%)	Leaf biomass (mg)
2,4-D + BAP (0.5)	66.0	1630	62.0	1380
2,4-D + BAP (1.0)	72.3	1810	71.0	1605
2,4-D + BAP (1.5)	80.0	2383	78.8	2230
2,4-D + BAP (2.0)	**98.6**	**2825**	94.6	2705
2,4-D + BAP (2.5)	90.5	2765	89.0	2634
2,4-D + KN (0.2)	65.0	1610	65.0	1680
2,4-D + KN (0.4)	76.7	1740	73.6	1815
2,4-D + KN (0.5)	88.5	1835	86.8	2085
2,4-D + KN (1.0)	93.5	2190	**97.6**	**2798**
2,4-D + KN (1.5)	90.7	2137	92.5	2705

inoculated under aseptic conditions onto two different media; Murashige and Skoog (1962) and Woody plant medium supplemented with different phytohormones: 2,4-dichlorophenoxy acetic acid (2,4-D) plus 6-benzylaminopurine (BAP) and kinetin (KN) with different concentrations individually and in combination.

Calli cultures thus raised were sub cultured on to fresh medium once in a week and the cultures were maintained under fluorescent light with 12 h photo period as well as in the dark at 25 ± 1ºC temperature with 50 - 60% relative humidity.

Isolation and characterization of berberine from calli cultures

The air dried calli culture (500 mg) was homogenized with a mortar and pestle then extracted with pure methanol repeatedly until all the yellow color is removed from the material. Methanol was evaporated and the concentrated extract was separated by preparative TLC by using ethyl acetate : methanol. The yellow fluorescent fraction was collected and was evaporated by dryness. The crude extract was loaded into a silica column and eluted with different fractions of low to high polar solvents. Yellow colored powder thus obtained was identified by comparing Rf- values on TLC, HPLC, ^{1}H NMR, ^{13}C NMR and MS spectra with relative value of authentic compound obtained from the Sigma Chemical company USA.

Dragendroff's test

Compound (1 mg) was dissolved in 2 ml of chloroform, 10% solution of Dragendroff's reagent was then added which gave brick red precipitation indicating the presence of alkaloid.

RESULTS AND DISSCUSION

In the present study an attempt has been made to analyze the response of two different media; Murashige and Skoog and Woody plant medium for callus induction. Among these two media MS medium showed maximum response of callus induction. Hence only MS medium was used through out this study. The quality and quantity of plant growth regulators initially present in the media or administered during the course of *in vitro* culture seems to have a significant effect on the metabolism of secondary metabolites (Gamborg et al., 1971). Since the production of secondary metabolites in plant cell culture is a function of both cell multiplication and division, therefore, growth regulators are bound to have a major role in determining the production potentiality of a given culture (Kurz and Constabel, 1979; Staba, 1980). Effect of the type of auxin on secondary metabolite synthesis has been studied in different medicinal plants. Production of thebaine from cell cultures of *Papaver bracteatum*, indole acetic acid (IAA) was found to be better than naphthalene acetic acid (NAA) or 2,4-D (Furuya et al., 1971). In general, 2,4-D has been found to be less suitable for triggering secondary metabolites in tissue culture than either IAA or NAA (Nair et al., 1992). There are only few reports wherein 2,4-D has been affective in enhancing the secondary metabolites. Carotenoid and ubiquinone contents of callus cultures were enhanced with high levels of 2,4-D in tobacco plant cells in suspension culture (Ikeda et al., 1976).

In the present study, MS medium supplemented with 2,4-D is ideally suited for callus induction. In combination treatment, the varying levels of 2,4-D in conjunction with BAP (0.5 to 2.5 mg/l) resulted in wide variation in callusing response as well as biomass (Table 1). However, with the increasing concentration of 2,4-D along with BAP, there was a concomitant increase in callus response and callus growth as assessed from biomass (Ikuta et al., 1975). In *Coptis japonica*, Ikuta and Itokawa (1982) have obtained callus on MS medium supplemented with 2,4-D and KN. In the present study, percent calli response from the petiole explants ranged from 66.0 to 98.6% whereas with leaf explants it ranged from 62 to 97.6%. Similarly biomass of petiole explants ranged from 1630 to 2825 mg and with leaf explants the biomass ranged from 1380 to 2798 mg. These results clearly indicate superiority of petiole explants over leaf in terms of percentage calli response and its biomass. It is obvious from the data presented in Table 2 that with an increase in concentration of BAP along with 2,4-D (2.0 mg/l), there was a concomitant increase in the percent dry weight of berberine from 1.124 to 1.788%. However,

Table 2. Effect of auxin and cytokinin during callus response for biomass and % dry wt of berberine from petiole explants of *C. fenestratum*.

Constant concentration 2.0 mg/l 2,4-D and varying concentration of BAP	Petiole biomass (mg)	Dry wt. of berberine (%)
2,4-D + BAP (0.5)	1630	1.124
2,4-D + BAP (1.0)	1810	1.322
2,4-D + BAP (1.5)	2383	1.427
2,4-D + BAP (2.0)	**2825**	**1.788**
2,4-D + BAP (2.5)	2765	1.614

the threshold concentration of BAP was noticed as 2.0 mg/l where the maximum percent dry weight of berberine (1.788%) was obtained. Increasing concentration of BAP beyond 2.0 mg/l had shown to have a negative affect (Table 2). Quantitative analysis of berberine done by High Pressure Liquid chromatography (HPLC) indicated by the presence of large peak of berberine with the retention time of 8.49 min similar to that of reference sample from sigma chemicals USA.

Conclusion

Media, pH of medium, plant growth regulators and sucrose concentration profoundly influence the biomass and berberine accumulation in the calli cultures of *C. fenestratum*. The content of berberine in the calli cultures was 17.88 mg/g dry wt. which is nearly 18 folds higher compared to the petiole of intact plant. Therefore, *in vitro* cultures can be used as an alternate source for berberine production. However, efforts are underway to yield maximum berberine content in cell cultures of *C. fenestratum* by adopting different culture techniques such as immobilization, elicitation and precursor feeding, as this medicinal liana is facing genetic erosion.

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