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IN- VITRO CONSERVATION OF *BACOPA MONNIERI* – AN ENDANGERED MEDICINAL PLANT

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ABSTRACT

The World Health Organization (WHO) estimated that 80% of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs. Plants provide the predominant ingredients of medicines in most medical traditions. While the demand for medicinal plants is growing, some of them are increasingly being threatened in their natural habitat. Plants are endangered by a combination of factors: over-collecting, unsuitable agriculture and forestry practices, urbanization, pollution, habitat destruction, fragmentation and degradation, spread of invasive alien species. Around 70% of India's medicinal plants are found in tropical areas mostly in the various forest types spread across the Western and Eastern ghats, the Vindhyas, Chotta Nagpur plateau, Aravalis & Himalayas. Although less the 30% of the medicinal plants are found in the temperate and alpine areas and higher altitudes they include species of high medicinal value. Several medicinal plants are already threatened, rare, or endangered. Plant tissue culture is an emerging tool for the propagation and conservation of some economically important crops that are listed as endangered, rare and threatened. This review aims to summarize the *in vitro* technique for conservation of *Bacopa monnieri* an important medicinal plant which is listed as endangered medicinal plant. *Bacopa* is a great neurotonic, immuno-modulator, adaptogen, tranquilizing, memory and learning enhancing, cerebral activator

KEY WORDS: Aflatoxin, HPLC, LC-MS *Bacopa monnieri*, tissue culture, growth regulators, conservation.

INTRODUCTION

The International Union for Conservation of Natural and National Resources has a long time ago listed *Bacopa monnieri* as a threatened species. *Bacopa monnieri* (L.) is a sprawling succulent ayurvedic tropical herb of the family scrophulariaceae found in fresh and brackish waters, wet and marshy lands throughout India, Nepal, Sri Lanka, China, Taiwan, and Vietnam (Anonymous 1988), and is also found in Florida, Hawaii and other southern states of the USA where it can be grown in damp conditions by the pond or bog garden. The herb can be found at elevations from sea level to altitudes of 4,400 feet and is easily cultivated if adequate water available. It is best known for its small but profuse white-to pink flowers and trailing habit. It grows 8 to 12 inches tall and wide and needs full sun to bloom abundantly. Flowers and fruits appear in summer and the entire plant is used medicinally. *Bacopa* spreads by producing new plants on above ground runners (Zimmerman, 1993).The new plants can be separated from the parent plant once they have taken root. The natural regeneration of this herb is hampered by death of seedlings at 2-leaved stage and specific habitat (marshy areas) requirements. *Bacopa* seems to be poor competitor and so it can colonize open spaces only (Tiwari *et al.,* 2000). In India it is commonly known as 'Brahmi'. It is medicinally very important as it contains alkaloids (nicotine, brahmine, herpestine), saponins (hersaponin, betulic acid, bacosides A, B, C and D) and other chemicals like stigmastanol, b-sitosterol and stigmasterol (Bose and Bose, 1931; Fransworth, 1966; Ramseh *et al.,* 2007). It is an ancient and renowned medicinal plant with legendary reputation as memory vitalizer (Anonymous, 1998). 'Brahmi' is found to be effective in cases of anxiety and neurosis. It possesses anti-inflammatory, analgesic and antipyretic activity (Vohra *et al.,* 1997). It is also used to treat asthma, insanity, epilepsy, hoarseness, enlargement of spleen, snake bite, rheumatism, leprosy, eczema and ring worm, it is also used as a diuretic, appetitive and cardio tonic (Basu and Walia, 1994). It can enhance immune function by increasing immunoglobulin production. It may regulate antibody production by augmenting both Th1 and Th2 cytokine production (Yamada *et al.,* 2011). *Bacopa* may also increase the effects of calcium channel blocker anti-hypersensitive drugs and their doses may need to be reduced if using *Bacopa* as a supplement. It may also cause a lower heart rate, and increase secretions in the stomach, intestines, and urinary tract. The increase in secretions may irritate ulcers and urinary tract obstructions. The herb requirement is rising rapidly in view of the popularity of the *Bacopa* based drugs. In view of wider market demand, there is need to conserve the wild stocks of *B. monnieri.* To conserve this crop and to meet the growing demand of raw material of medicinal plants, tissue culture techniques are being used as alternative methods for propagation in many countries. Most of the work has been carried on plant regeneration by adventitious organogenesis from shoot tip, leaf and other parts of the plant. Attention should be paid to conserve this crop and improve the technology to achieve 100% success in all species / cultivars to meet growing demands of the growers globally. From the literature, it is evident that *Bacopa monnieri* highly amenable to *in vitro* studies, as various explants were found to favorably respond to different culture media with different types and concentration of growth regulators. Figures 1 and 2 exhibit

the different categories of plants and different plant parts constituting herbal medicines respectively.

