

## Review Article

# Advancement in Plant Tissue Culture-Based Research for Sustainable Exploitation of Well-Known Medicinal Herb *Bacopa Monnieri*

Abhijith Vinod<sup>1</sup>, Shivika Sharma<sup>2</sup>, Vikas Sharma<sup>1\*</sup>

1)Department of Molecular Biology & Genetic Engineering, School of Bioengineering and Biosciences, Lovely Professional University, Phagwara-Jalandhar, India.

2)Biochemical Conversion Division, SSSNIBE-Kapurthala Punjab.

\* Corresponding author, email: biotech\_vikas@rediffmail.com, vikas.25269@lpu.co.in

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### ABSTRACT

The current review focuses on the plant *Bacopa monnieri*, one of prominent medical herbs in Indian ayurvedic system. The plant is well known for its cognitive and memory enhancing capabilities. The plant contains many useful alkaloids and secondary metabolites. Studies have shown that it has various promising pharmacological properties which have the potential to treat many illnesses and disorders such as asthma, bronchitis, rheumatism also in for renal disease, water retention, blood cleaning etc. This leads to the over exploitation of the plant which puts a stress on the naturally available stock of the plant, therefore, it becomes a necessity to find optimum methods for mass production of the plant and its important secondary metabolites. This review attempts to compile and to discuss the advancements in methods and techniques including type of culture vessels, plant growth regulators (PGRs), effect of stress, plant growth promoting rhizobacteria (PGPRs) interactions; for *in vitro* propagation of *Bacopa monnieri* and the enhanced production of its important bioactive (bacoside) for its sustainable exploitation.

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### INTRODUCTION

The *Bacopa*, also commonly referred to Water hyssop or the Brahmi plant, is a creeping herb mostly found in wetlands and muddy regions. Belonging to the *Scrophulariaceae* family, it is a key component in many Ayurvedic medicines. The plant leaves are thick and succulent in nature, flower's small and white. The entire plant is medically useful (Bone 1996). It is a perennial plant and distributed among tropical and subtropical regions such as India, China, Nepal, Pakistan, Viet Nam and Sri Lanka. Also, its occurrence has been reported in Florida, some states of USA, and Hawaii. Its presence has also been noted in the Mediterranean basin. The areas of the Arabian Peninsula where this plant has been observed include: Kuwait, UAE, Saudi Arabia, southern and northern parts of Oman, Yemen, Bahrain, and Socotra. Additionally, reports of its presence throughout the western and southern Peninsula have also been recorded. In India, the *Bacopa* plant has been found in many states including Delhi, Andhra Pradesh, Kerala, Goa, Assam, Orissa, Bihar, Gujarat, Andaman, Karnataka, Tamil Nadu, Manipur, West Bengal, Punjab, and Rajasthan.

Since ancient times, the *Bacopa* has been utilised in many Ayurvedic treatments. It has been valued as an important herb in many literatures since about 800 BC, one of its treatments for mental ailments has been documented in the text *Charaka Samhita* (Singh & Dhawan 1997). It is primarily used to improve cerebral capacities, but there are several reports where it has been used against inflammatory diseases including asthma, bronchitis, rheumatism and for treating of numerous kidney disorders like blood purification, water retention etc. (Singh & Dhawan 1982; Channa et al. 2006; Rao et al. 2012).

The presence of several compounds like alkaloids (nicotine and herpestine), flavonoids (apigenin and luteolin), glycosides (thanakunide, asiaticoside), and phytochemicals like wogonin, betulinic acid,  $\beta$ -sitosterol, betulinic acid, stigmaterol, oroxindin and brahmic acid, brahmoside, iso-brahmic acid, brahminoside, vallerine, volatile oil, pectic acid, ascorbic acid, fatty acids, asiatic acid, tannins, thanakunide etc. has been documented by (Chopra et al. 1956; Sivaramakrishna et al. 2005; Mathew et al. 2010). Of these, Bacoside-A (3-( $\alpha$ -L-arabinopyranosyl)-O- $\beta$ -D-glucopyranoside-10, 20-dihydroxy-16-keto-dammar-24-ene) is thought to be the key active compound which helps in assisting memory (Chatterji et al. 1965).

