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RESEARCH ARTICLE

Effects of Plant Growth Regulators and Activated Charcoal on Regeneration and Plantlet Development in Neer Brahmi (*Bacopa monnieri*)

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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 monniera*. 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 explants. 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,* Murashige and Skoog medium, plant growth regulators, activated charcoal, plant hormones.

Introduction

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). 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 approx. 80% of traditional medicine preparations are made up of plants or plant extracts (Dhyani and Kala, 2005; Divya *et al.,* 2014). However, the collection of medicinal plants on a mass scale from the natural habitats leads to depletion 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). 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). Activated charcoal (AC) is composed of carbon, arranged in a quasigraphitic form of small particle size.

AC 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). *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 Ayurvedic 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). 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).

In vitro clonal multiplication method of selected clones helps in propagation and *ex situ* conservation of endangered and threatened medicinal plants. Earlier researches revealed that propagation of *B. monniera* through seeds is slow due to short viability and frequent seedling death and its vegetative propagation is also slow due to poor performance of propagules (Volluri *et al.,* 2011). Therefore, in the present study, leaf and stem are selected for propagation and regeneration. Furthermore, very few reports are available on the use of activated charcoal for plant tissue culture. Herein, we report an effective, efficient and cost-effective method for large scale production of *B. monnieri* for the first time.

Materials and methods

Plant material: Leaves and nodes were collected from healthy grown plants, washed thoroughly with running tap water without damage the tissues. Thereafter, the plant parts were surface-sterilized with sodium hypochlorite (1%) for 15 min followed by mercuric chloride (0.05%) for 3 min and finally 3 to 5 times in sterilized distilled water under aseptic conditions to avoid bacterial and fungal pathogens. Leaves and nodes were then shortened 0.5-1 cm in length, and these base-containing pieces were cut longitudinally, producing one explants per leaf. Leaf pieces were placed on the medium adaxial-side down and nodes are 45% vertical placed on the culture medium.

Tissue culture and in vitro plant regeneration: The leaves and nodes were cut into small pieces (0.5-1.0 cm) and inoculated into Murashige and Skoog (MS) medium salt with 3.0% (w/v) sucrose as carbon source, MS basal medium supplemented with auxin and cytokinins (2,4-D, IBA, NAA, IAA and BAP) alone and different combinations (Murashige and Skoog, 1962). The pH was adjusted to 5.6±2.2 followed by gelling with 0.8% agar in case of solid medium. The media was autoclaved at 121^{o}C and 1.06 kg/cm^{2} pressure for 20 min. All the cultures were incubated in a growth chamber maintained at temperature of 25±2^{o}C, relative humidity 70-80% and 16/8 (L/D) h of photoperiod duration under photon flux density of 50 µE mol m^{-2}s^{-2} provided by day light fluorescent tubes. The callus, once formed on the MS medium (with agar), was sub-cultured on MS medium containing activated charcoal on the 4th week (Nagarajan *et al.,* 2009). All experiments were repeated at least three times and similar results were obtained with all replicates. At least 50 leaf explants and 50 stem explants were analyzed per treatment.

Acclimatization: Plantlets with developed roots were removed from culture media and washed with running tap water. Thereafter, the plantlets were transferred to plastic cups containing sterilized vermiculite and soil (2:1). The plants were kept under 16/8 h photoperiod and 1/2 strength MS salt solution as poured regularly under lab conditions. Initially, the hardened plantlets were closed with polythene for maintaining high relative humidity (80-90%). Under glass house condition, the survivability was observed to be 100%.

Statistical analysis: The data were subjected to One-way Analysis of Variance (ANOVA) to evaluate the significant of difference of means of various experiments using SPSS statistical software package (Version: 10). The values are presented as mean±S.D and $p<0.05$ were considered as significant.

Results and discussion

In vitro plant regeneration depends on the manipulation of both organic and inorganic constituents in the medium, plant environment, type of explants and species. Biotechnological tools are essential for multiplication and genetic enhancement of the medicinally important plants by adopting *in vitro* plant regeneration techniques. The establishment of a reliable *in vitro* regeneration protocol is necessary to apply genetic engineering techniques to enhance production of bioactive secondary metabolites (Nikam *et al*., 2009).

