Biochemical changes during adventitious root formation in *Bacopa monnieri (L.)* Wettst.

Dissertation

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the thesis entitled "**Biochemical changes during adventitious root formation in *Bacopa monnieri* (L.) Wettst.** ", in partial fulfillment of the requirement for the award of the degree of Master's of Science, Department of Biotechnology, Thapar University, Patiala, India, is an authentic record of my own work during the period of January 2016 to July 2016 under the supervision of Dr. Anil Kumar, Associate Professor, Department of Biotechnology, Thapar University, Patiala, Punjab. The matter embodied in this thesis has not been submitted in any part or full to any other university or institute for the award of any degree in India or abroad.

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CERTIFICATE

This is to certify that the dissertation entitled "**Biochemical changes during adventitious root formation in *Bacopa monnieri* (L.) Wettst.**" being submitted by **Ms. Aanchal Goel** in partial fulfillment of requirements for the award of degree of **M.Sc. biotechnology** and being submitted to the Department of Biotechnology, Thapar University, Patiala, is a bonafied work carried out by her under our supervision. The work has reached the standard necessary for submission. The contents of this dissertation has not been submitted for the award of any other degree or diploma.

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Chapter 1: Introduction

The Adventitious rooting involves the change in cell developmental process for the formation of root primordial that originates from morphogenetic pathway taking place in the shoot cells. This change leads to *de novo* rooting and a series of metabolic changes during different phases (induction, initiation and emergence) of root primordium formation (Batish et al., 2008). The process of adventitious root formation comprise of redifferentiation in which pre-determined cells changes from their morphogenic identity to act as mother cells for formation of root primordia (Aeschbachger et al., 1994). Rooting is amongst the three main routes of organ regeneration (other ones being somatic embryogenesis and shoot formation). During the rooting process, cells have ability to get back to meristematic stage after undergoing an apparent reversal of differentiation. Therefore adventitious rooting is used for vegetative propagation that are either selected from natural population or are found in breeding programmes. Efficient rooting treatment results in high quality root system and higher shoot to root ratio. Efficient root systems also recognized by the performance of plant after plantation in soil (De Klerk et al., 1997).

Adventitious roots developed by certain plant stems are of great economic importance, for instance, redwood trees develop adventitious buds at their lower trunk and their small pieces are sold as redwood burls. On the other hand, adventitious root formation also has importance in some difficult to root plant species. As adventitious buds often originate when stem gets wounded and replaces the lost branches.

Types of roots - Adventitious can be classified into various types such as roots with indefinite shape (tuberous roots), roots occurring at stem base (fasiculated roots), near tips (nodulose roots), stem nodes (stilt roots), lateral stems (prop roots) and roots attaching themselves to a support (climbing roots).

Formation of adventitious roots involves 3 successive, interdependent phases, all with different requirements and features (Hartman et al., 1990) that are induction, initiation

and elongation. Induction phase is marked by several molecular and biochemical changes without any visible morphological change followed by initiation where cell division, organization of root primordia and formation of root meristems occurs and finally root primordia is formed in expression phase. Third phase also involves root emergence out of stem cuttings. (Ford et al., 2001; Sebestiani et al., 2002; Kevers et al., 1997). Apparently, no change can be seen during induction phase, so initiation and expression phases are often considered as root formation phases.

The process associated with phase change of rooting can be understood by two approaches, epigenetic perspective and genetic perspective (Hackett and Murray, 1996) :-
Epigenetic approach refers to comparison of tissues having similar genotype but different ontogenetic states.
Further, two patterns have been recognized for adventitious root formation in both herbaceous and woody plants (Hartman et al., 1990). Direct pattern refers to development of adventitious root primordia from cells present in close proximity to the vascular system so that they can get associated with xylem and phloem. However, exact location is greatly varied. In newly formed stems, roots often originate from parenchyma, somewhere between the vascular bundles. Indirect process refers to adventitious root formation by proliferation or multiplication of undifferentiated cells (from callus), which generally begins in parenchyma of epidermal cells (near vascular cambium). In this process, few cells in undifferentiated tissue become organized and adventitious root primordia is originated.
Usually direct pattern of development is found in herbaceous species and easy-to-root species of wood and indirect pattern is found difficult-to-root species.