The *Bacopa* plant has been ranked second on the importance for its commercial worth, therapeutic value, and potential for future research and development, based on a study undertaken by The Export-Import Bank of India. Therefore, the estimated annual dry weight demand for the plant is about 12.700 tons, which is around Rs 15 billion (Ahmed 1993). Almost the entirety of this need is satisfied by the existing natural stock or by traditional methods of propagation.

This present study focuses on the effectiveness of secondary metabolites production from *Bacopa monnieri* using various *in-vitro* propagation methods.

The whole *B. monnieri* plant is medically significant (Bone 1996). Plants collected from wild, greenhouses or *in vitro* grown plantlets have been used for micropropagation. Stem, shoot tips, nodal and internodal segments from mature plants as well as from plantlets cultured in *in-vitro* has been used for the micropropagation of *Bacopa monnieri*. Even though different culture medias can be used for micropropagation, a comparative study between Murashige and Skoog (MS (Murashige & Skoog 1962)) and Gamborg's (B<sub>5</sub>) media, where no additional plant growth regulators (PGR) were used, showed that MS media is better suited for *B. monnieri* plant for both shoot and leaf multiplication *in vitro* (Koul et al. 2014).

The most widely used plant parts for *B. monnieri* clonal growth are shoot tips and nodes, which are generated from *ex-vitro* (~45% of reports) and *in-vitro* cultivated plants (~16% of reports), amongst which the nodal explants were reported to yield better results for enhanced *in-vitro* callusogenesis (Saha et al. 2020).

Impact of explant size has also been evaluated in one study where explants of size 0.5 cm and 20 explant/40 millimetre (1 explant/2 millimetre) yielded the best explant response in terms of number of shoots per explants regenerated and shoot length. Also, increase in size of explant did not make for an increment in the number of shoots in the same proportion (Jain et al. 2012).

### ***In-vitro* Callusogenesis**

The induction of callus using leaf explants on semi-solid MS basal media supplemented with various concentrations of Kinetin (Kn) or 6-Benzylaminopurine (BAP) alone; as well as varied quantities of 1-

Naphthaleneacetic acid (NAA) or Indoleacetic acid (IAA) in combination with BAP were tried by (Rout et al. 2011) in which it was observed that a combination of BAP ( $2.0 \text{ mgL}^{-1}$ ) with NAA ( $0.5 \text{ mgL}^{-1}$ ) gave maximum callusing rate of  $71 \pm 2.2\%$ . Callus of 1-2 cm in diameter were suitable for organogenesis after sub-culturing in the same media (Sheikh et al. 2015). The impact of growth regulators; cytokinin and auxin, when coconut milk was used as an adjuvant was studied by (Kumari 2019).

The callus obtained from explants (leaf and stem) were pale, with soft surfaces, curled to compact, and turned green when exposed to light. Maximum calli development was obtained from 1-Naphthaleneacetic acid ( $2.5 \text{ mg}^{-1}$ ) which gave a (94.22 %) of calli formation in leaf explants; and from nodal explants 2,4-D showed maximum calli formation (of 71.41 %) on  $2.5 \text{ mg}^{-1}$  and internodal explants on 2,4-D showed 65.21% on  $2.5 \text{ mg}^{-1}$  (Ali et al. 2020).

According to (Patra et al. 2018) leaf explants grown on MS medium +  $0.5 \text{ mg L}^{-1}$  1-Naphthaleneacetic acid +  $0.1 \text{ mg L}^{-1}$  6-Benzylaminopurine showed the fastest growth in green callus of  $01.83 \pm 0.23 \text{ g/L}$  biomass (on the basis of dry cell weight) in less than two weeks (12 days).

### Regeneration by Nodal Segment

Multiple shoot proliferations by using varied intensities of 6-Benzylaminopurine and Kinetin alone or with combination of Indoleacetic acid were demonstrated by (Sape et al. 2020). When shoot bud induction was tested on nodal explants cultured in MS media fortified with  $\text{N}^6$ -benzyl adenine (BA), it resulted in gradual swelling of the base and the emergence of multiple shoot buds from both above and below the medium, though only a small number of the shoot buds located above the medium was able to elongate in it. (Behera et al. 2015).

In an assessment conducted by (Chaudhry et al. 2019) with different concentrations of MS media and combinations BAP and NAA, it was reported that the use of MS basal media at full strength was able to produce 100% bud break after 3-4 days of culture. In addition, a maximum number of 12-13 shoots were produced per culture.