Callus formation from leaf and stem: Callus is an undifferentiated mass of parenchymatous cells in the plant. 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. Compared to the stem, the leaf explants showed better callus formation. A maximum callus formation by 92.23% was observed on NAA (3 mg/L) followed by 2,4-D (2.5 mg/L) which showed formation by 73.11%. The combination of 2,4-D and BAP showed 72.22% callus formation whereas compared to other hormones, IBA (3 mg/L) showed lowest callus formation. 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 combination of 2,4-D and BAP which was found to 34.65% (Table 1).

Sub-culture was performed on the 4th week after *in vitro* callus culture, with the medium having same and different composition as in case of MS medium. The callus formation was found to increase with increasing hormone concentrations up to 3 mg/L. However, hormone concentration above 3 mg/L reduced the callus formation due to high level of hormone accumulation in plant tissues. Hence, it could be recommended that the hormone concentration between 2.5 mg/L and 3 mg/L is most suitable for maximum callus formation in both leaf and stem explants. The results of callus formation and *in vitro* plant regeneration are shown in Fig. 1.

Table 1. Callus formation from leaf and node explants of *Bacopa monnieri*.

Type of hormone	Conc. of hormone (mg/L) with MS	Callus formation (%)			
		Leaf	Callusing ability in leaf	Node	Callusing ability in node
2,4-D	0.5	21.64±1.2	+	38.65±2.0	+
	1.0	33.75±1.5	+	51.52±1.5	++
	1.5	47.32±0.5	++	62.24±1.0	+++
	2.0	62.05±2.5	+++	77.65±1.7	+++
	2.5	73.11±3.2	+++	64.70±2.5	+++
	3.0	61.85±2.0	+++	53.58±1.4	++
	3.5	52.64±1.7	++	40.33±2.0	+
NAA	0.5	25.85±1.5	+	19.33± 1.2	-
	1.0	39.64±1.2	+	28.42±1.3	+
	1.5	58.43±2.5	++	40.59±1.7	+
	2.0	71.54±3.4	+++	53.60±2.0	++
	2.5	83.62±2.3	++++	42.76±0.5	++
	3.0	92.23±1.5	+++++	30.68±0.1	+
	3.5	81.24±0.6	++++	21.11±0.2	+
IBA	0.5	6.65±0.3	-	8.67±0.2	-
	1.0	13.45±0.1	-	16.55±0.1	-
	1.5	21.32±0.2	+	24.42±0.9	+
	2.0	29.55±1.1	+	36.34±0.7	+
	2.5	37.42±1.3	+	24.60±0.04	+
	3.0	45.03±2.4	++	17.54±0.03	-
	3.5	33.65±1.2	+	11.55±0.02	-
2,4-D + BAP	0.5	12.55±1.0	-	8.11±0.1	-
	1.0	24.74±1.5	+	16.20±0.3	-
	1.5	36.55±1.8	+	23.48±1.2	+
	2.0	48.33±2.1	++	34.65±1.5	+
	2.5	60.83±2.8	++	22.57±0.6	+
	3.0	72.22±3.5	+++	11.70±0.2	-
	3.5	61.4±2.4	+++	6.70±0.1	-

Column means (n = 3) sharing common letter are not significant at P<0.05 Number in parentheses shows standard deviation (SD). - Below 20, + - 21 to 40, ++ - 41 to 60, +++ 61 to 80, ++++ - 81 to 90, +++++ - above 90.