Many biochemical and physiological changes occur during different phases of adventitious rooting. The mechanism of lignification in the cell wall, catalyzed by specific peroxidases also occur during rooting (Sato et al., 1993). Further, knowledge of morphological and biochemical changes related to induction of roots and its formation may help in improving rooting procedures, which has possibility to reduce losses of production, specifically towards its final steps. Hence, it would be beneficial to identify

or recognize reliable biochemical markers with respect to rooting, which can be commercially applicable for *in vitro* systems (Schwambach et al., 2008). Many internal and external factors influence adventitious root formation from the stem cuttings such as its genotype, types of cuttings, endogenous factors linked to cuttings, stock plants and time of collection. They act as signals which are capable of inducing a group of cells to redefine their fate (Li and Xue, 2010).

Furthermore, histological studies provides a platform to recognize the cells from which the rooting process initiates and therefore are target for auxin and many other factors that induce rooting. It helps to know if root primordia is already present or a site for formation of root primordium need to be created, to identify whether low rooting capacity is associated with anatomical features and to associate biochemical and physiological data with anatomical steps of rooting (Altamura, 1996).

In vitro studies on rooting are now widely carried out to understand the rooting mechanism and role of various biochemical factors such as antioxidant enzymes, plant growth hormones in rooting process of economically important plants (De Klerk et al., 1999).

Auxins

Auxins have been known to be involved in the process of adventitious root formation (Weismann et al., 1988; Haissig and Davis, 1994) and also in interdependent physiological steps of rooting (Gasper et al., 1997). Auxins induce rooting by breakdown of root apical dominance (Cline, 2000). In many cases high levels of IAA are related with the promotion of adventitious root formation (Liu and Reid, 1992). During induction, higher concentration of auxins are required but auxins becomes inhibitory in the later phase of root formation (De Klerk et al., 1999). Major sources of endogenous auxins are shoot apexes (De Klerk et al., 1999).

Presently, IBA is the most widely used auxin for root formation because of its non toxicity to most of the plants. Use of IBA is the most prominant and effective method to

enhance rooting of cultures (Hartman et al., 1990; Leaky, 2004). There is a positive co-relation between root formation and IBA as it has strong capacity for the promotion of root initiation, low toxicity and good stability when compared to IAA and NAA. It has been found that explants respond more to auxin application during stage of de differentiation of adventitious root formation and less responsive to later or earlier applications (De Klerk et al., 1995; Smith and Thorpe, 1975; De Klerk et al., 1999; Luckman and Menary, 2002).

Phenols

Phenolic compounds provide protection to auxins from decarboxylation so that after their application more auxin is available for root induction (Wilson and Van Staden, 1990). Certain phenolic compounds accounts for the rooting capacity of the plant, which suggests that root formation depends on inhibition of IAA decarboxylation. The number of hydroxyl groups and their position on aromatic ring decides the effectiveness of phenolics (Bandurski et al., 1995). Phenols also protect plant from oxidative stress (Jaleel et al., 2009), for example, in genetically modified tobacco, premature cell death was observes along with reduced levels of phenolics. This is because cells are protected by phenols from active oxygen species (Tamagnone et al., 1998).

Further, phenols (e.g. Ferulic acid) serve as an important adjuvants during initial steps of adventitious root formation which increases the competence of target tissues to root formation. Effect of phenols is controlled by biochemical endogenous level in plant cuttings. They are the main substrate for peroxidases and inhibits peroxidase catalysed auxin oxidation (Racchi et al., 2001). In presence of oxygen, polyphenol oxidases catalyses phenolic compound oxidation.

Peroxidases

Peroxidase enzymes (H_2O_2 oxidoreductase; EC 1.11.1.7) consists of heme group which plays a role in catalyzation of oxidation of many organic compounds. Peroxidases are

present in cell wall as well as in cytoplasm. It is useful in lignin polymerization (Christensen et al., 1998).

Oxidation of many hydrogen donors is catalyzed by peroxidases. Peroxidases activity has been observed in many biological activities of plants. In some plant species, it has been seen that peroxidases show a pattern of activity during root formation that is its activity is found to be minimum at induction phase and maximum during initiation phase (Moncousin et al., 1988; Gasper and Thorpe, 1977; Fett-Neto et al., 1992). Also, peroxidase activity maintains IAA catabolism. Changes in peroxidase activities have been identified as molecular marker for root formation and also increases rooting mechanism (Beffa et al., 1990; Mark et al., 2004).