The BAP and NAA also were seen to be a crucial factor as  $0.5 \text{ mg L}^{-1}$  of BAP gave good results ( $10.2 \pm 0.1$  shoots per culture), but bud break and shoot regeneration decreased as BAP increased (Dixit & Thakur 2017; Chaudhry et al. 2019). Higher concentrations of NAA ( $1 \text{ mg L}^{-1}$ ) caused callus induction. Maximum shoot elongation is more than any other culture and combination in the study took place in simple MS basal media with full strength, where it produced a maximum shoot elongation of 9 cm and  $12.0 \pm 0.2$  shoots per culture, after 4 weeks of culturing and sub-culturing in the same media composition (Chaudhry et al. 2019). The combination of BAP, Kn and NAA, each  $1 \text{ mg./l}$ , also proved as very effective in obtaining a maximum mean number ( $18.4 \pm 0.8$ ) of shoots per explant (Pandiyaraj & Selvaraj 2012).

### Direct Regeneration by Leaf Explant

Direct regeneration has had the most succeed, as plants grown using direct organogenesis are significantly more stable than those grown through indirect organogenesis (callus) (Kamenickà & Rypák 1989). Based on a study conducted by (Nagella et al. 2009), leaf explants, although they were able to successfully regenerate shoot buds in culture, required a two-stage culture setup. In which the first stage involved growth of the explants in a static medium and in the second stage, they were transferred from the static to liquid medium. Here, the leaf explants

produced shoot buds which were too small and made it impossible to be counted and took longer time in becoming shoots.

There are existing numerous records by which successful leaf explant cultures have been established on different media, as well as with different media composition (Joshi et al. 2010; Koul et al. 2014; Umesh et al. 2014; Rahe et al. 2020) etc.

Leaf explants cultured in full strength MS basal media showed shoot bud break and shoot bud proliferation (90%) in 10 days along the leaf margins. As seen in the case of nodal explants, higher concentrations of NAA induced callus formation. Yellowing of shoot buds were observed in two-week-old cultures composed of MS medium + BAP + NAA combinations. Maximum number of shoots were observed full strength MS basal media with 4 weeks of culture, ( $10.9 \pm 0.3$ ). After 3 weeks of sub culture, maximum shoot elongation of  $8.5 \pm 0.11$  was observed in full strength MS media (Chaudhry et al. 2019) (Table 1).

### **In vitro Rhizogenesis**

Root induction was carried out by using different concentrations of Indole-3-butyric acid (IBA) and MS media (Jain et al. 2013; Dixit & Thakur 2017; Ali et al. 2020), of which half strength MS with  $0.2 \text{ mgL}^{-1}$  IBA showed maximum root formation with an average of 10.2 roots, with an average length root length of 4.2 cm in four days from shoot inoculation (Behera et al. 2015). When in IBA concentration was increased to  $0.3 \text{ mg L}^{-1}$ , it produced short and thick roots (Dixit & Thakur 2017).

### **Hardening and Acclimatization**

Plantlets grown *in vitro* via tissue culture cannot be planted directly into the field as they are grown in controlled environmental conditions and therefore, they need to be acclimatized, as to reduce the overall mortality rate (Chaudhry et al. 2019). This can be achieved by a number of means. The type of potting mixture used always serve as a vital component of this process. Several potting mixtures like sand, soil, cocopeat, cow dung, vermiculite, farmyard manure, soilrite, peatmoss etc. have been used in different ratios and combinations resulting in the hardening and acclimatization process of *B. monnieri* (Joshi et al. 2010; Rout et al. 2011; Bhusari et al. 2013; Umesh et al. 2014; Naik et al. 2014; Hegazi 2016; Sharma et al. 2018; Chaudhry et al. 2019) (Table 1).

Rooted shoots after sufficient period of culturing, has to be transferred to pots for hardening. These plantlets have to be carefully detached from their *in vitro* culture media. This is followed by thoroughly cleaning the roots with sterile distilled water under lab conditions, to remove any media remains attached to the roots. These washed plantlets are then transferred to small containers such as plastic cups with potting mixture composed of uncontaminated soil and vermiculture in the ratio (2:1 vol./vol.). They are maintained under (16/8 hr Light/Dark) photoperiods. Primary hardening is done in laboratory conditions by regularly pouring salt solution of half strength MS media. Hardened plantlets were initially encased in polythene bags to maintain a sufficient high humidity of 80%. In two weeks, the polythene covers were removed and direct light exposure was given to these potted plants. Sterile distilled water was used to water the plants under these conditions. A survivability rate of 100% was reported for these plants under glass house conditions (Sharma et al. 2016; Ali et al. 2020).

