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IN VITRO PRODUCTION OF BACOSIDES IN TISSUE CULTURES OF BACOPAMONNERI

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ABSTRACT

Bacopa monnieri is a perennial, creeping herb whose habitat includes wetlands and muddy shores. Quite frequent in mashes throughout India ascending to 1600m. A small, prostrate and fleshy herb. The leaves are sessile, soft, succulent, Reni form, obovate-oblong or spatulate, up to 2.5mm long, with obscure venation. The lower surface is punctuate and entire. The stem is 10-30cm long and 1-2mm thick, with soft ascending branches. Flowers blue or white with purple views, axillary and solitary on peduncles usually` longer than leaves, with linear bracteoles. Fruits ovoid capsule included in the persistent calyx. Leaf venation:-Both the epidermises with straight anticlinal walls are provided with thin cuticle sunken spherical multicellular sessile, glandular trichomes filled with yellowish green content, surrounded by the radiating row of cells. Stomata are annocytic type present on both surface of the epidermis, numerous in the lower surface. Stem provided with epidermis and cuticle. Ground tissue is parenchymatous. Xylem is in the form of a closed cylinder. Saponins and triterpenes- Dammaranes such as the bacosides and bacosaponins based on the bacogenins A1 – A5 are most important constituents. These include bacosides A, B, and C with bacoside 'A' constituting about 2.5 – 3%. Bacoside A produceebelin lactone on acid hydrolysis and yields jujubogenin on degredation. Bacopasaponin D, the Bacopasaponins E and F, Hersaponins and monnierin have also been reported, together with the triterpenes, betulic acid, bacosines B sitosterol, stigmastonol and

stigmasterol. Alkolids :Brahmine and herpestin are present in the aerial parts. Flavonoids such as glucuronyl -7-Epigenin and glucuronyl -7- luteolin are present along with luteolin -7-glucoside and luteolin. According to Ayurveda, it is bitter, pungent, heating, emetic, laxative, and useful in bad ulcers, tumors, enlargement of spleen, indigestion, leprosy, anemia etc. According to Unani system of medicine, it is bitter, good in scabies, leucoderma, syphilis etc. It is promising blood purifier and useful in diarrhea and fevers. Bacopa appears to strengthen memory and improve concentration by enhancing the conductive of nerve tissue. It is commonly found Indian herb commonly given to infants to boost memory power intelligence and mental health. It is said that the use of Bacopa for memory enhancement goes back years or more in India, when it was cited for its medicinal properties, especially the memory – enhancing capacity. Studies show that bacope has strong antioxidant properties, protects mental function in those with epilepsy who take the drug phenytoin, while a study on rate showed Bacopa administration improves learning skills. The explants of Bacopa monnieri cultured in M.S media with different PGR induced the shoots and callus formation. The shoots tips responded more towards the shoots formation and the nodes responded more towards the callus information. Among different explant used shoots tips showed maximum response towards the growth as compared to nodes and inter nodes. Different series showed different growth response for e.g. GB1, GB2, GB4 and GB7 induced the callus formation, while as GB2 induced the callus as well as shoot information and GB3, GB6 induced the shoot regeneration. The callus morphology, shoot formation and root formation was influenced by the type of PGR and concentration in the media. Here we observed that BAP in combination with NAA respectively did not evoked any shoot or root formation. Its influence on callus morphology was quite clear from observation. In the initial stages after sub culturing, the MS+BAP+IAA, MS+BAP+IBA, MS+NAA+Kinetin, MS+IAA+Kinetin and MS+IBA+Kinetin showed very less response towards the root formation but showed very good response in the latter state.

KEYWORDS: MS, PGR, KN, NAA, IAA, IBA, 2, 4-D, Bacopamonneri.

INTRODUCTION

The culture of plant cells and plants tissues in a synthesis culture medium under controlled aseptic conditions is known as tissue culture. It is also called *in vitro* culture. The culture medium provides all minerals and growth hormones necessary for the growing cells. The controlled conditions give the culture a suitable micro environment for the cell growth.

During tissue culture, cells of small segments of plant tissue undergo repeated divisions to form masses of cells called calla (sing callus). Each callus undergoes differentiated into shoots and roots to forms a plantlet. All plantlets regenerated from a plant material are identical and similar in the metabolic activities. Hence they are known as clones. The method of raising the clones is called the micro propagation or *in vitro* propagation. After proper acclimatization, the plantlets are used for planting. The beginning of plants cell and tissue culture was made as early as 1898, when a German botanist G. Haberlandt successfully cultured fully differentiated individual plant cells, isolated from different tissues in several plant species. For about thirty five years (i.e. up to 1934), little further progress in cell culture research was made although culture of embryos, roots and other tissues was successful achieved. During 1934-1939 due to discovery of the importance of axons and B-vitamins, the foundation of plant tissues culture was laid down by three scientists (Gathered, white and forecourt), even though only a small pieces of tissues and not individual differentiated cells could be grown in cultures. During the next twenty years of variety of chemicals (Hormones & Vitamins etc.) were identified for their effect on cell division, growth and differentiation. Thus by earth 1960's the methods of invitro culture of plant cells, tissues and organs were reasonably well developed. Plant tissue culture has emerged as a major discipline in the experimental biology. Plant tissue culture begins with the concept of the cell theory given independently by schlieden 1838 and Schwann 1839 which implied, that the cell is a functional unit. It was only during 1960 that Morel reported plantlets formation in orchids, which became commercially viable program. The last decade has seen a very rapid rise in the number of plant scientists using the technique of organ, tissue and cell culture in plant physiological researchers. It is also increasingly becoming popular in basic studies in plant sciences.