Previously, Joshi and Dhawan (2007) have been employed 0.5 g/L AC along with 1/2 MS medium supplemented with NAA for rooting in *Swertia chirayita* micropropagation. The combination of 2,4-D (2.5 µM) and BA (5.0 µM) produced slightly more callus than the combinations of BA/Kn (5.0 µM) with NAA (2.5 µM). However, a better callus growth, in the presence of BA/Kn (5.0 µM) and IAA (2.5 µM), has been reported by Nikam *et al*. (2009). According to Asghari and Lockwood (2002), the combination of cytokinins is significantly effective for callus proliferation in *Peganum harmala* (Zygophyllaceae). Callus formation at the basal cut ends of the node and shoot-tip explants of *Leptadenia reticulata* on medium enriched with BA or Kn is in concomitant with the report on *Gymnema sylvestre* and *Holostemma adakodien* (Komalavalli and Rao, 2000; Martin, 2002). Nguyen *et al.* (2007) found only 29% survival in *Sorghum bicolor* without AC, while it increased up to 80% in the presence of AC. In spite of this, the addition of AC prevented the embryos from producing callus in some cases.

Shoot formation and multiple shoot formation from callus: Well developed callus transferred to MS basal medium supplemented with auxin and cytokinins in different combinations, the highest shoot formation was observed to 76.43% and multiple shoot formation to 49.55% in combination of IBA+BAP (3 mg/L), followed by IBA (2 mg/L) which showed shoot formation by 73.23% and multiple shoot formation by 47.24%. Shoot formation and multiple shoot formation by 58.56 and 36.34%, respectively was achieved with BAB (2 mg/L), whereas, a combination of IAA+BAP (3 mg/L) produced lowest shoot and multiple shoot formation by 57.32 and 38.14%, respectively (Table 2). A previous report by Tejavathi and Shailaja (1999) revealed that IAA is effective for the shoot regeneration in *B. monnieri*. A similar report by Rout *et al.* (1992) suggested that MS medium supplemented with KIN (4.5 mg/L) and NAA (0.1 mg/L) was effective for leaf callus formation in *Cephaelis ipecacuanha*. MS medium supplemented with BAP (2 mg/L) showed the highest shoot regeneration frequency and number of regenerated shoots (6.0).

Fig. 1. Callus formation and *in vitro* plant regeneration: a. Callus formation from leaf explant; b. Callus formation from nodal explant; c. Shoot initiation without AC; d. shoot initiation with AC; e. Multiple shoot formation without AC; f. Multiple shoot formation with AC; f. Hardening under glass house condition.

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

Type of hormone	Conc. of hormone (mg/L) with MS	Shoot formation (%)	Multiple shoot formation (%)
BAP	0.5	32.33±1.2	17.32±0.4
	1.0	45.44±1.9	24.36±0.5
	2.0	58.56±2.1	36.34±1.2
IBA	0.5	51.33±1.6	24.33±1.3
	1.0	62.12±2.2	36.83±1.8
	2.0	73.23±2.8	47.24±2.3
IBA+ BAP	1.0+1.0	52.5±2.0	24.54±0.3
	2.0+2.0	63.21±2.5	37.47±1.8
	3.0+3.0	76.43±3.6	49.55±2.3
IAA+ BAP	1.0+1.0	33.50±0.6	16.12±0.2
	2.0+2.0	45.62±1.6	26.57±1.2
	3.0+3.0	57.32±1.1	38.14±1.4