### **Bacoside Production**

By using the Plackett-Burman (PB) method of study, four factors essen-

**Table 1.** Some methods used in micropropagation of *Bacopa monnieri*

| Explant-source/Type   | Observation   | Media (shoot induction/multiplication)  | Rooting Media  | Acclimatization   | Reference             |
|---|---|---|--|---|-----------------------|
| Leaf explant ( <i>ex vitro</i> )  | Multiple shoot formations; rooting.   | MS+6.0µM BAP+3% sucrose.  | Half Strength MS +1% sucrose+ 2.0 µM IBA.                      | Sand: soil (3:1)  | (Joshi et al. 2010)   |
| Leaf explant ( <i>ex vitro</i> )  | Callus induction; multiple shoot formations; rooting. (Mean no. of roots/ shoot 6.8±0.72)   | MS + 0.5 mg l <sup>-1</sup> NAA+ 2.0 mg l <sup>-1</sup> BAP.                  | Half MS + 2.00 mg L <sup>-1</sup> IAA                          | Mixture of soil: sterilized sand: Powdered dry cow dung (1:1:1) for 1 week. 86% survival. | (Rout et al. 2011)    |
| Stem and leaf explants ( <i>ex vitro</i> )  | Bioreactor as well as different culture vessels used; Multiple shoot formations; Phenolic determination.                                | MS+ IAA 00.01 mg l <sup>-1</sup> + BAP 02.5 mg l <sup>-1</sup> + 3 % sucrose. | –  | –   | (Jain et al. 2012)    |
| Axillary nodes, shoot tips and young leaves ( <i>ex vitro</i> )                               | Cost effective method for some culture components; Multiple shoot formations; rooting.  | Half semi solid MS +3.0 mgL <sup>-1</sup> Kn +00.5 mgL <sup>-1</sup> IBA.     | Half MS+ NAA 0.5 mgL <sup>-1</sup> + IBA 0.5 mgL <sup>-1</sup> | In pots containing soil: farmyard manure: sand (1:1:1).                                   | (Bhusari et al. 2013) |
| Leaf and nodal explants ( <i>ex vitro</i> )   | Multiple shoot formations; Bacoside production; detection by HPLC method.   | MS + 2.0 mg L <sup>-1</sup> Kn.   | MS.  | soil rite (Mixture of coco brick, vermiculite and cocopeat perlite).                      | (Umesh et al. 2014)   |
| Leaf, node and internode segments ( <i>ex vitro</i> )   | Multiple shoot formations; rooting.   | MS + 2.0 mg L <sup>-1</sup> Kn.   | MS + 2.0 mg L <sup>-1</sup> Kn                                 | soil rite. 95% survival.  | (Naik et al. 2014)    |
| Shoot cultures from nodal segments.   | Bioreactor based cultures; Growth index measured in terms of dry wet; Multiple shoot formations; bacoside production. (Maximum GI 5.84) | MS+ 3% sucrose+ 1mg L <sup>-1</sup> BAP                                       | –  | –   | (Sharma et al. 2015)  |
| Shoot tips ( <i>ex vitro</i> ) & <i>in-vitro</i> sub-cultured shoot tips (for encapsulation). | synthetic seeds; encapsulation, storage and recovery; rooting.  | MS+ 0.1 mg/ L myo-inositol+ 3% sucrose+ 00.53 µM NAA + 04.44 µM BAP.          | MS.  | Peatmoss:sand (1:1) 93% survival.   | (Hegazi 2016)         |
| Nodal segments with single axillary buds ( <i>ex vitro</i> )                                  | Multiple shoot formations; rooting.   | MS + IAA 00.5 mg L <sup>-1</sup> + BAP 1.0 mg L <sup>-1</sup>                 | MS+ 00.2 mg L <sup>-1</sup> IBA                                | –<br>70% survival in hardening.   | (Dixit & Thakur 2017) |

Table 1. Contd.

