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In vitro propagation and phytochemical assessment of *Berberis chitria*: An important medicinal shrub of Kumaun Himalaya, India

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Berberis chitria **is an important Himalayan shrub known for its diverse medicinal uses. A reproducible *in vitro* micropropagation protocol for *B. chitria* has been developed. Seeds were initially inoculated on water agar medium; contamination-free green seeds were transferred to Woody Plant Medium (WPM) with 78.89% seed germination. Initially, maximum multiple shoots (8.30±1.0) per explant were observed in WPM supplemented with 6-benzyl adenine (BA)+α-Naphthalene acetic acid (NAA) (8.88+1.34 µM). The shoot multiplication rate was increased up to 22.89±2.51 shoots per explant on successive subculture but shoot necrosis and concomitant decrease in shoot height was the big problem. Incorporation of casein hydrolysate (CH; 500 mg/L) and gibberellic acid (1.44 µM) in shoot multiplication medium not only facilitated shoot height, but the number of shoots per explant also increased without any necrosis. Microshoots were exposed to auxins in two different ways. The highest rooting percentage (100%) was observed after 8 weeks on WPM supplemented with indol butyric acid (IBA; 100 µM) for 7 days with 5.92±0.36 roots per microshoot. Well-rooted plants were transferred to thermacol pots containing nonsterile, sieved soil and farmyard manure and hardened successfully. This study also provides evidences that the methanolic extract of *in vitro* raised plants have strong antioxidant activities and higher phenol content than naturally grown plants.**

Key words: *Berberis chitria*, 6-benzyl adenine (BA), gibberellic acid (GA_3), micropropagation, antioxidant activity.

INTRODUCTION

The genus, *Berberis* L. of family Berberidaceae consists of spiny shrubs widely distributed in temperate and sub-tropical regions of northern hemisphere and temperate South-America (Chauhan, 1999). *Berberis* has about 650 species worldwide (Duke et al., 2002) out of which 54 have been reported from Indian Himalaya (Sharma et al., 1993) and 22 from Uttarakhand (Tiwari and Adhikari, 2011).

Berberis chitria Lindl.is an important medicinal shrub of family Berberidaceae and berberine is the major alkaloid found in roots and stem-bark of this plant; therefore, it is commonly used as a substitute/adulterant to *Berberis aristata*, that is, 'Daruharidra' (Srivastava et al., 2006). Berberine is an isoquinoline alkaloid known for its activity against cholera (Dutta and Panse, 1962), acute diarrhea (Lahiri and Dutta, 1967), to cure a variety of ailments

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such as eyes and ear diseases, rheumatism, diabetes, fever, jaundice, stomach disorders, malarial fever, skin disease and is used as tonic (Srivastava et al., 2006; Kirtikar and Basu, 1975; Chopra et al., 1958). Berberine has also been used to treat diabetes for thousands of years in China (Yin et al., 2008).

Free radicals and reactive oxygen species are thought to cause oxidative damage and other degenerative diseases, namely, Alzheimer's, cancer and cirrhosis in the body (Hue et al., 2011). Polyphenols are the major antioxidant constituents isolated from many medicinal and edible plants (Moon and Shibamoto, 2009). In recent years, there has been an increasing interest in finding natural antioxidants, which can protect the human body from free radicals and encounter the progress of many chronic diseases (Kaur and Kapoor, 2001; Kinsella et al., 1993). Srivastava et al. (2006) and Tripathi et al. (2010) reported that *B. chitria* possess higher amount of berberine in root tissue as compared to *B. aristata* and *B. asiatica*.

Due to diverse medicinal uses of berberine and its availability of high content in *B. chitria*, it is necessary to enhance the production of this species. Although, *Berberis* species are mainly propagated by seeds and stem cuttings, but seeds of barberry species possess embryonic dormancy (Dirr and Heuser, 1987) and only 22% of the seeds survival has been reported (Swingle, 1939). Vegetative propagation through stem cuttings requires macro part of stem for large scale production and it could lead exploitation of this species. Considering the aforementioned facts, this study was undertaken to develop an efficient *in vitro* propagation protocol for *B. chitria* and to evaluate the antioxidant potential and phenolic content of *in vitro* grown plants and their comparison with naturally grown plants (wild plants). On the basis of available literature till date, the complete *in vitro* micropropagation protocol for *B. chitria* is unavailable. So, the present study could be an important step towards the multiplication and conservation of this valuable medicinal species.