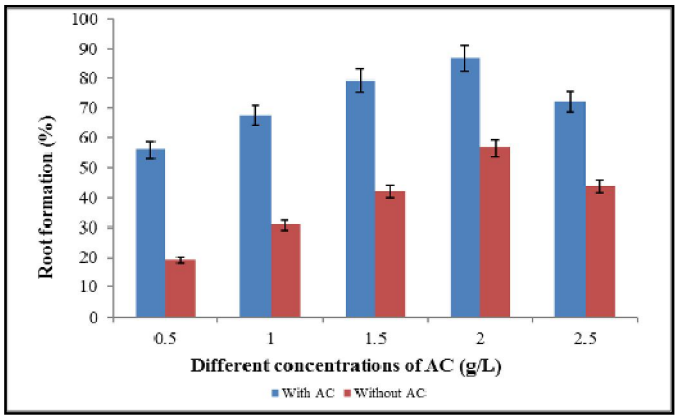
The frequency of shoot proliferation was relatively low and there were fewer shoots per explants when medium was supplemented with KIN (60%, 4.06) or TDZ (45%, 3.30) instead of BAP (Singh and Tiwari, 2011). The superiority of BAP over KIN for shoot regeneration has been reported in several systems including *Andrographis paniculata* and *Pterocarpus marsupium* (Chand and Singh, 2004; Purkayastha *et al.,* 2008). TDZ is very common and used for high frequency shoot regeneration from different explants in several species including Brahmi (Tiwari *et al.,* 2001), but it is not effective in case of *Clitoria ternatea* (Lata *et al.,* 2009). Medium supplemented with 1 mg/L or more TDZ failed to induce shoot regeneration from DEAs. Similarly, shoot buds developed on medium containing TDZ were stunted and often fascinated and subsequently failed to elongate in several plant species such as *Balanites aegyptiaca*, *Rhododendron* spp. and *Dalbergia sisso* (Preece and Imel, 1991; Pradhan *et al.,* 1998; Anis *et al*., 2010). Thus, BAP when added alone in the medium was the most effective plant growth regulator indicating the cytokinin specificity of DEAs of *C. ternatea* for multiple shoot regeneration (Singh and Tiwari, 2011).

Roots formation from shoots: The micropropagated shoots were transferred to the MS basal with 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. 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. The effect of activated charcoal on the roots formation is given in Fig. 2. In *Fortunella crassifolia* shrub, the shoots formed from epicotyl explants, exhibited maximum rooting (75%) on ½ MS medium supplemented with NAA, Kn and AC (0.5 g/L) (Yang *et al.,* 2006). Roots were induced on shoots of *Arachis hypogea* derived from regenerated virus free ELISA tested calli. According to Radhakrishnan *et al*. (1999), about 60-80% of the shoots rooted when cultured on MS medium containing AC (0.04 g/L) and CH (0.2 g/L), another extra additive commonly used in plant tissue culture.

Table 3. Root formation from *in vitro* shoots of *B. monnieri.*

Types of hormone	Con of hormone (mg/L) with MS	Root formation with charcoal (%)	No. of roots per shoot (%)	Root formation without charcoal (%)	No. of roots per shoot (%)
IAA	0.5	46.54±1.4	20.11±0.12	29.51±1.2	7.12±1.1
	1.0	63.38±2.2	26.46±0.38	42.23±2.3	12.33±1.8
	2.0	71.47±2.4	33.44±1.97	56.54±2.8	24.55±2.2
NAA	0.5	29.35±2.5	14.58±0.21	19.21±2.6	7.66±0.5
	1.0	43.23±2.6	15.55±0.24	30.32±2.4	12.66±1.1
	2.0	51.42±2.1	20.71±0.76	41.54±2.2	22.33±2.1
IBA	0.5	64.65±2.3	25.32±0.95	32.35±1.5	11.42±1.4
	1.0	70.46±2.6	32.12±1.24	45.44±1.8	21.75±2.2
	2.0	85.52±2.8	38.55±2.5	52.22±2.1	26.44±2.1

Fig. 2. Roots formation in *B. monnieri* with and without charcoal.



In *Thapsia garganica,* rooting of the stock culture kept on MS medium supplemented with BA and NAA were rooted on ½ MS medium fortified with IBA prior to AC (0.5 g/L) treatment and 50% of the plantlets rooted while an average number of 6 roots per shoot were observed in each shoot (Makunga *et al.,* 2006). Babu *et al.* (2003) reported rooting of *Cinnamomum camphora* (camphor tree) shoots derived from shoot tips and nodal segments on WPM medium supplemented with AC, IBA and NAA, in which 100% of the shoots rooted on IBA and AC (2 g/L) containing medium were achieved. Addition of AC was also found to increase roots formation in *Acer pseudoplatanus* (Barcelo-Munoz *et al.,* 1999), *Garcinia mangostana* (Techato and Lim, 1999) and *Lavandula vera* (Andrade *et al.,* 1999).

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* (Brahmi) has been achieved on the medium supplemented with PGRs with addition of activated charcoal.

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