| Explant-source/Type  | Observation                         | Media (shoot induction/ multiplication)                                 | Rooting Media                              | Acclimatization                         | Reference              |
|--|-------------------------------------|---|--|---|------------------------|
| Nodal segments with axillary buds ( <i>ex vitro</i> )                | Multiple shoot formations; rooting. | Semi solid MS+ 01.5 mg L <sup>-1</sup> Kn + 02.5 mg L <sup>-1</sup> BAP | Half liquid MS+ IBA 1.0 mg L <sup>-1</sup> | Sand + Soil+ FYM (1:1:1). 100% survival | (Sharma et al. 2018)   |
| Leaf explants, nodal segments with axillary buds ( <i>ex vitro</i> ) | Multiple shoot formations; rooting. | MS  | Half MS + 0.5 mg L <sup>-1</sup> IAA       | Soil: vermiculite mixture (50:50)       | (Chaudhry et al. 2019) |

BAP- 6-Benzylaminopurine; FYM- Farm Yard Manure; GI- growth index; IBA- Indole-3-butyric acid; IAA- Indoleacetic acid; Kn- Kinetin; MS- Murashige and Skoog medium; NAA- 1-Naphthaleneacetic acid.

tial for producing secondary metabolites were examined by (Patra et al. 2018). Four main factors: size of the inoculum, NO<sub>3</sub><sup>-</sup>/NH<sub>4</sub><sup>+</sup> ratio, sucrose concentration, and KH<sub>2</sub>PO<sub>4</sub> concentration were studied in high yield producing variety of plants (bacoside-A of content up to 10.0 mg g<sup>-1</sup> dry weight in whole plant, obtained from CIMAP- Lucknow, India). Here, as some reports had suggested that one-fourth strength of MS medium was more preferable to half-strength MS media for both bacoside and biomass production, one-sixth strength MS media was optimised optimized and used in this case (Wu et al. 2006; Fadel et al. 2010; Patra & Srivastava 2016). It was found that biomass production was affected by sucrose, KH<sub>2</sub>PO<sub>4</sub> and inoculum concentration (Patra et al. 2018). The limited effect of sucrose and KH<sub>2</sub>PO<sub>4</sub> on bacoside and biomass was also reported by (Seth et al. 2020).

Biomass production was not affected by NO<sub>3</sub><sup>-</sup>/NH<sub>4</sub><sup>+</sup> ratio (Patra et al. 2018). The inoculum size had a direct effect on the production of secondary metabolites and its growth rate, as the larger the inoculum size, the higher the production of secondary metabolites and the slower the rate of growth. (Patra et al. 2018). As shown (Trejo-Tapia et al. 2003) in *Lavandula spica*, it also imparted a positive impact on enzymes that regulates metabolic path ways.

The buffering ability of the culture medium determines how much cell biomass can increase, which accounts for the positive effect of KH<sub>2</sub>PO<sub>4</sub> (Patra et al. 2018). The positive impact of sucrose, as a crucial factor in biomass development because of how it affects osmo-regularity of the culture, as cell signalling molecule and as a carbon source for biomass production (Weathers et al. 2004; Patra et al. 2018).

To analyse and for the further optimization of the media, response surface methodology (RSM) was applied. In accordance to dry weight, 3.65 g/L of maximum biomass was obtained in one-sixth strength of MS medium + 0.1 mg L<sup>-1</sup> BAP + 0.5 mg L<sup>-1</sup> NAA with 30 grams per litre sucrose, 1.24 mM KH<sub>2</sub>PO<sub>4</sub> and 2.0 g/L inoculum. One-sixth strength of MS medium + 0.1 mg L<sup>-1</sup> BAP + 0.5 mg L<sup>-1</sup> NAA with 41.92 gram per litre sucrose, 0.22 mM KH<sub>2</sub>PO<sub>4</sub> and 2.0 g/L inoculum predicted the highest bacoside yield of 0.49 mg g<sup>-1</sup> (Patra et al. 2018). It was also found that as KH<sub>2</sub>PO<sub>4</sub> was only suitable for high biomass production and not in production of bacoside-A, as bacoside-A being a secondary metabolite which observes a non-growth associated production kinetics (Patra et al. 2018).