Historical events in plant tissue culture: During the last two decades plants cell, tissue and organ culture have developed rapidly and becomes a major biotechnological tool in agriculture, horticulture, forestry and industry. Those problems which were not feasible through conventional techniques, now have been solved via these techniques, for example, inter and intra specific crosses, micro propagation, soma clonal variation, encapsulated seeds etc. Each living cell of a multi cellular organism is capable of independent development, when provide with suitable conditions (White 1963). Morgan coined the term 'totipotency' to denote this capacity of cell to develop into an organism by the regeneration. However the concept of totipotency is important in tissue culture. **Application of tissue cultures:**-The

invitro culture of plants cells or tissues in the artificial medium is said to be plant tissue culture. It has many applications in crop improvement, preservation, & breeding in industries. **Tissue culture is employed in**: - (1) Micropropagation (2) Elimination of pathogens from plant material (3) Germplasm storage (4) Production of soma clonal variations (5) Embryo rescue (6) Production of haploids (7) Production of artificial seeds (8) Production of secondary metabolites (9) Production of Somatic hybrids (10) Production of transgenic plants.

Scientific classification of Bacopamonneri

Kingdom	**Division**	**Class**	**Order**
Plantae	Magnoliophyta	Magnoliopsida	Lamiales
Family	**Genus**	**Species**	**Botanical name**
Plantaginacea	Bacopa	B. Monnieri	Bacopa monnieri

Habitat:-*Bacopa monnieri* is a perennial, creeping herb whose habitat includes wetlands and muddy shores. Quite frequent in mashes throughout India ascending to 1600m. **Botanical Description**:-A small, prostrate and fleshy herb. The leaves are sessile, soft, succulent, Reni form, obovate-oblong or spatulate, up to 2.5mm long, with obscure venation. The lower surface is punctuate and entire. The stem is 10-30 cm long and 1-2 mm thick, with soft ascending branches. Flowers blue or white with purple views, axillary and solitary on peduncles usually` longer than leaves, with linear bracteoles. Fruits ovoid capsule included in the persistent calyx. Leaf venation:-Both the epidermises with straight anticlinal walls are provided with thin cuticle sunken spherical multicellular sessile, glandular trichomes filled with yellowish green content, surrounded by the radiating row of cells. Stomata are annocytic type present on both surface of the epidermis, numerous in the lower surface. Stem provided with epidermis and cuticle. Ground tissue is parenchymatous. Xylem is in the form of a closed cylinder. **Parts used:** Dried whole plant mainly leaves and stems. **Major Chemical Constituents:-** Saponins and triterpenes- Dammaranes such as the bacosides and bacosaponins based on the bacogenins A1 – A5 are most important constituents. These include bacosides A, B, and C with bacoside 'A' constituting about 2.5 – 3%. Bacoside A produceebelin lactone on acid hydrolysis and yields jujubogenin on degredation. Bacopasaponin D, the Bacopasaponins E and F, Hersaponins and monnierin have also been reported, together with the triterpenes, betulic acid, bacosines B sitosterol, stigmastonol and stigmasterol. Alkolids :Brahmine and herpestin are present in the aerial parts. **Flavonoids:** -Flavonoids such as glucuronyl -7-Epigenin and glucuronyl -7- luteolin are present along with

luteolin -7- glucoside and luteolin. **Medical and Pharmacological activities :-** (1) Improve intellect, consciousness and mental activity (2) Calms the mind and promotes relaxation, increases protein synthesis and activity in brain cells (3) Improves memory, metal density and longevity (4) Decreases anxiety, restlessness and senility (5) Most commonly used to improve mental alertness and enhance learning and academic performance. According to Ayurveda, it is bitter, pungent, heating, emetic, laxative, and useful in bad ulcers, tumors, enlargement of spleen, indigestion, leprosy, anemia etc. According to Unani system of medicine, it is bitter, good in scabies, leucoderma, syphilis etc. It is promising blood purifier and useful in diarrhea and fevers. Bacopa appears to strengthen memory and improve concentration by enhancing the conductive of nerve tissue. It is commonly found Indian herb commonly given to infants to boost memory power intelligence and mental health. It is said that the use of Bacopa for memory enhancement goes back years or more in India, when it was cited for its medicinal properties, especially the memory – enhancing capacity. Studies show that bacope has strong antioxidant properties, protects mental function in those with epilepsy who take the drug phenytoin, while a study on rate showed Bacopa administration improves learning skills. **What is Bacopa**? Two Saponins, designated as bacopaside I and II, are found in Bacopa monnieri. Additional Photochemicals betulinic acid, wogonin and Oroxindin have been isolated from the aerial parts of Bacopa monnieri. Bacopa also has the flavonoids apigenin and luteolin. **Cultivation: -** Bacopa monnieri can grow in wide range of temperature (15 – 40°C) and soil PH (5 – 7.5). The plants grow best in the high moisture location which offer partial shade during the past opt the day. The fresh collected propagules of 10 – 15cm length with internodes and rootlets are the best planning material for its cultivation. 30 kg/hector of Nitrogen. 50 kgs of phosphorus, 40 kgs of potash and 20 kgs of zinc are required to be mixed with soil before planting in the plains of North India. 70 kgs of fresh weight of propagules is sufficient for the planting the nursery for one hectare plantation. The plant is preferably kept in undated in 4 -5 cm depth of water throughout or more frequent irrigation (2 – days internal) depending on the type of soil and availability of water. The crop can be harvested from 75 – 90 days after planting. As a pure crop fresh yield is 22.5 tons/hectare. Dry weight yield is app. 5.5 tons per hectare.