Carbohydrates

Carbohydrates serves the purpose of providing energy during root formation. These are ‘building blocks’ for plant tissues. They are the main component for carbon metabolism (Acosta et al., 2009). Sugars have important regulatory effect on photosynthesis thus providing energy and essential carbon for formation of new tissue. It represses the transcription of photosynthetic genes. It also interacts with ethylene signalling and abscisic acid (Leon and Sheen, 2003). Sucrose or its monosaccharides glucose and fructose are used for cell differentiation and cell growth in root formation. In general, frequency of regeneration increases with increase in sugar concentration until an optimum level is achieved and reduces at its higher concentration. High ratio of C/N of cutting enhance rooting but do not exactly predict the frequency of rooting response.

Ascorbate peroxidase

Ascorbic acid is important for antioxidant system of plant. It carries out scavenging of hydrogen peroxide. Ascorbate peroxidase (APx) (EC 1.11.1.11) (family oxidoreductases) uses ascorbate as substate to carry out the reaction. APx has been observed to be a part of regulation of plant meristematic activity, cell division and cell elongation (De Gara et al.,

2000). High levels of APx activity is known to be involved in actively dividing cells. On other hand, its activity decreases during cellular de-differentiation. There is rapid increase in its activity during induction phase of rooting followed by decrease during initiation phase for a short period and again increased during emergence (Tyburski et al., 2006). APx activity was decreased in IBA treated tissues (Li et al., 2009).

Catalase

Catalase (EC 1.11.1.6) is an iron containing enzyme (Warburg, 1923). In almost all aerobic cells, catalases are present in peroxisomes. It catalyzes the break down of hydrogen peroxide to water and oxygen thus suppressing the toxicity of hydrogen peroxider.

Present study will focus on varous biochemical changes in plant after IBA application during rooting process in *Bacopa monnieri*. Further changes at morphological level will also be studied through histology.

Objectives

- induction of roots on basal MS and IBA supplemented medium in *Bacopa monneiri*.
- investigate biochemical changes during root formation on basal MS media and MS medium supplemented with IBA.

Chapter 2: Review of literature

Formation of adventitious roots is a multicellular process that reactivates the cell division in cells that are not directly involved in root meristemoids formation (Altamura, 1996). *In vivo* studies have been carried out to differentiate the successive phases of adventitious root formation and its regulation (Freidman et al., 1985; Jarvis, 1986; Erikson, 1974). Phases of formation of adventitious rooting is based on histology (Sircar and Chatterjee, 1973; Jasik and Klerk, 1997) and on physiology (Gasper et al., 1994; Erikson, 1974; Friedman et al., 1985; De klerk et al., 1999; Moncousin and Gasper, 1983; Kevers et al., 1997). The mechanism of rooting involves cytological processes for the estimation of physiological status of cells in the process of formation of root primordium (Molnar and Lacroix, 1972a,b). For example, this method have already been used to study development of adventitious roots in apple microcuttings (Zhou et al., 1992; Hicks, 1987).

A good quality root system and high frequency of rooted shoots can be yielded by optimal rooting treatment (De Klerk et al., 1997). Good quality root system consists of absence of callus, higher number of roots per shoot cutting, longer length of roots. Researchers have studied *in vitro* performance of rooting of cultivars, or clones of several genotypes with high rooting abilities (Baraldi et al., 1995; Collet et al., 1994; Jay-Allemand et al. 1995). The cutting position in the donor plant, effect of age of donor plant, have been studied in association with adventitious root formation (Wilson, 1999). Some authors have experimented a stimulus for root induction to a single genotype at particular developmental stage (Hausman, 1993; Berthon et al., 1989; Heloir et al., 1996; Garcia-Gomez et al., 1995). Role of external and internal factors in root initiation have been widely reported (Jasik and Klerk, 1997).

Polyamines and auxins are reported to have a play role in induction phase of rooting (Nag et al., 2001; Davis et al., 1988). It has been found that adventitious rooting can be induced with the help of auxins (Blakesley, 1994; Jarvis, 1986; Osterc et al., 2009; Ludwig-Muller, 2005). Light conditions have also been observed to effect auxin metabolism and also

tissue sensitivity, and further auxins play an important role for determination of rooting ability (Reid et al., 1999). High frequency of root induction is associated with high levels of endogenous auxins (Jay Allemand et al., 1995; Blazkova et al., 1997; Caboni et al., 1997). In contrast, Feito et al., (1996) noticed low rooting rate with high endogenous auxin level. In some plant species, rooting can be achieved by applying exogenous auxin (Henrique et al., 2006; Diaz sala et al., 1996; Hunt et al., 2011; Ballester et al., 2009). After the application of exogenous auxin for root induction, the level of endogenous auxin generally reaches the peak for sometime, hours or may be days which can increase rooting (Gatineue et al., 1997; Gasper et al., 1996).

