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In vitro regeneration of *Bacopa monnieri* (L.) from leaf and stem explants

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Abstract
The effects of plant growth regulators (PGRs) and activated charcoal (AC) on plant regeneration and plantlets development were assessed *in vitro* using the leaf and stem explants of *Bacopa monnieri* Leaf. and stem explants were cultured on Murashige and Skoog (MS) basal medium supplemented with different concentrations of PGRs (Auxins and cytokinin) with and without Activated charcoal AC) (0.5 to 2.5 g/L). Plant regenerated successfully through calli derived from stem and leaf plants. Compared to stem, leaf explants showed good callus formation. Maximum callus formation (92.23%) was recorded for naphthalene acetic acid (NAA) (3 mg/L) whereas, 73.11% callus formation was recorded for 2, 4-D (2.5 mg/L). A combination of IBA+BAP (3 mg/L) showed highest shoot formation and multiple shoot formation by 76.43% and 49.55%, respectively whereas, addition of IBA (2 mg/L) showed 73.23% and 47.24% shoot formation and multiple shoot formation, respectively. Addition of AC with IBA (2 mg/L) exhibited maximum root formation by 85.52% and number of roots per shoot by 38.55% whereas, with IAA (2 mg/L), the root formation was found to be 71.47% and number of roots per shoot to be 33.44% The present study concluded that addition of AC promotes roots induction and formation from *in vitro* shoots in Brahmi.

Keywords: *Bacopa monnieri*; callus; regeneration; organogenesis; leaf; stem

Introduction
Bacopa monnieri (L.) Wettst. Belongs to Scrophulariaceae family and is an amphibious herb of tropics, generally grown on the banks of the rivers and lakes. *B. monnieri* is a widely known medicinal herb and is well reputed to the name of "Brahmi" in India. The herb is mainly effective for CNS disorders and therefore, played a very important role in Ayurveda therapies for the treatment of cognitive disorders including epilepsy and insanity. In addition, Brahmi has capacity to treat inflammation, fever, pain, cancer, cells oxidation, asthma, snakebite, rheumatism, leprosy, eczema, kidney and cardiac disorders and ringworm (Tripathi *et al.*, 1996; Russo and Borrelli, 2005) [1, 2]. The saponins present in the plant, namely bacoside A, B, C and D have been indicating for memory enhancing properties and hence, called memory chemicals (Jain and Kulshreshtha, 1993; Rastogi *et al.*, 1994) [3, 4].
B. monnieri is a perennial, creeping herb native to the wetlands of southern India, Australia, Europe, Africa, Asia, and North and South America. It is a non-aromatic herb. The leaves of this plant are succulent, oblong and 4-6 mm (0.16-0.24 in) thick. Ability to grow in water makes it a popular aquarium plant. It can even grow in slightly brackish conditions. Propagation is often achieved through cuttings. It commonly grows in marshy areas throughout India, Nepal, Sri Lanka, China, Pakistan, Taiwan, and Vietnam. It is also found in Florida, Hawaii and other southern states of the United States where it can be grown in damp conditions by a pond or bog garden. This plant can be grown hydroponically.
Since time immemorial, humans have been depended on plants for their daily needs, in which medicine is one of the important and primary needs. Moreover, the plants are still found as a fundamental source of modern medicines (Kala, 2005) [5]. Nowadays, medicinal plants are important to the global economy, as most of the drug industries depend, in part, on plants for their raw material and approximately 80% of traditional medicine preparations are made up of plants or plant extracts (Dhyani and Kala, 2005; Divya *et al.*, 2014) [6, 7]. However, the collection of medicinal plants on a mass scale from the natural habitats leads to repletion of plant resources which causes a serious effect on environment.

Propagation and conservation of plants through conventional methods like vegetative and seed propagation have many limitations. Among them, the major ones are variations in edaphic and climatic factors, low percentage of seed set and seasonal dormancy (Savangikar, 2002) [8]. Plant tissue culture is important in terms of aseptic culture of cells, tissues, organs and their components under defined *in vitro* physical and chemical conditions. It is also an important tool for various applied studies and commercial applications (Thrope, 2007) [9]. Activated charcoal is composed of carbon, arranged in a quasi-graphitic form of small particle size. It is a porous and tasteless material distinguished from elementary carbon by removal of all non-carbon impurities and the oxidation of carbon surface. It is an essential component of many plant tissue culture media, which prevents browning of cultured tissues and media by adsorption of toxic compounds like polyphenols released by cultured tissues (Thomas, 2008) [10].