### Upscaling Using Bioreactors

Different culture vessels have been used in the micropropagation of the *B. monnieri* plant, large scale cultivation of shoots is possible by using bioreactors. It is one of the best available methods for commercial use, in

the propagation and production of phytomedicines. This method of propagation has been known to increase the rate of multiplication of cultures and thereby making the overall process more costs and the energy becomes efficient. It also reduces labour which makes commercial production using bioreactors a more feasible approach (Bhanja et al. 2007; Khan et al. 2009; Koul et al. 2015). Among which, maximum growth index (GI) on the basis of dry weight was recorded in air lift bioreactor system (5.84), which is followed by Growtek bioreactor (4.22) (Sharma et al. 2015) then by magenta box, 100 ml conical, glass jars, 250 ml conical flask. Variations were observed in the results, in depending on the number of shoots per litre and the type of vessels used (Jain et al. 2012). In which air lift bioreactor system showed an increase in shoot number of ~48.33 to ~443.33, while Growtek bioreactor showed an increment from ~9.00 to ~42.67 (Sharma et al. 2015).

### Effect of Plant Growth Promoting Rhizobacteria (PGPRs)

Plant growth promoting rhizobacteria (PGPR) are rhizosphere bacteria helping in the promotion and enhancement of plant growth by the use of different mechanisms like biological nitrogen fixation, phosphate solubilization, phytohormone production, rhizosphere engineering, antifungal activity etc. (Bhattacharyya & Jha 2012). PGPRs can also be inducted into various stages of plant tissue culture such as in hardening and rooting, to increase the overall health, survivability and productivity of the plant (Ahmad & Kibret 2014). When halotolerant species of PGPRs were utilised to observe the effects of salt stress in *B. monnieri* plant, it was found that the rhizobacteria *E. oxidotolerans* (GenBank accession no: JQ804988) produced a higher plant yield and greater bacoside-A content than that of non-inoculated plants (Bharti et al. 2012). Here, primary and secondary salinity stresses were established. The primary salinity was achieved by mixing 4 g of sodium chloride per kg of soil with sterilized field soil, followed by irrigation using non-saline sterilised water. Secondary salinity level was achieved by the by irrigating pots with saline solution. The desired concentration of 4 g of sodium chloride kg<sup>-1</sup> soil was achieved by the gradual incrementation of sodium chloride concentration every seven days at a gradient of 50 mM, to avoid osmotic shock (Kohler et al. 2010). A 50% increase in fresh weight was reported in inoculated plants in non-saline (control) conditions in comparison to non-inoculated plants. Plants inoculated with *E. oxidotolerans* when exposed to primary and secondary salinity, gave 109 and 138% better herb yield respectively than non-inoculated plants. Non-salinized *E. oxidotolerans* inoculated plants showed a 36% increase in bacoside-A content. Plants inoculated with *E. oxidotolerans* produced 44 and 76 % more bacoside-A content in salinized plants under primary and secondary salt stress, respectively, while non-inoculated plants had a reduction of 33-50% in bacoside-A content (Bharti et al. 2012).

An increase of 1.5% in bacoside-A production was also reported in bacopa plants treated with chitinolytic microbes namely, *Streptomyces* sp. MTN14 and *Chitinophilus* sp. MTN22 alone or in combination. In plants, that were treated with the microbial combination, its bacoside biosynthetic pathway genes were upregulated, and helped in the plant's defensive mechanism by enhancing chlorophyll-a, and defensive enzymes and phenolic compounds like cinnamic acid, ferulic acid, gallic acid and syringic acid; against the pathogen *Meloidogyne incognita* (Gupta et al. 2017).

PGPR inoculation can have strain-specific impacts on the secondary metabolites production in plants as shown by (Walker et al. 2011) which implies the existence of a fine-tuned interaction mechanism. And

as reported by (Bharti et al. 2012), that PGPR inoculated plants, both under stressed condition (salinity stress) as well as under non-stressed condition (non-saline condition) exhibited a higher level of bacoside-A content, which could mean their potential role in secondary metabolite pathway. The increased bacoside content could also be because of the improved leaf-stem ratio of the inoculated plants (Phrompittayarat et al. 2011).

### Effect of Abiotic Stress on *B. monnieri*

The effect of drought (mannitol) and salinity (NaCl) was studied on *B. monnieri* plant, it was found that the growth rate was decreased in cultures under both kind of stress. Also, elevated amount of proline content was found in mannitol induced osmotic stress and in salinity stress. Whereas protein content increase was reported in lower concentration of NaCl and mannitol stress, and a reduction of protein content in higher concentrations of the same (Debnath 2008; Dogan 2020).