MATERIALS AND METHODS (General Requirements and methods):- Following items are requirement for tissue culture.

(1) Area for medium preparation: - A separate area is necessary for the preparation of the culture medium. It must be situated just away from the working room to avoid interference.

(2) A sterile room: - A sterile rom or sterile air cabinet or laminar flow hood is required for the laboratory. It is used for (A) Distribution of medium into flasks or plates (B) Aseptic transfer of medium and cultures (C) Sub- culturing. Sterile room or sterile air cabin:-It is a small chamber that has facilitates to create sterile environment. It should have following items. Laminar flow hoods: - Laminar flow hoods are sterile air cabinets designed in such a way that sterile air passes across the working area continuously. Laminar flow hoods in which air filters are kept in vertical position are called vertical laminar flow hoods. If air filters are placed in horizontal position. Then they are known as horizontal laminar flow hoods.

(3) Glass ware and other instruments: - (a) A large flask to hold a large amount of nutrient medium (b) Conical flasks for the distribution of medium and culture (c) Measuring cylinders, test tubes, pipettes of different contents and Petriplates. They are useful in preparation of the medium and culture of tissues (d) Scissors, forceps and scalpels for the preparation of explants and aseptic transfer of explants and callus (e) A PH meter for adjusting the PH of the medium (f) A spirit lamp for the aseptic transfer of explants and callus near its flame (g) A balance with appropriate weights to weigh nutrients for the preparation of media (h) An autoclave to sterilize all the glass wares scissors, forceps etc.

(4) A Constant temperature room: - A constant temperature room or a normal microbiological incubator is essential for the culture of callus and its maintenance. It provides a constant temperature of 25 c for cell growth. Further it supplies enough light to the growing plant cell or tissues.

(5) A shaker system: - A good shaker system is essential for maintaining individual cells in suspension culture. Due to the agitations given by the shaker system callus break into many small pieces or individual cells. It provides good aeration to the cells.

Tissue Culture Medium: - Plant cells and tissues require a proper nutrient medium for their growth and development. The medium must contain the following components,

Carbon Sources	Sucrose
Macronutrients	Nitrogen, Phosphorous, Potassium, Magnesium, Calcium, Sulphur.
Micronutrients	Iron, Manganese, Zinc, Boron, Copper, Molybdenum, and Chlorine.

Organic Supplements	Coconut milk, Tomato juice, Potato Extracts or Yeast Extracts.
Vitamins	Nicotinic-acid, Pyridoxine Hcl, Thiamine Hcl, Glycine
Hormones	IAA, NAA, 2, 4-D and Kinetin.

The PH between 5.5 and 5.8 is suitable for cell growth, so PH of the medium should be adjusted to 5.5 – 5.8 Different media are used for plant tissue culture. They are often named after the discoverers who formulated the media. MS medium was formulated by Murashige and Skoog in 1962. B5 medium was formulated by Gamborg et al in 1968. The S.H. medium was formulated by Schenk and Hilderbandt in 1972. The L.S medium was formulated by Linsmaier and skoog in 1965.

Composition of MS Medium: - M.S medium is widely used to culture plant tissues its composition is given below.

Stocks	Components	Stock Solution (gm/lit)	Amount (ml/lit)	Final concentration (mg/lit)
Major stock (I) (20X)	NH4NO3 KNO3 MgSO4 7H2O KH2PO4	33.0 38.0 7.40 3.40	50	1650.0 1900.0 370.0 170.0
Major stock (II) (20X)	CaCl2 2H2O	8.80	50	440.0
Major stock (III) (20X)	FeSO4.7H2O Na2EDTA	0.557 0.745	50	27.85 37.25
Minor stock (100X)	KI H3BO3 MnSO4 4H2O ZnSO4-7H2O Na2MoO42H2O CuSO4 5H2O CoCl2 6H2O	0.083 0.62 2.23 0.86 0.025 0.0025 0.0025	10	0.83 6.2 22.3 8.6 0.25 0.025 0.025
Vitamins (100X)	Nicotinic-acid Pyridoxine Hcl Thiamine Hcl Glycine	0.05 0.05 0.01 0.2	10	0.5 0.5 0.1 2.0

Plants Growth Regulators or Hormones

Organic Substances which in low concentration regulate Physiological activities of plants are called hormones. Hormones are produced in one site and transported to the site of action. Plants produce growth hormones such as axons, gibberellins, cytokinins and ethylene in small proportions. These hormones are mainly used in plants tissues culture. **Auxins: -** Auxins are natural growth hormones found in plants, which promote the shoots elongation in plants.