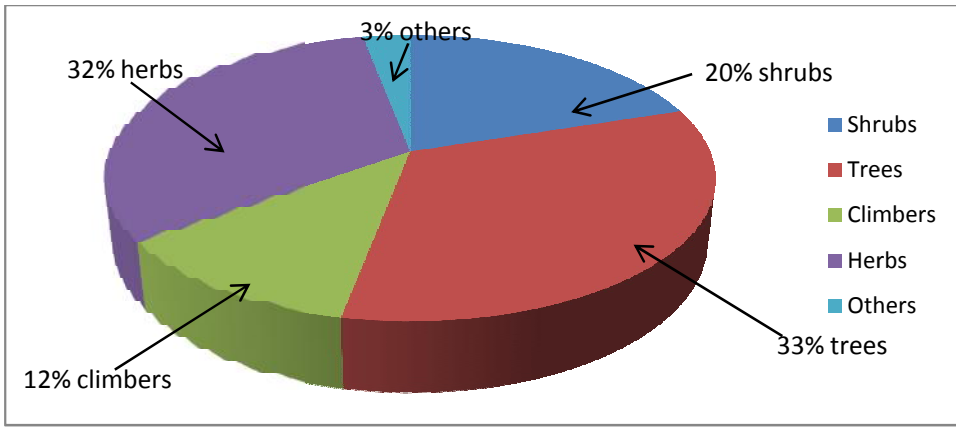

FIGURE 1. Distribution of medicinal plants by habitats

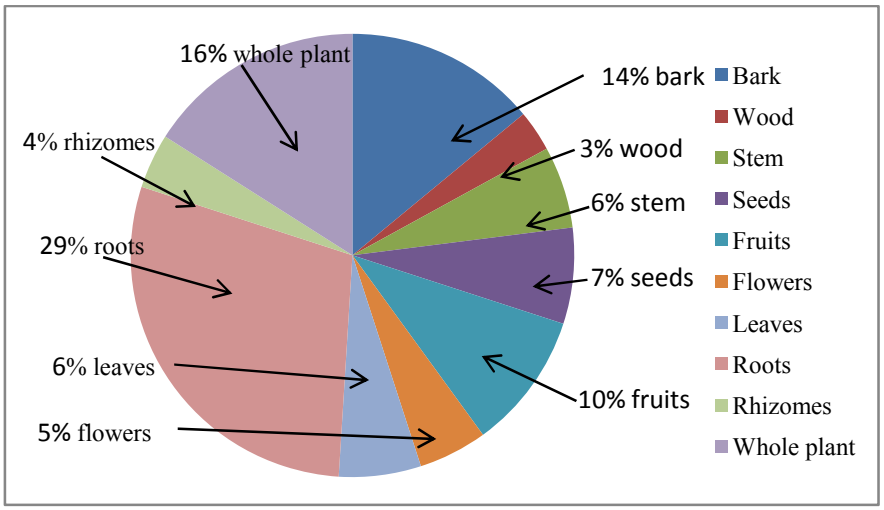

FIGURE 2. Different parts used as herbal medicine

TISSUE CULTURE

Tissue culture techniques are very widely used and its most important application is the micro-propagation. The micro-propagation technique has been a miraculous tool for the restoration of those flora and fauna, which are threatened and are at the verge of extinction. Tissue culture procedure has been proven to be commercially practical in *Bocopa* propagation.

The term *in vitro* cultures cover a wide range of techniques involving the growth under sterile condition of plant germplasm (especially shoot tips, meristem, somatic embryos or embryonic callus) on artificial culture media. Although each plant species and tissues requires an optimum nutrient medium for their luxuriant growth *in vitro*. Therefore the proper choice of medium is very important and it is based on plant morphology and their nutrient requirements. In plant tissue culture, there are number of culture medias e.g. MS medium (Murashig & Skoog medium), White Medium, Gambog etc. MS medium (Table 1) was successfully used by many workers for *in vitro* regeneration of *Bacopa* (Sharma *et al.,* 2010).