MATERIALS AND METHODS

Ripened, dark purple, fruits of *B. chitria* were collected from wild habitat of Nainital, India (29° 23' N, 79° 30' E), in December 2010. Seeds were taken out from outer flashy pericarpic layer of fruit, washed with tween 20 and subjected to fungicide treatment (bavistin, 0.5% w/v, 30 min) and shifted to laminar air flow bench for further steps. The seeds were surface sterilized with mercuric chloride ($HgCl_2$, 0.1% w/v, 12 min) followed by five rinses of sterile double distilled water and were dried for 2 min in blotting paper (autoclaved) then cultured on 100 ml water agar medium (WA; 8 g/L agar, 30 g/L sucrose) (Himedia, India) in 250 ml conical flasks/culture bottles. Inoculated seeds were kept in the culture room at 25±2°C. Contamination-free green seeds after 10 days of inoculation on WA medium were transferred to different media, that is, Murashige and Skoog (MS; Murashige and Skoog, 1962) and Woody Plant Medium (WPM; Lloyd and McCown, 1980), supplemented with 8 g/L agar and 30 g/L sucrose in a sterile culture bottles for *in vitro* seed germination and seedling growth. All cultures were maintained under 16-h light/8-h dark photoperiod (60-µmol m^{-2} s^{-1} fluorescent and radiant light) at 25±2°C temperature.

Shoot multiplication

Nodal segments from *in vitro* generated seedlings (<2.5 cm) were prepared and used as explants for shoot multiplication. To investigate the responses of explants to different concentrations of plant growth regulators (PGRs), 6-benzyl adenine (BA; 0.00 to 8.88 µM) either alone or in combinations with indol acetic acid (IAA; 0.00 to 2.86 µM) or α-Naphthalene acetic acid (NAA; 0.00 to 2.68 µM) were used in WPM for shoot multiplication (Table 1). Each treatment consisted of at least six flasks with three explants in each. Data on growth rate were recorded after 30 days. The growth rate was defined by the number of shoots and length of shoots (Table 1).

Effects of gibberellic acid (GA_3) and casein hydrolysate (CH)

Different concentrations of GA_3 (0.00 to 2.89 µM) and CH (100 to 1000 mg/L) were tested out to optimized culture medium and to evaluate their effects on phenolic production, antioxidant capacity, and plant growth.

In vitro rooting and acclimatization

Well rejuvenated microshoots (>3 cm in length) (Figure 3e) were transferred to rooting medium. The rooting medium included WPM supplemented with 30 g/L sucrose, 2.4 g/L clarigel and different concentrations of auxins (NAA and indol butyric acid (IBA); 0 to 200 µM). Microshoots were exposed to auxins in two different ways, either prolonged exposure of low concentrations (IBA, 0 to 9.18; NAA, 0 to 10.75 µM) or short period exposure of high concentrations of IBA and/or NAA (50 and 100 µM) for 3 or 7 days (Tables 2 and 3). After 8 weeks, rooted plantlets were taken out from culture bottles and roots were gently washed under running tap water to remove the traces of clarigel. The plantlets were then transferred to small thermacol pots (8 cm width and 12 cm height) containing nonsterile sieved soil and farmyard manure in the ratio of 3:1, and covered with plastic covers to maintain humidity. Potted plantlets were placed in a growth chamber (16-h light/8-h dark period, at 25±2°C). After 2 weeks, the plastic covers were removed to reduce humidity. After 4 weeks, plantlets were transferred to bigger pots (15 cm width and 20 cm height) and were kept inside polyhouse for further growth at 25±2°C.

Extract preparation

Plant extracts were prepared as described by Al-Khateeb et al. (2012) with some modifications. The fresh leaves from *in vitro* grown three year-old cultures and plants grown in natural habitat were collected and washed individually with sterile water. Ten grams of leaves (each condition) were crushed with liquid nitrogen into a fine powder and extracted with 12.5 ml of 95% methanol. The slurry was heated to 60°C for 2 h in water bath. The mixture was then filtered through 8 layers of muslin cloth and centrifuged at 10,000 ×g for 10 min. The supernatant was collected, filter sterilized and stored at -20°C till further use.

Determination of free phenolic compounds

Total phenolic content (PC) was determined by Folin-Ciocalteu's

Table 1. Effects of different plant growth regulators on shoot multiplication in *in vitro*-derived nodal segments of *B. chitria* on WPM.