Auxin is a generic term for all compounds capable of inducing elongation of shoot tips. The free form of Auxins is indole-3-acetic acid. **Cytokinins**:-Cytokinins are degradation products of DNA. They are made up of an adenine and a furfural ring. This basic structure is known as 6-furfuryl amino-purine. Cytokinins stimulate cell division and enlargements of cells. The deficiency of cytokinins results in stunted growth in plants. Types of Cytokinins- (a) Zeatin (b) Kinetin (c) N-6 Isopentenyl Adenine (d) t-RNA Ribosyl Zeatin. **Gibberellic Acid: -** There are growth regulators found in plants. The deficiency of these acids causes marked symptoms like the shortening of internodes, stunted growth, production of seedless fruits etc. in plants. It was first isolated from the fungus Gibberella Juji Kuroi. Cleland (1969) defined Gibberellic acid as "a compound which is active in gibberellin bioassay and possessing gibbane ring skeleton." Each Gibberellic acid molecule consists of a gibbane ring. The Gibberellic acid differs among themselves by the nature of the distribution of different atoms or groups (R- groups) on the Gibban ring. 'R' may CH_3, OH or COOH group. **Ethylene: -** Ethylene is metabolic product released from some fungi. Curtis et al (1968) reported that 228 species of fungi produce ethylene when they are culture in nutrient media. Some important applications of PGRs are as- **(1) Auxins** :- (a) Horticulturists use IAA to induce rooting from cutting of vegetatively propagating plants (b) IAA is used to induce callus production form explant materials in tissue culture. **(2) Cytokinins: -** (a) Induce cell division in plant tissues growing in nutrient media (b) Used to break dormancy of some seeds. **(3) Gibberellic Acids: -** (a) Production of seedless fruits (b) Apical dominance in some crop can be reduced by applying Gibberellic Acid. **(4) Ethylene**: - Ethylene is used to induce ripening of harvested fruits.

Preparation of M.S Medium

The macronutrients are weighted correctly and dissolved in 200ml distilled water in a flask. Similarly micronutrients are weighted and dissolved in 200ml distilled water in another flask. The macronutrients solution and micronutrients solution are mixed together and stirred well. Sucrose is then added to the solution and stirred well. The volume of the solution made up to 950ml by adding distilled water. PH of the medium is adjusted to 5.7 using 0.1 moles NaoH normal Hcl. The final volume is made up to 1 liter by adding distilled water. The mouth of the vessel is plugged with non-absorbent cotton and vessel is covered with chess cloth. The vessel containing medium is autoclaved in an autoclaved at 121°C for 40 minutes. The medium is allowed to cool. The vitamins, amino acids and hormones are added to it. The resulting medium is used for tissue culture. If solid medium is required, the prepared medium

is distributed into flasks and agar is added to it. Then the medium is sterilized in an autoclave. The sterilized medium is distributed to culture flasks and allowed to cool at room temperature. As a result, semi-solid nutrient. Medium is formed in culture flasks.

Collection of Explants: An excised portion of plant body which is utilized for initiation of cultures is called explant. The following portions may be used as Explant materials for initiation of cultures- (1) Apical shoot tips (2) Stem (3) Leaf (4) Nucellus (5) Germinating grains (6) Embryo (7) Stamen. Explants material are taken from standing crops in green houses or from wild plants. They are cut just below the desired portion with a sharp knife and put in a screw cap bottle to bring them to the laboratory.

Surface Sterilization of explants: The process of killing microbial contaminants on the surface of explants material is called as surface sterilization. It is carried out by treating with some chemical agents called surface sterilants. E.g., ethyl, alcohol, silver nitrate and mercuric chloride. Explant materials are surface sterilized in the following way:- (a) The explants material is cleaned with running water (b) It is then transferred to a vial containing detergents like tween - 80 and the vial is shaken two or three times (c) Thereafter, it is kept dipped I n 70% ethyl alcohol for 5 minutes (d) It is again washed with distilled water to remove the ethanol (e) It is then submerged in 1% $HgCl_2$ solution for 10 minutes (f) After this plant material is washed well with distilled water (g) Now the explants material is free from microbial contaminants and growth inhabiting chemical.

Preparation of explants and inoculation: The sterile cabinet is properly sterilized by using ethanol and UV lamp. All glassware's are sterilized by autoclaving and brought to the cabinet. The sterilized medium is distributed to conical flasks near flame of a spirit lamp and mouths of the flasks are plugged with non-absorbent cotton. Theme dim in the flasks is allowed to solidify at room temperature so as to from semi-solid medium. The explants material is cut into small pieces of desirable size with the help of a sterile forceps and Knife. The suitable pieces of explants material from which calluses are cultured are called explants. The explants may be pieces of leaf, stem, basal plate meristem, root, endosperms or embryo. Mouth of the conical flask is open near flame of a spirit lamp, the explant is transferred to the semi-solid medium in the flasks by holding in forceps and the explant is pressed gently in the medium. The process of transferring explants to culture medium under aseptic conditions is called inoculation. In such a way 3-5 explants are inoculated in the culture flasks. The mouth of the flasks in then plugged with the cotton.

Incubation of culture flasks: The explant-inoculated flasks are kept in a constant temperature room or incubator for a considerable time. This process is called incubation. The temperature inside the incubator is adjusted to 25+2c which provide a constant temperature around 25c. a small illumination is given to the culture to ensure better growth of callus. Cells of the explants divide repeatedly and grow into a mass of parenchyma cells. The mass of cells which divide actively is called callus. Callus is irregular is shape due to irregular proliferation of explant cells. It appears as a soft and brittle mass of cells liable to breakdown by mechanical disturbances. Its cells are closely arranged without intercellular spaces. In most cases explants produce callus within 3-8 days of incubation.

Sub-Culture of Callus:-Callus growths till enough nutrients are available in the culture medium. When the nutrient level comes down the callus growth also decreases simultaneously. So the callus is cut into small pieces and each piece is transferred to a fresh medium. The maintenance of fragmented callus is fresh medium is called sub-culture.

Regeneration of plants from callus: - The rearing of callus into young plants looking like seedlings is called regeneration. The young plant has a distinct shoot system and few roots. The young plant is named plantlet. The plantlets are transferred to pots in a green-house for proper acclimatization and then planted in main fields (1) Organogenesis (2) Embryogenesis.