There is an evidence that IBA is the most effective inducer of rooting process (Lyndon, 1990). IBA is found to be most useful for hardwood and softwood cuttings (Christov and Koleva, 1995; Polat et al., 2000; Yildiz, 2001; Koyuncu and senel, 2003; Sebestiani and Tognetti, 2004; Tworkoski and Takeda, 2007; Delargy and Wright, 1979). Some studies have shown that *Agrobaterium, Streptomyces, Alcaligenes, Bacillus* and *Pseudomonas* bacteria are capable of root induction in stem cuttings because they can produce IBA and further research have proved that cuttings inoculated with these bacteria, their rooting can be enhanced by application of exogenous IBA (Ercisli et al., 2003, Esitken et al., 2003; Falasca et al., 2000)

IAA controls different phases of differentiation and growth. Their free levels, esterification and enzymatic degradation is catalyzed by IAA oxidase (IAAO) (Schneider EA. F Wightman, 1974). In some cases, adventitious rooting is enhanced by high levels of IAA, and in other cases, development of roots have also occur by decrease in endogenous IAA level (Hausman, 1993). It has been observed that root initiation can occur without any change in level of IAA in rooting region (Nordstrom and Eliasson, 1991). Auxins being the main part in rooting, role of carbohydrates, light and nutrition cannot be neglected (Bennett et al., 2003; Kevers et al., 1997).

Carbohydrates are the key components of the plant that supply energy to the plant (Kozai, 1991). Once the adventitious rooting mechanism is initiated, it requires energy to sustain

cell division at root generation site (Ahkami et al., 2009, Okoro and Grace, 1976; Veierskov, 1988; Haissig, 1984). In tissue culture, sucrose is generally used as a carbohydrate source because it is only translocatable sugars in angiosperms (Zimmermann and Zeigler, 1975; Smith et al., 1995).

Sucrose is mostly used for *in vitro* evaluations due to high solubility in water. Sugar act as a signal molecules to influence metabolic activity, photosynthesis and respiration (Rolland et al., 2002). Haissig (1974) stated that sugar metabolism gets activated on application of exogenous auxin to the rooting region to release energy and for the provision of carbon for production of other compounds like proteins. Various carbon sources like glucose, sucrose, mannitol, fructose, maltose and sorbitol also influence rooting in microshoots (Correa et al., 2005). Thompson and Thorpe (1987) said that sucrose may not be necessarily an apt carbon source for plant regeneration and somatic embryogenesis induction, but it is an important molecule in rooting of cuttings. There are evidences that sucrose promotes the action of auxins on plants (Caboni et al., 1992; Anonymous, 2008). Carbohydrates type used along with auxin treatment is an important factor influencing rooting (Pawlicki and Welander, 1995; Uosukainen, 1992).

Since biochemical reactions continuously takes place in the living organism, they constantly produce reactive oxygen species (ROS) such as super oxide radicals, hydrogen peroxide and singlet oxygen (Inze, D and Van Montagu, 1995). The ROS produced gets utilized in metabolic processes like cell senescence, lignin formation and flowering (Mehlhorn et al., 1996) but their overproduction can harm cellular components like proteins, DNA and lipids (Pellinen, RI, Palva, 2002). The plants have their natural defense mechanism to control ROS, which can be enzymatic or non enzymatic. Enzymatic mechanism involves enzymes like ascorbate peroxidise (APx), peroxidase, catalase etc. Catalase in peroxisomes and APx in chloroplasts and cytosol are key enzymes for hydrogen peroxide scavenging (Willkens et al., 1995; Davletova et al., 2005)

Ascorbic acid is an important component of antioxidant system of plant known for reduction of hydrogen peroxide to water in presence of APx (a heme containing protein)

which uses ascorbate as electron donor (Noctor and Foyer, 1998; Shigeoka et al., 2002). It helps in growth of plant and its development (Arrigoni, 1994; Navas et al., 1994; Arrigoni et al., 1997; Potters et al., 2000). Ascorbic acid serves as a co-factor for enzymes that helps in the synthesis of ethylene, gibberellins and abscisic acid (John and Prescott, 1996; Arrigoni and De Tullio, 2000; Qin and Zeevaart, 1999). It plays role in plant cell elongation (Gonzales et al., 1994; Kato and Esaka, 1999) and is required for cell cycle progression in meristems (Liso et al., 1984; De Tullio et al., 1999). Kerk and Feldman, 1995 reported that ascorbic acid facilitates quiescent cells for division, after 48 hours of incubation. It was also found that ascorbic acid is present in less amount in dormant regions of root meristems which results in their low proliferation rate whereas it is rich in distal and proximal regions of meristems, resulting in their active proliferation.