Materials and Methods

Explant preparation

Diseases and pest free bramhi plants were collected from the Sai nursery located at Walminaka, Paithan road, Aurangabad. Leaf and stem segments were excised from bramhi plants. For sterilization, explants were first washed with tap water. These explants were further treated with 1% v/v sodium hypochlorite for 15 min and washed with distilled water twice. after that, explants were treated with 0.05% w/v mercuric chloride for 3 min and washed with sterile distilled water for 5-6 times. Explants were dried on a sterile blotting paper to remove excess water and used for all experiments.

Callus Induction

MS basal medium (Murashige and Skoog, 1962) [11] with different concentrations of 2,4-D (1, and 3mg/l), NAA (1, 2 and 3mg/l), IBA (1, 2 and 3mg/l) and combination of 2,4-D (1, 2 and 33mg/l) and IBA (1, 2 and 3mg/l) was used for callus induction. Media was prepared using appropriate concentrations of components and pH of the media was adjusted to 5.8±0.05 using 1N NAOH and 1N HCl. Media was autoclaved at 121°C and 15psi for 20 min and poured in sterilized glass vessels. Sterilized explants were inoculated on medium after two days of preparation. After inoculation of explants, plates were sealed with Parafilm and incubated at 25±2°C, relative humidity 70-80% and 16/8 (L/D) h of photoperiod duration provided by day light fluorescent tubes. After 28 days, culture were sub-cultured on similar medium and incubated for further 28 days. Cultures were observed on daily basis but number callus inducing explants were counted after 8 weeks of incubation.

Shoot formation and multiple shoot formation from callus

Well developed callus were transferred on MS medium supplemented with different concentrations of BAP (0.5, 1 and 2mg/l), IBA (0.5, 1 and 2mg/l), combination of BAP with IBA (0.5, 1 and 2mg/l each) and combination of BAP with IAA (0.5, 1 and 2mg/l each) for multiple shoot induction. Cultures were incubated on same conditions for one month. Number of callus showing organogenesis and number of shoots per callus were counted after one month.

Root induction and effect of activated charcoal

Regenerated shoots were transferred to root induction medium. Root induction medium consist of MS medium fortified with IBA (0.5, 1 and 2mg/l), IAA (0.5, 1 and 2mg/l), and NAA (0.5, 1 and 2mg/l), either with or without activated charcoal. Cultures were incubated in similar conditions for 21 days.

Statistical Analysis

Each experiment was repeated three times and mean values and standard deviation were calculated. All data obtained were subjected to the single factor analysis of variance (ANOVA) using Microsoft excel. The critical difference (C.D.) values were calculated at p=0.05 level to find out the significant difference between the means of different treatments.

Results and Discussions

Callus formation from leaf and stem

Callus is an undifferentiated mass of parenchymatous cells in the plant. The success in callus formation depends on the selection of a suitable plant part to serve as explants. Morphogenic response of explants cultured *in vitro* is largely result of interplay between the innate physiological status, precisely the endogenous levels of phytohormones of the donor tissue and the influence of the exogenously added plant growth regulators (PGRs) in the culture medium. The callus formation was achieved by placing the segments of surface sterilized leaf and stem explants in to the semi-solid MS basal medium supplemented with different concentrations of auxins. On day 11 onwards, the callus formation was observed from the leaf and stem explants (Fig. 1A). Compared to the stem, the leaf explants showed better callus formation. A maximum callus formation (92.23%) was observed on 2,4-D (3 mg/L) followed by 2,4-D (2 mg/L) which showed formation by 73.11% from leaf explants. Showkat *et al*. (2010) [12] also obtain maximum callus induction from leaf explants using 2,4-D. The combination of 2,4-D and BAP showed 72.22% callus formation whereas compared to other hormones, IBA (1 mg/L) showed lowest callus formation from leaf explants. However, in the case of stem explants, the highest callus formation was recorded on 2,4-D (2 mg/L) by 77.65%, followed by NAA (2 mg/L) with 53.60%. The least callus formation was achieved with a MS medium containing IAA (3mg/l) which was found to 34.65%. *In vitro* callus induction in *B. monnieri* was reported by few workers like Jain *et al.* (2013) [13], Vijayakumar *et al.* (2010) [14] and Parale *et al.* (2010) [15].