Treatment (µM)			Average number of shoots/explant ±SE	Average shoot length (cm) ±SE	Average length of longest shoot (cm) ±SE
BAP	NAA	IAA			
0.00	0.00	0.00	0^{a}	0^{a}	0^{a}
1.00	-	-	1.42 ± 0.08^{bc}	0.97 ± 0.08^{ab}	1.00 ± 0.09^{ab}
4.44	-	-	1.50 ± 0.14^{bc}	1.13 ± 0.12^{ab}	1.30 ± 0.08^{ab}
8.88	-	-	1.75 ± 0.14^{bc}	1.04 ± 0.11^{ab}	1.07 ± 0.07^{ab}
1.00	-	1.43	2.08 ± 0.17^{bc}	1.60 ± 0.16^{ab}	1.73 ± 0.16^{ab}
4.44	-	1.43	3.58 ± 0.85^{d}	1.72 ± 0.11^{ab}	2.23 ± 0.17^{bc}
8.88	-	1.43	2.58 ± 0.33^{c}	1.68 ± 0.12^{ab}	1.94 ± 0.18^{bc}
1.00	-	2.86	5.42 ± 1.80^{e}	1.72 ± 0.08^{ab}	2.38 ± 0.12^{bcd}
4.44	-	2.86	6.42 ± 1.76^{ef}	2.08 ± 0.36^{ab}	2.59 ± 0.01^{bcd}
8.88	-	2.86	7.50 ± 0.51^{fg}	2.86 ± 0.03^{bc}	2.83 ± 0.20^{bcd}
1.00	1.34	-	6.05 ± 1.23^{ef}	2.97 ± 0.12^{bc}	3.23 ± 0.15^{bcd}
4.44	1.34	-	6.40 ± 1.22^{ef}	2.07 ± 0.30^{ab}	4.17 ± 0.61^{cd}
8.88	1.34	-	8.30 ± 1.00^{gh}	3.26 ± 0.14^{bc}	3.60 ± 0.61^{bcd}
1.00	2.68	-	6.73 ± 0.75^{ef}	2.73 ± 0.27^{ab}	3.40 ± 0.10^{bcd}
4.44	2.68	-	6.20 ± 1.17^{ef}	2.87 ± 0.33^{bc}	4.00 ± 0.29^{bcd}
8.88	2.68	-	6.07 ± 1.09^{ef}	2.27 ± 0.44^{ab}	3.17 ± 0.20^{bcd}

Values are mean ± standard error; mean values followed by the same letter(s) in a column are not significantly different (P≤0.05). Each treatment consisted of twelve explants in each. Data were recorded after 45 days.

Table 2. Effects of prolonged exposure of auxins on *in vitro* rooting in *B. chitria* microshoots

Treatment (µM)		Rooting (%)±SE	Average number of roots/shoot ±SE	Average length of roots (cm)±SE	Average length of longest root (cm)±SE
NAA	IBA				
-	-	0.00^{a}	0.00^{a}	0.00^{a}	0.00a
2.68	-	33 ± 00^{c}	1.40 ± 0.31^{c}	1.30 ± 0.06^{c}	1.43 ± 0.12^{c}
5.37	-	44 ± 11^{d}	2.22 ± 0.78^{e}	1.53 ± 0.07^{d}	1.97 ± 0.19^{d}
10.74	-	44 ± 11^{d}	4.45 ± 0.40^{g}	1.93 ± 0.09^{e}	2.82 ± 0.34^{e}
-	2.45	22 ± 11^{b}	1.00 ± 0.58^{b}	0.80 ± 0.40^{b}	0.933 ± 0.48^{b}
-	4.90	44 ± 11^{d}	1.43 ± 0.30^{d}	2.97 ± 0.73^{f}	3.37 ± 0.74^{f}
-	9.18	55 ± 11^{e}	2.78 ± 0.11^{f}	2.99 ± 0.36^{g}	4.67 ± 0.98^{g}

Values are mean ± standard error; mean values followed by the same letter(s) in a column are not significantly different (P≤0.05). Each treatment consisted of twelve explants in each. Data were recorded after 60 days.

colorimetric method by following the method described by Singleton and Rossi (1965) with minor modifications. In 0.200 µl of methanolic extract, 2.5 ml ten time diluted Folin-Ciocalteu's reagent was added and allowed to react for 5 min. This mixture was neutralized by 2 ml of 7.5% sodium carbonate (w/v) and incubated at room temperature in the dark for 90 min. The absorbance was measured at 765 nm using UV-VIS spectrophotometer (Thermo scientific UV 1 V7.09). Quantification was done on the basis of standard gallic acid curve and results were expressed in mg gallic acid equivalent (GAE) per gram fresh weight of respective leaves.