Organogenesis Method:-Organogenesis refers to development of adventive roots and shoots directly from the callus. The roots and shoots don't have common axis. They are physically separated by callus tissue. Organogenesis from callus is depending upon the concentration of axons and cytokinins in culture media. High proportion of axons (3mg/lit) and low proportion of cytokinins (0.02mg/lit) induce root development from callus. So the rooting medium formulated so as to contain more IAA or NAA and less Kinetin or BAP. Low proportion of auxins and high proportion of cytokinins (1-2mg/lit) in the medium induce shoot development from the callus. So the shoot regeneration medium contains less IAA or NAA and more kinetin or BAP. If the medium contains less cytokinins and no axons, neither root induction nor shoot induction form the callus takes place. For plant regeneration a callus is first grown in a shooting medium for 4-8 weeks. Many small meristems develop at the periphery of the callus. They are called meristemoids. Some meristemoids grow into shoots. After shooting, individual shoots are cut along with a portion of the callus and grown in the rooting medium. A few adventive roots develop from the shooted callus. As a result a plantlet is formed.

Embryogenesis Method: - Production of embryo like structure from callus is known as embryogenesis or somatic embryogeny. The embryo like structure is called embryoid or asexual embryo. Embryoids develop from a single periphery cell or masses of cells of the callus. The embryoid initial cell contains dense cytoplasm and a large nucleus. It divides repeatedly to from a globular mass of cell called globular embryo. The latter becomes heart shaped and then torpedo like. The torpedo-like embryoid is transferred to top of a filter paper bridge that is in contact with a liquid medium in a tube. The medium contains kinetin (0.2mg/lit), Sucrose and the usual nutrients. The embryoid develops into a plantlet within few weeks. Well-grown plantlets are then transferred to pots in green house. In some cases, single cells or explants as-such develop into Embryoids with callus phase. This method is often called direct somatic embryogenesis e.g. citrus etc.

Establishment of culture of Bacopa monnieri

Shoots tips, Nodes and some internodes collected from field grown plants of Bacopa monnieri were utilized as explants source material for the establishment of multiple shoot culture and subsequent plantlet regeneration there from: - (A) The explant material were surface sterilized which were carried out by treating the explant material with some surface sterilants e.g. Ethyl alcohol, silver nitrate and Mercuric Chloride (B) All the glassware and other instruments were sterilized by autoclaving and brought to the sterile cabinet where the inoculation is done and the MS media was prepared by using all the essential components needed for the growth. The MS media containing the following components was prepared:- (i) Sucrose, (ii) Macro and micronutrients, (iii) Vitamins, (iv) PGR, (v) Organic Supplements, (vi) Agar-Agar.

- The PH of the medium was adjusted to 5.5 – 5-8 before autoclaving.
- The inoculation was done in the inoculation room under aseptic condition.
- The explant material was transferred to the media near the flame of the spirit lamp by holding in the forceps and gently pressed in the medium.
- The mouth of the test tubes was then plugged with the non-absorbent cotton.
- The explant inoculated tubes were under constant light and temperature region for a period of 4 – 6 weeks.
- Observations were taking time by time and recorded.
- The cells of the explant material divide repeatedly and grew into callus and shoots.
- The callus and shoots were sub-cultured into the conical flasks.

- The sub-culturing was done by cutting the callus and shoots into small pieces and transferred to the fresh medium and provided with the same condition described above.

Extraction isolation and quantitative determination of bacosides

Extraction and isolation of bacosides:-The multiple shoot culture and in vitro regenerate from first and second generation were harvested and dried at 50 +2^{o}C. Overnight. Powdered plant material (10gm) was extracted in a soxhlet with methanol (150ml) for 4 hours. The extract was concentrated to 60ml under vacuo and made up to 90ml with water and successively extracted with n hexane (100ml) and n-butanol (50ml) Butanol extracts were dried under vacuum to obtain total bacosides contents. The procedure was repeated for in vitro raised regenerated plants growth in the green house and those in the fields.

Quantitative determination:- Bacosides were quantified by LC – MS carried out on Agilent – 1100 series HPLC system hyphenated to Iron trap Mass spectrometer (Esquire 3000, Bruker). HPLC analysis was conducted using Acetonitrile water (48:52) as the mobile phase with a flow rate of 0.4ml minute at wavelength of 210nm on a RP (Merck, 5m, 4 X 125mm column). The column temperature was maintained at 30°C for maximum peak efficiency. The analysis was carried out using the positive electro spray mode of ionization. The MS data obtained was in agreement with that reported in literature (Rastogi et al., 1994; Rastogi & Kulsherstra, 1998). Bacosides A3 and A2 eluted at retention times of 8.452 and 9.470 minutes respectively which exhibited molecular ion peaks at m/z951 [M+Na] and 921 [M + Na] in the positive mode. HPLC grade acetonitrile and methanol (Rankem make) purchased from Ranbaxy Chemicals Ltd. (Mohali, Punjab, India) were used without further purifications. The samples and standards were prepared in HPLC grade methanol and filtered through 0.45m Millipore filter. Stock solutions of the standard reference mixture were prepared and working solutions in the concentration range of 4.77 to 20.32gm were utilized for plotting of calibration curves for standard markers-bacosides A3 and A2. Concentrations of bacosides A3 and A2 were found to be in the ratio of 41:9 as determined by HPLC at 210m a purity of 0.99985 and 0.99782 respectively. On the basis of the standard curves obtained percentage of bacosides A2 and A3 were determined in samples of intact plant, shoot culture regenerated plants.

RESULTS AND OBSERVATION: Initiation and establishment of mother culture of Bacopa monnieri with different PGRs were using various explants. (Shoots tips, Nodes and Internodes).

