

Research

Production of bergenin, an active chemical constituent in the callus of *Bergenia ciliata* (Haw.) Sternb.

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Abstract

In vitro culture of *Bergenia ciliata* (Haw.) Sternb. was carried out for the examination of bergenin content. Leaf explants were cultured in MS (Murashige and Skoog) basal media supplemented with or without phytohormones. The hormonal series maintained were in the range of 0-2 mg l⁻¹ for BAP (6-benzyl amino purine) and 0-1.5 mg l⁻¹ for NAA (α -naphthalene acetic acid). Bergenin content of *in vitro* grown tissues of *B. ciliata* was compared with that of wild plants collected from three different localities of Nepal. The best growth of callus and plantlets occurred in the media containing BAP 1.0 mg l⁻¹ + NAA 1.0 mg l⁻¹ and BAP 1.5 mg l⁻¹ + NAA 1.0 mg l⁻¹. Production of bergenin was high in the media supplemented with 1.0 mg l⁻¹ BAP + 1.5 mg l⁻¹ NAA (3.40 μ g g⁻¹) and 2.0 mg l⁻¹ BAP + 1.5 mg l⁻¹ NAA (3.05 μ g g⁻¹) under experimental condition. The bergenin content in the wild plants collected from Langtang, Jumla and Godawari was found to be 4.28 μ g g⁻¹, 4.53 μ g g⁻¹ and 3.64 μ g g⁻¹ respectively. This study shows that the *in vitro* cultured callus of *B. ciliata* is capable of synthesizing bergenin in quantity comparable to that of the wild plant.

Key-words: callus, chemical content, HPTLC analysis, medicinal plant, tissue culture, Nepal Himalaya.

Introduction

Bergenia ciliata (Haw.) Sternb. (Saxifragaceae) is a high value medicinal plant of the Himalaya. It is a perennial herb with thick, stout, creeping rhizomes. It is found on moist rock ledges and rocky cliff in forest and shady banks from an altitude of 900 to 3600 m asl in the Himalaya. It is locally known as *Pakhanbhed* (in Nepali), the name derived from Sanskrit word – *Pashanabheda* which signifies its growth habit and habitat (*Pashan* meaning rock and *bheda* meaning piercing). *B. ciliata* is a threatened species in Nepal (Shrestha and Joshi 1996) due to commercial harvest and has been prioritized for conservation and economic development by the Government of Nepal (DPR 2008).

Rhizomes of *B. ciliata* have variously been used as medicine for different diseases (Sinha *et al.* 2001; Baral and Kurmi 2006; DPR 2007). The active chemical has been identified as bergenin and various methods have been developed for its quantitative and semi-quantitative estimation (Anonymous 2002). High performance thin layer chromatography (HPTLC) has been used as the quantitative tool for the determination of bergenin content in the rhizome of *Bergenia* spp. (Bahl *et al.* 1974).

It has been shown that under some suitable conditions a plant cell can be activated to produce chemicals of medicinal value *in vitro* by means of tissue culture technique (Flores and Filner 1985; Chattopadhyay *et al.* 2004; Paudel and Pant 2006). This may have important implication, in the future, for the production of medicinally active secondary compounds in commercial scale (Goldstein 1980; Zenk 1991; Verpoorte

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et al. 1993; Ahmad *et al.* 2007). The yield of secondary products in cell culture has been reported to be equal to those of intact plants (Kreis and Reinhard 1989; Charlwood and Rhodes 1990; Andrade *et al.* 2004; Ahmad *et al.* 2007), rarely reaching as high as 10% (Trease and Evans 2002). However, in most cases, the secondary metabolites in the tissue or cell culture remained in quantities lesser than that produced by intact plants (Zieg *et al.* 1983; Asai *et al.* 1994; Trease and Evans 2002).

Although tissue culture technique has been used to propagate *B. ciliata* by some workers (e.g., Karki and Rajbahak 2008), the analysis of the callus of this species for bergenin content has not been reported. The present study was aimed to examine the potency of the callus formation of various parts of *B. ciliata* and assess the content of bergenin, the active principle, in callus grown in various combinations of BAP and NAA.

Materials and Methods

Healthy leaf segments (3-5 mm × 3-5 mm), shoot tips (1-2 mm) and root tips (3-5 mm) of *B. ciliata* were taken from *in vitro* raised plants under aseptic condition following Karki and Rajbak (2007). Then, the explants were inoculated in the MS media supplemented with or without hormones. Hormonal media comprised of 0.0-2.0 mg l⁻¹ BAP (6-benzyl amino purine) and 0.0-1.5 mg l⁻¹ NAA (α -naphthalene acetic acid) and their combinations. All the cultures were maintained at 25 ± 2° C and daily photo period of 16 h. Subculture was done only on the fraction of culture at the interval of three months as the callus grew very slowly.