Antioxidant assay using the β-carotene bleaching method

The antioxidant activity (AA) was evaluated by following the β-carotene bleaching method as described by Mitsuda et al. (1996) with modifications. One milligram of β-carotene was dissolved in 10 ml of chloroform, and an emulsion was prepared as follows: 1 ml β-carotene solution, 10 µl linoleic acid, and 200 mg tween 40 were added, then the chloroform was removed by nitrogen gas. 100 ml of oxygenated water was added slowly to the semi-solid residue with a vigorous shaking, to form an emulsion. An absorbance at 470 nm was immediately recorded after adding 200 µl of the sample to three ml of β-carotene/ linoleic acid emulsion, which was regarded as t = 0 min. The tubes were capped and incubated in a water bath (50°C) for 120 min. Oxidation of the emulsion was monitored by measuring the absorbance at 470 nm over 120 min. Control samples contained 200 µl of methanol instead of sample, and water was used as the blank. The antioxidant activity was expressed using the following equation:

Antioxidant activity = 100 (DRc – DRs)/DRc

where DRc is the degradation rate of the control; DRc = (ln (A/B)/120), DRs is the degradation rate in the presence of the sample; DRs = (ln (A/B)/120), A is initial absorbance at time 0 min,

Table 3. Effects of short time exposure of higher concentrations of auxins on *in vitro* rooting in *B. chitria* microshoots.

Treatment (µM)	Time (days)	Rooting (%)±SE	Average number of roots/shoot ±SE	Average length of roots (cm)±SE	Average length of longest root (cm)±SE
control	-	0.00[a]	0.00[a]	0.00[a]	0.00[a]
NAA 50	3	75.0±16.06[b]	1.42± 0.30[b]	2.12±0.39[b]	2.19±0.32[b]
NAA 100	3	83.00±09.81[c]	2.00±0.25[f]	2.33±0.39[c]	2.65±0.45[c]
NAA 50	7	91.05±08.05[e]	1.83±0.51[e]	2.79±0.27[e]	3.13±0.43[e]
NAA 100	7	83.25±16.75[d]	1.67±0.30[c]	3.00±1.03[f]	3.38±1.02[f]
IBA 50	3	83.25±16.75[d]	1.75±0.67[d]	3.99±1.01[g]	4.43±1.20[h]
IBA 100	3	83.00±09.81[c]	1.83±0.55[e]	2.44±0.43[d]	2.95±0.58[d]
IBA 50	7	91.67±08.05[f]	2.08±0.58[g]	5.35±1.88[i]	6.17±0.08[i]
IBA 100	7	100±0.00[g]	5.92±0.36[h]	4.69±0.36[h]	3.58±0.61[g]

Values are mean ± standard error; mean values followed by the same letter(s) in a column are not significantly different (P≤0.05). Each treatment consisted of twelve explants in each. Data were recorded after 8 weeks.

and B is the absorbance after 120 min.

Statistical analysis

Experiments were performed in a completely randomized design to determine the effect of the treatments. Each treatment consisted of at least twelve explants. All experiments were repeated at least twice. Level of significance was determined by analysis of variance (ANOVA) and Fisher's least significant difference (LSD) among means (P = 0.05) was estimated according to Snedecor and Cochran (1967).

RESULTS AND DISCUSSION

Seeds became green within 5 days of inoculation on WA medium and 60% contamination-free seeds were available after 10 days of inoculation. These contamination-free green seeds started germinating 6 days after transfer to WPM and up to 78.89% germination was scored after 14 days. (Figure 3a) in comparison to MS medium where germination started 15 days after transfer and only 50% germination was recorded even after 30 days. The fastest seedling growth and the highest seedling length (Figure 3b) were also recorded in WPM in comparison to MS (data not shown). Therefore WPM was used for further study.