1st observation (Nature of response) (Date of inoculation-11/07/15 Date of observation-24/07/15):

S. No	Media Code	Media + PGR	Number of Explants	Response of Explants	% Response	Morphogenetic Response
1.	GB_1	MS=BAP+NAA	10	7	70%	Explants turn into callus.
2.	GB_2	MS+BAP+IAA	10	5	50%	Explants turns into callus and shoots
3.	GB_3	MS+BAP+IBA	10	6	60%	Regeneration of shoots.
4.	GB_4	MS+BAP+2,4-D	10	5	55.5%	Explants turn into callus.
5.	GB_5	MS+NAA+Kinetin	10	5	50%	Explants turn into callus.
6.	GB_6	MS+IAA+Kinetin	10	7	70%	Explants turn into callus.
7.	GB_7	MS+IBA+Kinetin	10	5	50%	Explants turn into callus.
8.	GB_8	MS+2,4-D+Kinetin	10	4	40%	Explants turns into callus and shoots.

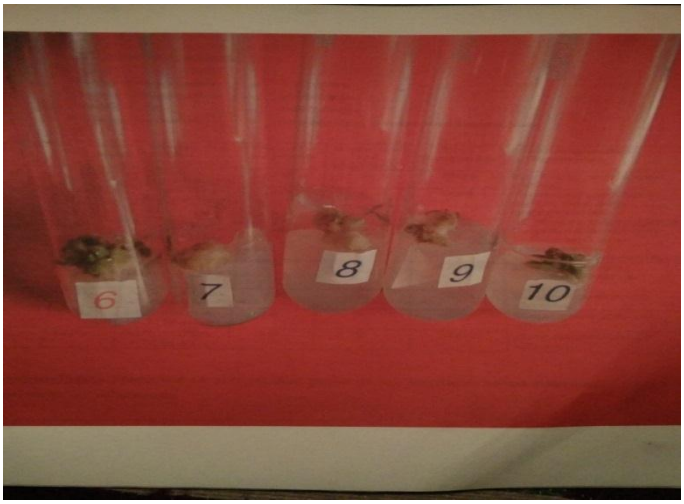
Observation: - Here we observed that most explants more towards the callus formation.

2nd observation (Date of inoculation - 11/07/15**,** Date of observation - 31/07/15**):**

S. No	Media Code	Media + PGR	Number of Explants	Response of Explants	% Response	Morphogenetic Response
1.	GB_1	MS+BAP+NAA	10	10	100%	Explants turn into callus.
2.	GB_2	MS+BAP+IAA	10	5	50%	Explants turns into callus and shoots
3.	GB_3	MS+BAP+IBA	10	7	70%	Regeneration of shoots takes place.

4.	GB_4	MS+BAP+2,4-D	10	5	55.5%	Explants turn into callus.
5.	GB_5	MS+NAA+Kinetin	10	7	70%	Explants turn into callus.
6.	GB_6	MS+IAA+Kinetin	10	7	70%	Explants turns into callus and shoots.
7.	GB_7	MS+IBA+Kinetin	10	7	70%	Explants turn into callus.
8.	GB_8	MS+2,4-D+Kinetin	10	6	60%	Regeneration of shoots takes place.

Observation: - Out of different explants used, shoots tips showed maximum response towards the growth compared to nodes internodes.



3rd observation (Date of inoculation - 11/07/15, Date of observation - 29/07/15): -

S. No	Media Code	Media + PGR	Number of Explants	Response of Explants	% Response	Morphogenetic Response
1.	GB_1	MS+BAP+NAA	10	5	50%	Explants turn into callus.
2.	GB_2	MS+BAP+IAA	10	6	60%	Explants turn into callus and also regeneration of shoots takes place.
3.	GB_3	MS+BAP+IBA	10	6	60%	Explants turn into callus and also regeneration of shoots takes place.
4.	GB_4	MS+BAP+2,4-D	10	4	40%	Explants turn into callus.

5.	GB_5	MS+NAA+Kinetin	10	5	50%	Regeneration of shoots
6.	GB_6	MS+IAA+Kinetin	10	4	40%	Regeneration of shoots
7.	GB_7	MS+IBA+Kinetin	10	5	50%	Regeneration of shoots

Observation: - Here we observed that most of the explants used showed regeneration of shoots.

Sub culture- Observation-I (Date of Inoculation - 01-09-15 Date of observation -20-09-15):-