Ascorbic acid is used as substrate by enzyme APx. Ascorbate peroxidase regulates meristematic activity (De Gara et al., 1996). This enzyme produces ascorbate free radical that facilitates cell cycle process and involved in dormancy regulation (De cabo et al., 1996; Hidalgo et al., 1989). High levels of ascorbic acid and APx activity are characteristic features of actively dividing cells whereas low APx activity has been observed during differentiation (De Gara et al., 1996; Pinto et al., 2000). APx also plays a role in cell division because of effects on hydrogen peroxide availability for other enzymes like peroxidases and also on ascorbate availability for prolyl-hydroxylase (De Tullio et al., 1999).

Several other studies have shown that root formation is significantly affected by changes in phenolic compounds, protein and sugar content (Kaur et al., 2002; Sivaci and Yalcim, 2007; Satish et al., 2008; Kevresan et al., 2007). It has been suggested that IAA-oxidase and peroxidase combined activity (as stimulators or inhibitors) can control levels of phenols (Gasper et al., 1992; Pal, 1990; De Klerk et al., 1999). Further it has been found that phenols would not directly help in rooting but is involved due to changes in IAA-oxidase activity (Gasper et al., 1992). Also, there is an inverse relation between peroxidases and phenolic compounds during process of rooting (Gasper et al., 1992;

Moncousin, 1986). Phenolic compounds also serve as the substrates for peroxidases enzymes. Peroxidase facilitated oxidation of auxins is prevented by phenolic compounds (Racchi et al., 2001). Moreover, stimulatory or inhibitory effect of exogenous phenols depends on its application during different stages of rooting and also on the phase through which the explant or microshoot is undergoing (Berthon et al., 1990, 1993).

Many researchers have noticed that peroxidase activity and IAA content or elongation growth are inversely related where the peroxidase activity effects IAA metabolism (Chanda and Singh, 1997; Jupe and Scott, 1989; Mato and Vieitez, 1986). Peroxidases are known to be involved in rooting where it occurs when the microshoots have reached a particular peak of activity (Gasper et al., 1992). They serve as the marker for initial two phases of rooting in plants (Rout et al., 1999; Yilaz et al., 2003; Syros et al., 2004; Kaveresen et al., 2007). Primary cell wall gets stiffened due to peroxidases (Fry, 1986). Cell wall lignifications, may occur during rooting, that is catalyzed by specific peroxidase (Sato et al., 1993). They also synthesize suberin (Chao et al., 2001).

Chapter 3: Material and Method

Plant material and glassware

Cultures of *Bacopa monnieri* accession number BM-1 maintained at Plant tissue culture laboratory at TIFAC-CORE, Thapar University were used as source of explants during experimentation. Cultures were multiplied using nodal segments on basal Murashige and Skoog medium (1962) containing 3% sucrose and 0.7% agar as solidifying agent and kept at temperature 25±1°C under 16h light and 8h dark photoperiod and light intensity of 42 µmol m^{-2} s^{-1} provided by cool white florescent lamp (Phillips). After 4-5 weeks, microshoots were used for experimentation. Nodal explants were cut and used for root initiation on basal MS medium and medium supplemented with 1µM IBA. The pH of the medium was adjusted to 5.8 and plant growth regulator (IBA) was added to media prior to autoclaving at 121°C for 15 min. All the experiments were conducted in 300 ml culture vessels (Kasablanka Ltd. Mumbai, India) each one containing 30 ml of medium. All the plant tissue culture grade chemicals were purchased from Himedia Laborataties Pvt. Ltd, Mumbai.

Effect of IBA on rooting

Effect of IBA on rooting was observed using nodal segments. Nodal segments were cultured on MS media supplemented with different concentrations of indole-3- butyric acid (IBA) (0µM , 1µM, 2.5µM, 5µM). The cultures were incubated under controlled conditions for 7-10 days till the first root promiordia was visible. Number of roots and their length was recorded.