Carbon source and organics

The most popular carbon source in plant tissue culture is sucrose. While autoclaving the medium sucrose is converted into glucose and fructose. First glucose is used than fructose. Plants cells and tissue lack autotrophic ability while they are in the culture medium, therefore they required external carbon source for energy. Plant synthesis vitamins endogenously, When plant cells and tissues are grown in vitro they synthesized some essential vitamins but only in suboptimal quantities. Thiamine (B1), Nicotinic acid (B3), Pyridoxine (B6), Calcium pentothenate (B5) and myo-inositol are used more often, thiamine is the basic vitamin required by all cells and tissues.

Gelling agent (agar)

In liquid cultures the tissue cells submerged and die due to lack of oxygen. For any culture types that require the plant

cells or tissues to be grown on the surface of the medium, it must be solidified. Agar produced from seaweed, is the most common type of gelling agent, and is ideal for routine applications. pH of the medium plan crucial role, it should be 5.74-5.8.

Growth regulators

Plant hormones are not nutrients, but chemicals, that in small amounts promote and influence the growth (Öpik 2005). There are five main classes of plant growth regulator used in plant tissue culture viz. auxins; cytokinins; gibberellins; abscisic acid; ethylene. The concentration and selection of growth regulator is the deciding step in any experiment of plant tissue culture. The concentration of growth regulators is expressed in terms of milligrams (mg), parts per million (ppm) or micromoles.

In vitro regeneration

In vitro cultivation of plant cells or tissues is basically applied to solve the two main problems. Firstly, maintenance of microorganisms (bacteria and fungi) free culture and secondly, to ensure the desired growth in cell and tissues by providing nutrient rich medium and aseptic cultural conditions.

Most plants which may reproduce by vegetative reproduction may be easily conserved in the laboratory without any concern for critical population size or genetic diversity (Kondo, 1996). *Bacopa monnieri* is a vegetatively reproducing species, and thus is an excellent candidate for conservation in a small laboratory space using tissue culture methods (Fig. 3).

FIGURE. 3 Various stages of *in vitro* regeneration of *B. monnieri*

Sterilization of explant

Surface sterilization is most important step before inoculation of explant. Srivastava and Rajani (1999) described the use of HgCl2 for 2 min followed by rinsing with autoclaved distilled water for sterilization of explants of Bacopa. Explants were washed with teepol and surface sterilized with 0.1% HgCl2 (w/v) followed by thoroughly washing (Joshi et al., 2010 ; Tanveer et al., 2010; Narayan, 2011 and Gurnani *et al*., 2012). Sharma *et al*. (2010) used (1 - 2% cetavelon) solution to remove dust particles and 0.1% (w/v) HgCl2 for surface sterilization. Different sterilization treatment was followed by Mathur and Kumar (1998), in which leaves and stem explants shaken for 10 min. in tween 20 and savlon in water for 10min, rinsed in running water for 10 min, treated with 0.1% HgCl2 for 3-4 and washed several times with sterile water. Banerjee and Modi (2010) treated explants with liquid detergent labolene (5% v/v) followed by washing with tap water and final rinse with autoclaved distil water to remove dust particle and applied 0.1% HgCl2 for surface sterilization. Vijayakumar *et al*. (2010) were able to establish contamination free explants from Bacopa by washing the explants, under tap water followed by bavistin (0.2-0.5%) and 0.03% streptomycin solution for 10 min. Again explants were immersed in aqueous solution of savlon (1.5% v/v chlorohexidine gluconate sol. and 3% w/v cetrimide) for 10 min. After this treatment, the explants were surface sterilized with 0.01% HgCl2 sol. for 1 min. Shrivastva and Rajani (1999) reported blackening of tissues with excessive treatment of HgCl2, Hence limited treatment of 0.1% mercuric chloride was given to plants 4 - 5 min.

Culture initiation

Micropropagation of Bacopa is being used in many countries from a range of explants. In tissue culture studied so far, plant regeneration was uniformly achieved with different explant as the source material (Murashige, 1977).

A number of studies have been carried out on shoot regeneration from different explants. The plant were produced from explant of leaves (Binita *et al*., 2005; Mohapatra and Rath (2005; Praveen *et al*., 2009; Joshi *et al*., 2010; Vijayakumar *et al*., 2010), axillary node (Banerjee and Modi, 2010), nodal segments (Tiwari *et al*., 1998; 2000; 2001; 2006; Binita *et al*., 2005; Debnath, 2008 Ramesh *et al*., 2009; Sharma *et al*., 2010; Vijayakumar *et al*., 2010; Mehta *et al*., 2012), internodes (Binita *et al*., 2005), shoot apex (Binita *et al*., 2005;

Debnath, 2008; Narayan, 2011), root (Vijayakumar *et al*., 2010), stem (Vijayakumar *et al*., 2010).