Shoot multiplication

Results of effect of different PGRs on shoot multiplication are given in Table 1. Approximately all the treatments induced multiple shoots. BA alone was not able to produce high number of shoots, but in combination with NAA or IAA, the results were promising and the number of shoots per explant was quite high (Table 1). However, high frequency shoot multiplication was achieved in WPM supplemented with BA+NAA, followed by BA+IAA (Table 1 and Figure 3c). WPM supplied with BA+NAA (8.88+1.34 µM) was most effective for induction of multiple shoots in comparison to other treatments and was used as the shoot multiplication medium (SM) for further study. Maximum number of shoots (8.30±1.0 shoots per explant) with maximum average shoot length (3.26±0.14 cm) was produced in this treatment while the average length of the longest shoot (4.17±0.61 cm) was recorded maximum in BA+NAA (4.44+1.34 µM). WPM without PGRs was unable to induce shoots even after one month and explants died. The results of ANOVA shows that PGRs combinations significantly (P=0.01) improved shoot number and shoot length. Regenerated microshoots were harvested after 6 weeks in culture and were further multiplied on the same medium. This could be repeated through three subcultures.

On successive subcultures, frequency of multiple shoot formation increased rapidly and up to 11.78±1.78 shoots per explant in the 1st subculture, 20.56±2.31 shoots per explant in the 2nd subculture and 22.89±2.51 shoots per explant in the 3rd subculture could be produced; but after first subculture, there was a concomitant decrease in shoot height (Figure 1). Same trend was reported in *Quercus semecarpifolia* by Tamta et al. (2008). In the present study, on subsequent subculture, decrease in shoot height was not only the problem, but the basal end of the regenerated shoots also showed the exudation of phenolic compounds, so the medium became yellowish brown and due to this, necrosis of shoots occurred.

Therefore, after the 3rd subculture, microshoots were transferred to the shoot multiplication medium (WPM+BA+NAA; 8.88+1.34 µM) supplemented with a growth adjuvant (CH, 100 to 1000 mg/L) and/or length augmenter (GA_3, 0.00 to 2.89 µM), that is, either alone or in combination to overcome these problems. Results of this experiment are presented in Figure 2 (detailed data were not shown). After the 3rd subculture incorporation of 500 mg/L CH to the shoot multiplication medium (2) facilitated the growth of shoots and number of shoots increased up to 27.67±1.45 shoots per explant without necrosis. Same simulative effect of CH has also been reported in *Psoralia corylifolia* (Anis and Faisal, 2005),

Table 4. Total phenolic content and total antioxidant capacity in leaves of *in vitro* raised and wild plants of *B. chitria*.

S/N	Leave	Total phenol (mg GAE/g fresh weight)	Antioxidant capacity
1	Wild	28.94±0.74[a]	197.20±0.004[a]
2	*In vitro*	31.94±0.79[b]	197.090±0.006[a]
3	BHT	-	54.94±0.027[b]

Values are mean ± standard error; mean values followed by the same letter(s) in a column are not significantly different (P≤0.05).

Quercus rubra (Vengadesan and Pijut, 2009) and *Cuphea procumben* (Fatima et al., 2011). Although, shoot length was more (3.37±0.26 cm) in this treatment as compared to SM (1) but was not much improved (Figure 2). Application of GA_3 (1.44 µM) in shoot multiplication medium (3) improved the shoot length (up to 5.50 cm± 0.61) after 2 months (Figure 3d), although number of shoots per explant was less (26.67±2.91) in treatment (3) in comparison with SM +500 g/L CH (2) (Figure 2). Addition of different concentrations of GA_3 increased the rate of proliferation and favored shoot elongation in *Camellia saluensis* and *Camellia japonica* shoot cultures (Beretta and Eccher, 1987) and some *Quercus* species, *Q. rubra* (Vengadesan and Pijut, 2009), *Q. leucotrichophora* and *Q. glauca* (Purohit et al., 2002). When the combination of CH and GA_3 in SM was employed (4), this treatment does not only increased average length of shoots (6.5±0.85 cm), average length of longest shoot (8.87±0.91cm) but frequency of shoot number per explant was also increased significantly (up to 28.07±1.69 shoots/explant).