Callus mass and proliferated shoots from each hormonal concentration were analyzed for bergenin content by HPTLC method (IDMA 2002; ICMR 2003). The tissue mass was shade dried and then ground to powder. Two grams of dried tissue was refluxed with methanol. After drying, the methanolic extract was again dissolved in 50 ml of methanol. The extract was analyzed first by thin layer chromatography (TLC) using the pre-coated plates with silica GF₂₅₄. Solvent used to develop the plate was toluene: ethyl acetate: acetone (2: 4: 4). The spots were visualized by spraying concentrated sulphuric acid and heating for 80° C in an oven (the spots were visible as blue spots). The plates were observed in daylight where bergenin was visible as pink spots at R_f = 0.36. Reference

solution was prepared by dissolving 2.5 mg of reference material (bergenin) obtained from Sigma (Ref. No. B6776) in 5 ml of methanol.

For HPTLC, 5 µl of the prepared extract was applied in thin layer plates (with silica GF₂₅₄). It was then developed with the solvent system toluene: ethyl acetate: acetone (2: 4: 4). Standard of bergenin was also placed along the sample for quantitative analysis. TLC plates were scanned in Desaga CD-60 Densitometer at wavelength 254 nm.

Rhizomes of *B. ciliata* were collected from natural populations at three different regions of Nepal: Langtang National Park (2280-3000 m asl) and Godavari Community forest (2300-2760 m asl), and Jumla (2370-3000 m asl). Langtang and Godavari lies in central Nepal and Juma in western Nepal. Rhizome samples were powdered, and 2 g of which were refluxed with methanol. Finally methanolic solution was prepared and analysed for bergenin content following the same methods as described above.

Results

IN VITRO CULTURE AND THE FORMATION OF CALLUS

The root tip and shoot tip failed to show any response in any of the culture media used, but leaf explants responded variously in growth and development of callus (Table 1). In the absence of NAA, hormonal media containing only BAP (at all concentrations) failed to initiate callus formation from leaf explants. However, hormonal media containing only NAA initiated callus growth with root like structures. However, stable cultures were not produced. In 0.5 mg l⁻¹ NAA alone (media no. 6), a slight evidence of callusing of the leaf explants was observed in 2 weeks and it took nearly a month for complete callusing.

At higher concentrations (≥0.5 mg l⁻¹) of both BAP and NAA in MS medium, the explants responded variously in callus formation. However, the optimal concentrations of these hormones for callusing and organogenesis were 1.0 mg l⁻¹ NAA and 1.5 mg l⁻¹ BAP. These hormonal concentrations were most appropriate for the plantlet proliferation. The cultures were maintained by sub-culturing the callus at the time interval of three months. Increase in NAA concentration promoted root differentiation and increase in BAP promoted shoot differentiation. However, high concentration (2.0 mg l⁻¹) did not support formation of callus and rejuvenation.

Table 1. Growth of callus at different hormonal concentrations.

	BAP	0 mg l ⁻¹ *	0.5 mg l ⁻¹	1.0 mg l ⁻¹	1.5 mg l ⁻¹	2.0 mg l ⁻¹
NAA						
0 mg l ⁻¹ *		Callus 1 -	Callus 2 -	Callus 3 -	Callus 4 -	Callus 5 -
0.5 mg l ⁻¹		Callus 6 +	Callus 7 ++	Callus 8 ++	Callus 9 +++	Callus 10 ++
1.0 mg l ⁻¹		Callus 11 +	Callus 12 +++	Callus 13 +++	Callus 14 +++	Callus 15 +
1.5 mg l ⁻¹		Callus 16 +	Callus 17 ++	Callus 18 +++	Callus 19 +++	Callus 20 ++

*MS basal medium

- no callusing, + slight callusing (<5mm in 1 month), ++ moderate callusing (5 mm to 10 mm in 1 month), +++ profuse callusing (>10 mm in 1 month). Culture condition: 25 ± 20° C and 16 h photo period daily.

Table 2. Presence of bergenin in callus cultured in different hormonal condition.

	BAP	0 mg l ⁻¹ *	0.5 mg l ⁻¹	1.0 mg l ⁻¹	1.5 mg l ⁻¹	2.0 mg l ⁻¹
NAA						
0 mg l ⁻¹ *						
0.5 mg l ⁻¹		Callus 6 +	Callus 7 +	Callus 8 +	Callus 9 +	Callus 10 +
1.0 mg l ⁻¹		Callus 11 ++	Callus 12 +	Callus 13 +++	Callus 14 +	Callus 15 ++
1.5 mg l ⁻¹		Callus 16 +	Callus 17 +	Callus 18 ++++	Callus 19 +	Callus 20 ++++

*MS basal medium

+ presence of bergenin in trace amount, ++ presence in low amount (<0.5 µg g⁻¹), +++ presence in moderate amount (>0.5 to <2.0 µg g⁻¹), ++++ presence in amount comparable to natural sample (>2.0 to 4 µg g⁻¹).