According to another study conducted by (Dogan 2020), salt stress also affected callus formation and its density. With increase in salt concentration, callus density decreased and loss in callus colour (browning and yellowing) was also reported. Even though low levels of salt stress did not have much of an effect on *in-vitro* culture, high salinity has had an impact on many physiological and biochemical parameters due to decreased chlorophyll content in shoots from applying salt stress, which lead to conditions like chlorosis, lipid peroxidation, and protein degradation (Ashraf & Bhatti 2000; Dogan 2020). Lipid peroxidation can be caused by the reactive oxygen species found in the cell membrane, which can affect the cell permeability, composition, and structure as well as membrane integrity of the plant (Bose et al. 2014). Chlorosis or reduced chlorophyll content in plants under salt stress is caused by membrane degradation due to lipid peroxidation as well as increased chlorophyllase enzyme activity, which degrades chlorophylls, as well as a decrease in chlorophyll production (Ashraf & Bhatti 2000; Santos 2004). The increase in proline content was thought to be causes of more proline productions or because stress conditions decreased the incorporation of proline into other macro molecules like protein. The increased activity of Pyrroline 5-carboxylate reductase (P5CR), proline oxidase, and ornithine amino transferase (OAT) could also give high proline content (Debnath 2008).

Secondary metabolites production increased gradually when treated with Cd up to 10.0  $\mu\text{M}$  which then showed a decrease at higher concentrations of 50.0  $\mu\text{M}$  and 100.0  $\mu\text{M}$ . It indicates that the abiotic stress, which in this case the Cd treatment, increased the secondary metabolite production to a certain limit and then decreases due to Cd at higher concentrations. Increase in bioactive compounds like bacoside A and toxicity bacoside I in all Cd treated plants were found by the use of TLC fingerprinting (Gupta et al. 2014).

The effect of abiotic stress on bacoside production was studied using abiotic elicitors like salicylic acid (SA), jasmonic acid (JA) and copper sulphate ( $\text{CuSO}_4$ ), which were used in different concentrations, it was found that a maximum concentration in bacoside of 08.73  $\text{mg g}^{-1}$  dry weight was given by  $\text{CuSO}_4$  (45  $\text{mg L}^{-1}$ ) in an elicitation period of 6 and 9 days. Both JA and SA produced higher concentration of bacoside than that of control but less than  $\text{CuSO}_4$  induced cultures. JA (1  $\text{mg L}^{-1}$ ) gave a bacoside yield of 08.46  $\text{mg g}^{-1}$  DW and SA (50  $\mu\text{M}$ ) gave 08.14  $\text{mg g}^{-1}$  DW of bacoside yield (Sharma et al. 2014).

Seasonal variation i.e., the influence of temperature on wild varieties of *B. monnieri* plant on bacoside production was estimated by collect-

ed samples from different regions (in India) and growing them in a uniform environment for one year. It was found that even though there were variations due to genotypic differences, the greatest amount of bacoside-A content (6.82 mg/plant) was documented during the summer, when the mean temperatures reached 40.0° C and the lowest values (0.34 mg/plant) during winter which recorded a mean temperature lower than 5.0° C (Bansal et al. 2016).

## CONCLUSION

Throughout the years, there has been a significant amount of research done on various parameters of micropropagation of *Bacopa monnieri*. Cultures were mostly set up by extracting explants from *ex vitro* plants rather than from *in vitro* ones. Nodal explants were used more extensively than leaf explants for *in vitro* propagation.

The use of bioreactors as a culture vessel have aided in acquiring increased biomass and bacoside production. Several key factors like size of the inoculum, sucrose concentration,  $\text{NO}_3^-/\text{NH}_4^+$  ratio,  $\text{KH}_2\text{PO}_4$  concentration, PGRs etc. play a significant role in biomass and bacoside production directly or indirectly. Plant growth promoting rhizobacteria's (PGPR) like *E. oxidotolerans*, incorporated plants were able to successfully produce higher results in herb yield and in bacoside production compared to non-inoculated plants. Temperature, salinity, and other abiotic elicitors like Cd, JA,  $\text{CuSO}_4$ , SA were also found to have an impact on bacoside production in *in vitro* cultures. Even though some crucial factors and parameters have been identified, further studies are still required so as to incorporate the current research parameters to find optimum requirements to enable the mass production of *Bacopa monnieri* as well as its important secondary metabolites.

## AUTHOR CONTRIBUTION

A.V. has written the manuscript. S.S. has done the review, editing and proof-reading of the manuscript. V.S. has conceptualized and supervised the study.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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