In vitro rooting and acclimatization

Although both tried experiments for root induction gave positive results in terms of rooting, but they differ in rooting frequency and time taken for root induction. The prolonged exposure of low concentrations of auxins showed poor response as these treatments took more time for root induction (>45 days) and percentage of rooting was also low (Table 2). However, in prolonged exposures, rooting percent (55±11), average root length (2.99±0.36 cm), average length of the longest root (4.67±0.98 cm) were maximum in WP+IBA (9.18 µM), while average root number was maximum (4.45±0.78 microshoot^{-1}) in WP+NAA (10.74 µM). Significantly, better results were recorded in short time exposure of higher concentration of same auxins. The highest rooting percentage (100%) was observed at 8 weeks on WPM supplemented with IBA (100 µM) for 7 days with 5.92±0.36 roots per microshoot. Induced root length (5.35±1.88 cm) and average length of the longest root (6.17±0.08 cm) were found superior when IBA 50 µM for 7 days was subjected to microshoots (Table 3 and Figure 3f and g). Well rooted plantlets were transferred to soil for acclimatization initially inside culture room conditions and after 45 days, they were exposed to polyhouse conditions for further hardening (Figure 3h to j). IBA was also found suitable for root induction in *B. aristata* stem cuttings (Ali et al., 2008) and *in vitro* root induction in *Ruta graveolens* (Faisal et al., 2005) and *Q. leucotrichophora* (Pandey and Tamta, 2012). *In vitro* raised plantlets exhibited high rate of survival (95%) under polyhouse conditions.

Total phenolic content and total antioxidant capacity

In present study, results of total phenolic content and antioxidant activity in leaves of *B. chitria* are shown in Table 4. Total phenolic content was found as 28.94±0.74 mg GAE/g in leaves collected from natural habitat and relatively higher amount, that is, 31.94±0.79 mg GAE/g was recorded in the leaves of *in vitro* grown plants. Since the accumulation of phenolics is directly influenced by light irradiance (Bakhshi and Arakawa, 2006; Ghasemzadeh et al., 2010) and in natural habitat, *B. chitria* is an under-story shrub while during micro-propagation these plants were irradiated with light intensity which may be a reason for high phenolic content in *in vitro* grown plants. Antioxidant activity also revealed promising result and it was observed that in leaves from both sources extract possess significantly higher antioxidant activity in comparison to standard antioxidant used (BHT) (Table 4). Generally, free radicals are known to maintain homeostasis at the cellular level and work as signaling molecules, but in excess they cause various degenerative diseases due to oxidative stress (Prakash et al., 2007). In this context, antioxidants can play an important role to protect the human body from free radicals and retard the progress of many chronic diseases (Fatima et al., 2011) by stopping the formation of reactive oxygen species (ROS) (Rawat et al., 2011). Nowadays, there has been an increasing demand of natural antioxidants and the antioxidant activity of plant materials closely correlated with the content of their phenolic compounds (Skerget et al., 2005).

However, Arena et al. (2000) were able to develop an *in vitro* propagation protocol for *Berberis buxifolia* (a native of Patagonia) in MS medium supplemented with

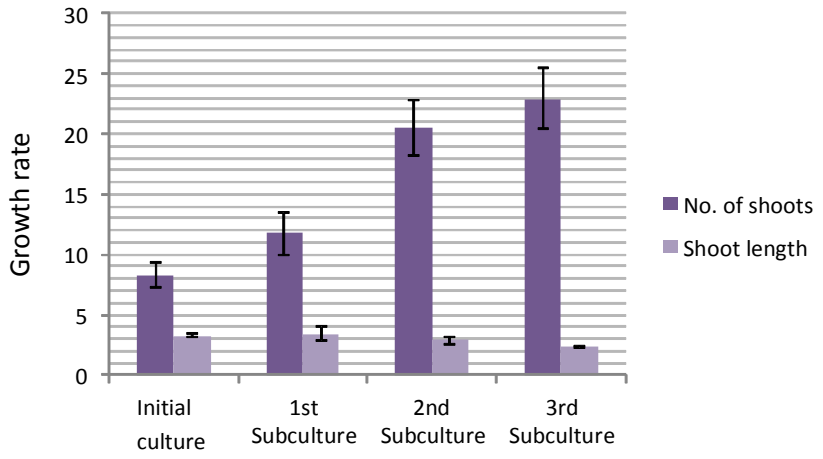

Figure 1. Effect of successive subcultures on growth rate of *B. chitria in* SM medium (WPM+8.88 µM BA+1.34 µM NAA); graphical columns with error bars of no. of shoots indicating continuous increase in shoot number after subculturing; while shoot length increased up to 1st subculture and decreased on further subculturing.