In vitro shoot multiplication

MS medium supplemented with sucrose (3%) along with different concentrations of BAP (2 to 12 µM) was used to establish cultures Joshi *et al*. (2010). Shoot proliferation was achieved on MS media supplemented with various growth regulator viz. BAP, Kinetin, IBA, AS, 2,4-D. The efficiency of BAP for shoot culture initiation and multiplication in B. monnieri, reported by (Tiwari *et al*., 2000; Shrivastava, 1999; Tiwari *et al*., 2007).These multiple shoots became dwarfish and excellent form to fit to culture tubes. Cytokinins are known to be very effective in promoting shoot proliferation and their role in shoot organogenesis is well established (Evans *et al*., 1983). Several studies also showed that media supplemented with NAA & BAP have also useful for production of shoots (Gurnani *et al*., 2012). Success of regeneration depends not only on the type of the explant chosen, but also the way explants are placed on the culture medium (Duzyaman *et al*., 1994).

Tiwarei *et al*. (2000) proposed an efficient and rapid method using liquid shake cultures for in vitro propagation of Bacopa. Tiwari *et al*., 2001, reported use of range of cytokinins for multiple shoot induction for Bacopa, with node, internode and leaf explants. Yang and Read, 1997; Bhuyan *et al*. (1997) used BAP (9-10mM) for shoot regeneration. Multiple shoots were obtained on MS medium supplemented with auxins or / and cytokinins with or without coconut milk. Maximum number of plants was obtained on medium containing Kin/2-ip (0.1 mg/l) and Kin (1 mg/l) in shoot tip and nodal cultures respectively (Tejavathi *et al*., 2001). Praveen *et al*. (2009) tried semisolid medium supplemented with 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l (BAP) or Kinetin (Kn) or thidiazuron (TDZ) for shoot. Highest rate of shoot regeneration was observed by Praveen *et al*. (2009) for leaf explants cultured on medium with 2 mg/l BAP or Kn or thidiazuron (TDZ). Mohapatra and Rath (2005) reported maximum shoot multiplication on MS medium supplemented with BAP and NAA.

Effectiveness of MS medium for optimum shoot multiplication in different species have also noted by various workers (Tewari *et al*., 1998, 2000; Tejavathi *et al*., 2001; George, 2004; Binita *et al*., 2005; Escandon *et al*., 2006; Sharma, 2007; Banerjee and Shrivastava, 2008).

In vitro rooting

In medicinal plants roots of microshoots have been obtained in MS medium with IAA, IBA, NAA used singly or in combination or when transferred to hormone free medium. The role of auxin in root development was established and reviewed by Torrey (1965, 1976). Singh *et al*. (1999) reported rooting in B. monnieri on MS medium supplemented with BAP (0.5 mg/l). Root induction has also been reported in Bacopa (Tiwari *et al*., 2011) using MS medium supplemented with a concentration of 1.0 mg/l of IAA and 1.0mg/l IBA. Sharma *et al*. (2010) reported best rooting in B. monnieri with IBA when incorporated in MS at different concentrations (0.1 - 0.3 mg/l). Narayan et al. (2011) maximum number and length of roots with IBA and IAA. Tiwari et al. (2000) tried rooting on different media in Bacopa, i.e. MS media with or without hormones and found that rooting was highest (90%) on full-strength MS medium containing 2.46 mM IBA.

Hardening and acclimatization

Attempts have been made for hardening and acclimatizing Bacopa plantlets, one of the most important aspects of in vitro raised tissue cultured plants. A few reports are available where different soil media were used and standardized with 50-100% success depending upon the soil mixture.

The rooted plants were hardened in sand/soil (3:1, v/v) mixture (Joshi *et al*., 2010). Sharma *et al*. (2010) transferred rooted plantlets to polybags containing a mixture of sand, farmyard manure and soil in a ratio of 1:1:1. Narayan *et al*. (2011) reported 90% plant in clay soil and clay + red soils respectively.

CONCLUSION

Herbs are being used since ancient time to maintain health, to treat disease and regain the healthy state of mind and body. However, due to over exploitation they are on the verge of extinction. Bacopa monnieri L. Penn., commonly known as Brahmi, has been used in Indian System of Medicine for centuries for everything from snakebite to headache. It is used most often as a brain tonic and a memory enhancer. The demand of Bacopa is met from natural population, which leads to put heavy strain on existing natural population and hence slow depletion of this important herb. Tissue culture techniques can be used to attain rapid multiplication of the elite clones and germplasm conservation of Bacopa monnieri.

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