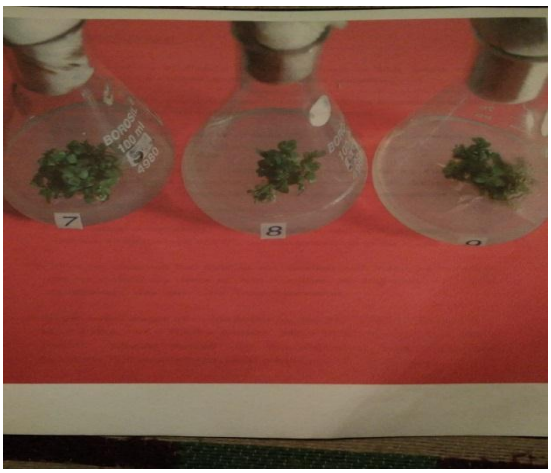
S. No	Media	Media +PGR	Level of Response	Callus Formation (type)	No. of shoots & Length	No. of roots and length
1.	GB_1	M.S+BAP+NAA	Moderate	Hard compact	No- response	No- response
2.	GB_1	M.S+BAP+NAA	Moderate	Hard compact	No- response	No- response
3.	GB_1	M.S+BAP+NAA	Moderate	Hard compact	No- response	No- response
4.	GB_1	M.S+BAP+NAA	Moderate	Friable	No- response	No- response
5.	GB_1	M.S+BAP+NAA	Very good	Semi Friable	No- response	No- response
6.	GB_2	M.S+BAP+IAA	Very good	No response	8 - 10 (0.5 -1cm)	5-6 (0.2 -0.5 cm)
7.	GB_2	M.S+BAP+IAA	Very good	Yellowish Green	10-15 (1-2 cm)	No- response
8.	GB_2	M.S+BAP+IAA	Very good	Green	5-8 (1-2 cm)	No- response
9.	GB_2	M.S+BAP+IAA	Very good	No response	6-9 (0.5 -1 cm)	No- response
10.	GB_2	M.S+BAP+IAA	Good	No response	5 -7 (0.5- 1cm)	2-5 (0.2-0.8cm)
11.	GB_2	M.S+BAP+IAA	Moderate	Friable	2 -4 (0.1-0.3cm)	No- response
12.	GB_2	M.S+BAP+IAA	Good	Yellowish	No- response	No- response
13.	GB_2	M.S+BAP+IAA	Moderate	Brownish	3-5 (0.2-0.5cm)	No- response
14.	GB_2	M.S+BAP+IAA	Moderate	Hard compact	No- response	No- response
15.	GB_2	M.S+BAP+IAA	Good	Friable	5-8 (0.2-0.5 cm)	No- response
16.	GB_3	M.S+BAP+IBA	Moderate	Friable	4-6(0.5-1cm)	2-5 (0.2-0.4cm)
17.	GB_3	M.S+BAP+IBA	Moderate	Hard compact	No- response	No- response
18.	GB_3	M.S+BAP+IBA	Moderate	Hard compact	4-7 (0.2-0. 4cm)	No- response
19.	GB_3	M.S+BAP+IBA	Low	Hard compact	No- response	No- response
20.	GB_3	M.S+BAP+IBA	Good	Hard compact	5-8 (0.5-1cm)	No- response
21.	GB_3	M.S+BAP+IBA	Very good	Hard compact	8-10	2-5

					(1-2cm)	(0.2-0.5)
22.	GB_3	M.S+BAP+IBA	Good	Hard compact	5-6 (0.3-0.5cm)	No- response
23.	GB_5	M.S+NAA+ Kinetin	Moderate	Brownish	No- response	No- response
24.	GB_5	M.S+NAA+ Kinetin	Very good	Yellowish	2-5 (0.2-0.4cm)	No- response
25.	GB_5	M.S+NAA+ Kinetin	Good	Brownish	1-3 (0.1-0.3cm)	No- response
26.	GB_5	M.S+NAA+ Kinetin	Moderate	Friable Brownish	No- response	No- response
27.	GB_6	M.S+IAA+ Kinetin	Very good	Yellowish green	5-8 (1-2cm)	5-9 (0.2-0.5cm)
28.	GB_6	M.S+NAA+ Kinetin	Very good	Hard compact	20-25 (2-3cm)	5-10 (0.2-0.5cm)
29.	GB_6	M.S+IAA+ Kinetin	Good	Yellowish	5-6 91-2cm)	No- response
30.	GB_6	M.S+NAA+ Kinetin	Very good	Hard compact	10-15 (1-2cm)	2-5 (0.2-0.4cm)
31.	GB_6	M.S+NAA+ Kinetin	Good	Hard compact	5-8 (0.2-1cm)	1-2 (0.1-0.3cm)
32.	GB_7	M.S+IBA+ Kinetin	Good	Hard compact	5-8 (0.2-0.5cm)	No- response
33.	GB_7	M.S+IBA+ Kinetin	Good	Yellowish brown	8-12 (0.5-1cm)	No- response
34.	GB_7	M.S+IBA+ Kinetin	Good	Hard compact	5-10 (0.5-1cm)	No- response
35.	GB_7	M.S+IBA+ Kinetin	Very good	Yellowish green	20-25 (0.5-1cm)	No- response
36.	GB_7	M.S+IBA+ Kinetin	Very good	Hard compact	10-15 (0.5-1cm)	No- response

Sub culture- Observation-II (Date of Inoculation - 01-09-15 Date of observation 09-10-15):-