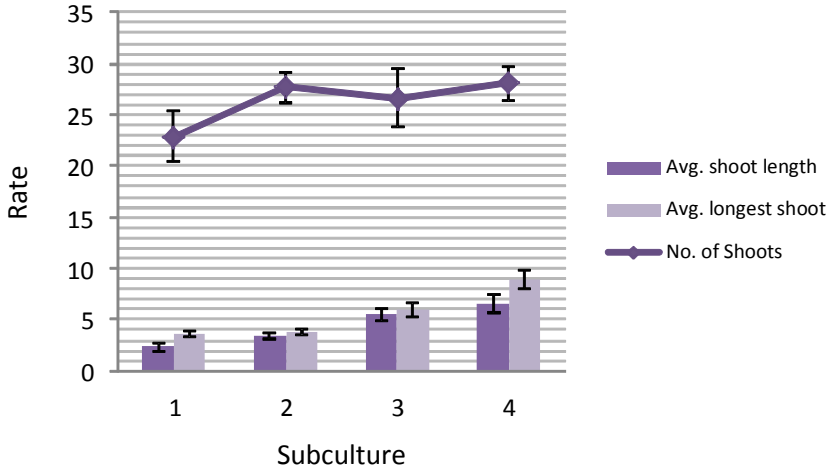

Figure 2. Effect of CH and GA_3 on shoot multiplication and shoot length of *B. chitria* after 3rd sub culture*;* 1: SM (8.88 µM BA+1.34 µM NAA), 2: SM+ 500 g/L CH, 3: SM+1.44 µM/L GA_3, 4: SM+500 g/L CH+1.44 µM/L GA_3

0.55 µM BA, but the multiplication rate was 4.7 shoots/explant after 63 days. Considering all growth parameters of the present study, WPM supplemented with 8.88 µM BA+1.34 µM NAA was found to be the best treatment for shoot proliferation in *B. chitria,* the multiplication rate was increased significantly on successive subculture. However, a serious problem of decrease in shoot height and necrosis on successive subculture was there. These problems were eliminated successfully by incorporating CH and GA_3 in to shoot multiplication medium. Addition of growth adjuvant (CH) and length augmenter (GA_3) do not only removed the problem of necrosis and decreased the height, but also increased the rate of multiplication. Microshoots exposed to higher concentration of IBA leads to 100% rooting in comparison to prolonged exposure. Well rooted plantlets were acclimatized by transferring to soil initially inside culture room conditions and then to polyhouse conditions. This study also provides evidences that the methanolic extract of *in vitro* raised plants have strong antioxidant activities, four times to BHT (used standard), but slightly lower than that of naturally grown plants (wild plant). The total phenol was found higher in *in vitro* raised plants than naturally grown plants. These results endorse the therapeutic potential of this plant species and opens scope for production of antioxidants in commercial level

Figure 3. *In vitro* propagation of *B. chitria*. (a) Initiation of *in vitro* seed germination on WA media after 15 days. (b) Well grown seedling in WP medium 30 days after subculturing. (c) Shoot multiplication after 90 days of subculture in SM (8.88 µM BA+1.34 µM NAA). (d) *In vitro* shoot elongation in SM+1.44 µM/L GA_3 in comparison to SM. (e) Well elongated shoots in root induction media for short time exposure of high auxin concentrations. (f) Rooting in ½ strength WPM supplemented with IBA (100 µM) for 7days, after 8 weeks. (g) Rooting response in (i) short time exposure and (ii) in prolonged exposure. (h-i) Acclimatization inside culture room conditions for 30 days with/without plastic covering. (j) Well growing plant in earthen pots ready to field transfer after 60 days under poly house condition.

under *in vitro* conditions.

Conclusion

From this study, it was concluded that micropropagation technique is very useful for this medicinally important plant and on the basis of available literature, this is the only report regarding to the propagation of this valuable plant species through *in vitro* propagation. This *in vitro* propagation study is not only useful for large scale production of *B. chitria*, but can also be used for the propagation and conservation of other overexploiting *Berberis* spp. of this region.

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ABBREVIATIONS

CH, Casein hydrolysate; **GA_3,** gibberellic acid; **BA,** 6-benzyl adenine; **NAA,** α-Naphthalene acetic acid; **IAA,** indol acetic acid; **IBA,** indol butyric acid.

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