CHEMICAL ANALYSIS OF THE CALLUS

The qualitative analysis showed the presence of bergenin in all the calli (Table 2). It was indicated by the blue spot when the TLC plates were developed with concentrated sulphuric acid. However, the quantitative analysis by densitometer (HPTLC) showed only trace amount of bergenin in most of the treatments, except in calli 11, 13, 15, 18 and 20 (Table 3). In those calli, bergenin content ranged 0.27-3.40 µg g⁻¹. Bergenin content in the rhizomes of *B. ciliata* from natural populations, however, varied from 3.64 to 4.53 µg g⁻¹. It was found that bergenin produced by the callus 18 (treated with MS + 1.5 mg l⁻¹ NAA + 1.0 mg l⁻¹ BAP), and callus 20 (treated with MS + 1.5 mg l⁻¹ NAA + 2.0 mg l⁻¹ BAP), the amount being 3.40 and 3.05 µg g⁻¹, respectively, were comparable with the amount of bergenin present in the natural sample collected from Godawari (3.64 µg g⁻¹) (Table 3).

Discussion

This study showed that NAA (auxin) and BAP (cytokinin) are essential for the induction and development of callus in *B. ciliata*. The most effective culture media for callus formation and proliferation of plant was MS media supplemented with 1.0 mg l⁻¹ NAA and 1.5 mg l⁻¹ BAP. These results were similar to Karki and Rajbahak (2007). They reported regeneration of plantlets of *Bergenia ciliata* using MS media supplemented with 0.5 mg l⁻¹ BAP, 0.1 mg l⁻¹ NAA and 100 mg l⁻¹ Adenine sulphate. Brar and Khush (1994) and Brown and Thorpe (1994) also reported that at higher concentrations both NAA and BAP did not support formation of callus and rejuvenation. The higher concentration of NAA and BAP might have inhibitory effect in the tissue differentiation (Brown and Thorpe 1994).

Table 3. Bergenin content in *in vitro* grown tissues of *B. ciliata*.

Callus no	Treatment	Amount of bergenin ($\mu\text{g g}^{-1}$)
11	MS + 1.0 mg l ⁻¹ NAA	0.28
13	MS+ 1.0 mg l ⁻¹ NAA + 1.0 mg l ⁻¹ BAP	1.81
15	MS + 1.0 mg l ⁻¹ NAA + 1.5 mg l ⁻¹ BAP	0.27
18	MS+ 1.5 mg l ⁻¹ NAA + 1.0 mg l ⁻¹ BAP	3.40
20	MS+ 1.5 mg l ⁻¹ NAA + 2.0 mg l ⁻¹ BAP	3.05

Table 4. Bergenin content in the rhizomes of *Bergenia ciliata* collected from three different regions of Nepal.

Geographical location	Altitude (m asl)	Amount of bergenin ($\mu\text{g g}^{-1}$)
Langtang	2280-3000	4.28
Jumla	2370-3000	4.53
Godawari	2300-2760	3.64

We observed only trace amount of bergenin in most the *in vitro* grown calli. However, bergenin contents in callus no. 18 and 20 were comparable with that in the samples collected from the natural habitats. Earlier studies have shown that the yield of secondary compounds produced in cell culture would be either much lower (Zieg *et al.* 1983; Asai *et al.* 1994; Trease and Evans 2002), equal (Kreis and Reinhard 1989; Charlwood and Rhodes 1990; Andrade *et al.* 2004; Ahmad *et al.* 2007), or higher (Trease and Evans 2002) than those in intact plants. Ahmad *et al.* (2007) studied production of podophyllotoxin in callus culture (MS media with combinations of NAA and BAP) of *Podophyllum hexandrum* and reported low yield (up to 0.79% dry weight) in the callus compared to natural root sample (5.48%). Jhang *et al.* (1974) found panquinin content in callus and suspension culture of *Panax quinquefolium* and *P. ginseng* to be 1.3% of fresh weight, representing about half of the amount of panquinin present in ginseng root of natural populations. Hence, from this study it can be concluded that bergenin can be produced in *in vitro* grown callus in the amount comparable to the samples from natural habitat. This could be an alternative to minimize the exploitation of natural population of *B. ciliata*.

As an attempt to increase the concentration of secondary metabolites in *in-vitro* culture callus, precursors have been used during tissue culture. For example, addition of coniferin (a polypropane) in the culture of *Podophyllum hexandrum*

improved podophyllotoxin synthesis by 12.8 folds (Chattopadhyay *et al.* 2003). Similarly, quinoline yield has been increased by L-tryptophan precursor feeding to *Chichona ledgeriana* (Trease and Evans 2002). However, various authors have also indicated the instability of cell culture line. Tissue cultured cells may produce the compounds not found in the plant itself. A new caumarin, rutacultin has been isolated from suspension culture of *Ruta graveolens* (Trease and Evans 2002). Two new chalcones have been characterized from tissue culture of *Glycyrrhiza echinata* (Charlwood and Rhodes 1990; Trease and Evans 2002). Similarly, Fedoreyev *et al.* (2008) found significant amount of isoflavonides produced by calus of *Maackia amurensis* sub-cultured from stable cell line. Thus, further study is needed to investigate whether the callus culture can yield a cell line that may produce bergenin in significantly higher concentration similar to callus culture of *Maackia amurensis* as done by Fedoreyev *et al.* (2008).

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