Shoot formation and multiple shoot formation from callus

The results obtained for multiple shoot induction in different treatments were presented in Table 2. The highest shoot formation (76.43%) with multiple shoot formation (49.21 shoots per callus) was observed on MS medium fortified with combination of IBA+BAP (3 mg/l), followed by 73.23% shoot formation with 47.83 shoots per callus on MS medium with 2 mg/l BAP (Fig. 1 B and C). Several reports suggested that cytokinin is required in maximum quantity for shoot induction but addition of a low amount of auxin along with cytokinin enhances the frequency of shoot multiplication (Tsay *et al*., 1989; Shasany *et al*., 1998; Sharma and Singh, 1997) [16, 17, 18].

Roots formation from shoots

The micro propagated shoots were transferred to the MS basal with and without addition of charcoal medium supplemented with different hormone concentrations. The addition of AC in the combination of IBA (2 mg/l) showed better root formation by 85.52% and number of roots per shoot by 38.55 whereas; the combination of IAA (2 mg/l) showed 71.47% root

formation and 33.44% number of roots per shoot (Fig. 1 D). The lowest root formation by 64.65% and number of roots per shoot by 25.32% was recorded with the addition of NAA (2 mg/l). However, without AC, maximum root formation and number of roots per shoot by 56.54 and 24.55%, respectively was achieved with IAA (2 mg/l) followed by IBA (52.22 and 26.44%) and NAA (41.54 and 22.33%). Results from Table 3 showed that addition of AC, at concentration ranging from 0.5 to 2 g/l, promotes more roots formation. However, a decrease in roots formation was observed with AC. concentration of more than 2 g/l. Hence, in the present study, AC played an active role in root induction and formation from *in vitro* shoots. IBA is highly effective auxin for rooting of *in vitro* regenerated shoots in several plants species (Gururaj *et al*, 2007) [19].

Fig 1: Indirect regeneration of *Bacopa monnieri* (L.) (A) Callus induction from leaf explant. (B) Induction of multiple shoots from callus. (C) Multiple shoots from callus cultures. (D) Root induction from multiple shoots of *Bacopa monnieri*.

Table 1: Effects of different hormones on Callus formation from leaf and node explants of *B. monnieri* (L.)

Type of hormone	Conc. of hormone (mg/l)	Callus formation from leaf (%)	Callus formation from Node (%)
NAA	1.0	33.92	28.71
	2.0	62.69	53.60
	3.0	61.49	30.29
2,4-D	1.0	39.52	51.64
	2.0	73.11	77. 65
	3.0	92. 23	52.31
IBA	1.0	13.28	28.71
	2.0	29.28	36.47
	3.0	45.30	17.27
2,4D+BAP	1.0	24.48	46.54
	2.0	48.20	54.24
	3.0	72.22	41.98

Table 2: Effects of different hormones on shoot and multiple shoot formation from callus of *B. monnieri* (L.)

Types of hormone	Conc. of growth regulator (mg/l)	Shoot formation (%)	Average shoots/ callus
IBA	0.5	32.29	17.68
	1.0	45.74	24.39
	2.0	58.36	36.59
BAP	0.5	51.40	24.65
	1.0	62.58	36.84
	2.0	73.23	47.83
IBA+BAP	1.0+1.0	52.51	24.38
	2.0+2.0	63.29	37.93
	3.0+3.0	76.43	49.21
IAA+BAP	1.0+1.0	33.38	16.87
	2.0+2.0	45.82	26.49
	3.0+3.0	57.61	38.25

Table 3: Effects of different hormones on Root formation from *in vitro* shoots of *B. monnieri* (L.)

Types of hormone	Conc. of hormone (mg/l)	No. of roots per shoots with Activated Charcoal	No. of roots per shoot without Activated Charcoal
IAA	0.5	20.39	7.29
	1.0	26.29	12.39
	2.0	33.44	24.55
NAA	0.5	14.96	7.47

	1.0	15.49	12.71
	2.0	20.92	22.33
IBA	0.5	25.32	11.35
	1.0	32.74	21.29
	2.0	38.55	26.44

Conclusion

The present investigation demonstrated that the addition of AC increases the roots growth and improves the *in vitro* morphogenic response of tissues in several ways. An improved vigorous growth and development of *B. monnieri* has been achieved on the medium supplemented with PGRs with addition of activated charcoal.

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