S. No	Media	Media +PGR	Level of Response	Callus Formation (type)	No. of shoots & Length	No. of roots and length
1.	GB_1	M.S+BAP+NAA	Moderate	Hard compact Yellowish white	No- response	No- response
2.	GB_1	M.S+BAP+NAA	Moderate	Friable	No- response	No- response
3.	GB_1	M.S+BAP+NAA	Moderate	Hard compact yellowish white	No- response	No- response
4.	GB_1	M.S+BAP+NAA	Moderate	Friable Yellowish brown	No- response	No- response
5.	GB_1	M.S+BAP+NAA	Moderate	Semi Friable	No- response	No- response
6.	GB_2	M.S+BAP+IAA	Very good	No response	15-20 (1-2cm)	5-10 (0.3-0.6 cm)
7.	GB_2	M.S+BAP+IAA	Very good	Hard compact Yellowish brown	15-20 (2-3cm)	2-3 (0.1-0.3cm)
8.	GB_2	M.S+BAP+IAA	Very good	Yellowish brown	8-12 (1-3cm)	2-3 (0.3-0.5cm)
9.	GB_2	M.S+BAP+IAA	Very good	Hard compact	10-15 (1-3cm)	No- response
10.	GB_2	M.S+BAP+IAA	Very good	Yellowish	8-12(1-2cm)	5-7 (0.3-0.5 cm)
11.	GB_2	M.S+BAP+IAA	Moderate	Friable	5-7 0.3-0.5cm)	No- response
12.	GB_2	M.S+BAP+IAA	Very good	Friable Yellowish brown	5-8 (0.1-0.3cm)	5-7 (0.1-0.3cm)
13.	GB_2	M.S+BAP+IAA	Moderate	Brownish	3-6 (0.2-0.5cm)	No- response
14.	GB_2	M.S+BAP+IAA	Moderate	Friable yellowish brown	8-12 (0.3-0.6cm)	No- response
15.	GB_2	M.S+BAP+IAA	Good	Friable	5-8 (0.5-1cm)	No- response
16.	GB_3	M.S+BAP+IBA	Good	Friable brownish	5-9 (0.7-1cm)	3-7 (0.3-0.6cm)
17.	GB_3	M.S+BAP+IBA	Good	Hard compact yellowish brown	2-3(0.1-0.3cm)	1-2 (0.1-0.2cm)
18.	GB_3	M.S+BAP+IBA	Good	Hard compact brownish	5-7 0.3-0.6cm)	1-2 (0.1-0.3cm)
19.	GB_3	M.S+BAP+IBA	Low	Hard compact brownish	No- response	No- response

20.	GB_3	M.S+BAP+IBA	Very good	Semi friable callus	10-15 (1-1.5cm)	2-3 (0.1-0.3cm)
21.	GB_3	M.S+BAP+IBA	Very good	Semi friable callus	10-14 (2-3cm)	5-6 (0.3-0.6cm)
22.	GB_3	M.S+BAP+IBA	Very good	Semi friable yellowish	10-14 (1-1.5cm)	2-3 (0.1-0.3cm)
23.	GB_5	M.S+NAA+ Kinetin	Good	Brownish	3-5 (0.3-0.7cm)	5-9 (0.5-1cm)
24.	GB_5	M.S+NAA+ Kinetin	Very good	Friable Yellowish	5-8 (0.3-0.6cm)	5-9 (0.5-1cm)
25.	GB_5	M.S+NAA+ Kinetin	Very good	Hard compact Brownish	4-5 (0.2-0.4cm)	2-3 (0.1-0.3cm)
26.	GB_5	M.S+NAA+ Kinetin	Very good	Friable yellowish brown	5-7(0.2-0.5cm)	7-8 (1-1.5cm)
27.	GB_6	M.S+IAA+ Kinetin	Very good	Hard compact Yellowish	8-12 (1-3cm)	8-12 (0.5-1cm)
28.	GB_6	M.S+IAA+ Kinetin	Very good	Semi friable	30-35 (3-4cm)	10-12 (0.5-1cm)
29.	GB_6	M.S+IAA+ Kinetin	Very good	Hard compact yellowish	8-15 (2-3cm)	2-3 (0.1-0.3cm)
30.	GB_6	M.S+IAA+ Kinetin	Very good	Hard compact	15-20 (2-3cm)	5-8 (0.3-0.5cm)
31.	GB_6	M.S+IAA+ Kinetin	Very good	Semi friable	10-15 (1-2cm)	2-3 (0.1-0.3cm)
32.	GB_7	M.S+IBA+ Kinetin	Very good	Hard compact yellowish	10-12 (0.5-1cm)	1-2 (0.1-0.3cm)
33.	GB_7	M.S+IBA+ Kinetin	Very good	Yellowish brown	10-15 (1-1.5cm)	2-4 (0.1-0.3cm)
34.	GB_7	M.S+IBA+ Kinetin	Very good	Hard compact	8-12 (0.6-1cm)	No- response
35.	GB_7	M.S+IBA+ Kinetin	Very good	Yellowish green	25-30 (1-2cm)	2-3 (0.1-0.3cm)
36.	GB_7	M.S+IBA+ Kinetin	Very good	Hard compact yellowish	15-20 (1-2cm)	5-6 (0.1-0.5cm)



CONCLUSION

- The explants of Bacopa monnieri cultured in M.S media with different PGR induced the shoots and callus formation.
- The shoots tips responded more towards the shoots formation and the nodes responded more towards the callus information.
- Among different explant used shoots tips showed maximum response towards the growth as compared to nodes and inter nodes.
- Different series showed different growth response for e.g. GB1, GB2, GB4 and GB7 induced the callus formation, while as GB2 induced the callus as well as shoot information and GB3, GB6 induced the shoot regeneration.
- The callus morphology, shoot formation and root formation was influenced by the type of PGR and concentration in the media.
- Here we observed that BAP in combination with NAA respectively did not evoked any shoot or root formation. Its influence on callus morphology was quite clear from observation.
- In the initial stages after sub culturing, the MS+BAP+IAA+ MS+BAP+IBA, MS+NAA+Kinetin, MS+IAA+Kinetin and MS+IBA + Kinetin showed very less response towards the root formation but showed very good response in the latter state.
- Among the different combination, MS+ IAA + Kinetin, and MS+ IBA + Kinetin showed the maximum growth response towards the callus, shoots and root formation.
- It is clear from the data that the sub-cultured material transferred to various combination of PGRs resumed growth in all tried combination except in MS + BAP + NAA Combination where it showed little response.
- The *in vitro* culture established retained the inherent capability to synthesize bacosides under tissue culture conditions. The identity of bacosides A3, A1 was conjoined on the basis of HPLC RT and MS data.

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