In another experiment, pulse treatment of IBA 25 µM was given to nodal segments of *Bacopa monnieri* for 12 and 24 h. Solid and liquid basal MS media supplemented with 25µM IBA was prepared. Shoot cultures from previously multiplied plant were harvested to obtain nodal cuttings and cultured them on both type of media. Explants cultured on

solid media were maintained at suitable conditions and those cultured on liquid media were kept at shaker at 100 rpm at 25±1°C. After 12 and 24 hours, the explants were then transferred to basal MS media. Number of roots and their length were recorded after 7-10 days when root primordia was visible.

Protein estimation (Lowry et al. 1951)

Reagents – 1. solution A was 2% Na_2CO_3 in 0.1 M NaOH prepared by adding 2 gm of Na_2CO_3 in 0.1M NaOH.

2 .Solution B was 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartarate prepared by dissolving 1 gm of disodium tartarate and 0.5 gm of $CuSO_4.5H_2O$ in 100 ml of distilled water.

3. Solution C was freshly prepared by mixing 50 ml of solution A with 1 ml of solution B.

4. Solution D was prepared by diluting Folin Ciocalteu phenol reagent with equal volume of distilled water. 20% TCA was prepared by dissolving 10 gm in 50 ml of water.

Proteins were estimated by homogenizing fresh tissue (1 gm) with pestle and mortar with 8 ml of freshly prepared 50 mM phosphate buffer (pH- 6.8) containing 100 mM sucrose and 1 gm cystein. Centrifugation of homogenate was carried out (12,000xg, 45 min) at 4°C after adding 200 mg of PVP in the extraction mixture. Supernatant was taken and the total volume was made upto 10 ml with the extraction buffer. 5 ml of supernatant was separated out in a test tube for enzyme assay. To other 5 ml extract, equivalent volume of 20% chilled TCA was added to precipitate proteins. The solution was incubated at 4°C for 4 hours. The extract was then centrifuged (12,000xg, 45 min) at 4°C. Supernatant was discarded and the pellet was dissolved in 3 ml of 1N NaOH. 1 ml from above solution was taken and total soluble protein content was determined using Lowry method.

To 1 ml of NaOH extract, 5 ml of solution C was added and incubated at room temperature for 10 min. 0.5 ml of solution D was then added and incubated again at room temperature for 30 min. Absorbance was then taken at 750/500nm. Determination of protein concentration was determined using standard curve prepared taking known concentrations of bovine serum albumin (20-200µg).

Peroxidase assay (Mc Ewen, 1971)

Reagents – 1**.** O-dinisidine solution was prepared by dissolving 10 mg in 1 ml methanol.

2. Phosphate buffer (pH-7) was prepared.

Peroxidase activity was determined by homogenizing 1 gm of sample in 7 ml of 50 mM phosphate buffer (pH-7). Samples were centrifuged at (12,000xg, 45 min) at 4°C. Supernatant was taken and total volume was made upto 10 ml using extraction buffer. Reaction mixture comprised of 2.75 ml of extraction buffer, 0.1 ml of O-dinisidine solution (1% w/v in methanol) and 0.05 ml of 1% H_2O_2. Reaction was started by adding 100 µl of enzyme extract. Change in absorbance was recorded at 470 nm from 0 to 2.5 min and activity was expressed in terms of change in optical density mg^{-1} fresh weight min^{-1} .

Phenol estimation (Swain and Hillis, 1959)

Reagents – 80% methanol in 0.3 N HCl was prepared by adding 80 ml of concentrated methanol and 830 µl of concentrated HCl in 20 ml of disltilled water. 10% folins reagent was prepared by diluting Folin Ciocalteu phenol reagent in 90 ml of distilled water. Saturated Na_2CO_3 was prepared by dissolving 7.3 gm of Na_2CO_3 in 100 ml of distilled water.

Determination of total phenol content was carried out by homogenizing 1 gm fresh tissue in pestle and mortar with 80% chilled methanol consisting of 0.3N HCl and incubated at 4°C for 12 hours. The homogenate was then centrifuged at (10,000xg, rpm, 15 min) at 4°C. Collected the supernatant in a test tube and the residue was re-extracted with the fresh solvent. Reaction mixture was prepared using 0.5 ml methanolic extract, 2.5 ml folin ciocalteu reagent (10%), 2.5 ml saturated sodium carbonate. Incubated the mixture at 45°C for 45 min. Absorbance was recorded at 765 nm. Phenol concentration was determined using gallic acid as standard.

Carbohydrate estimation (Dubois et al., 1956; Sumner, 1935)

Reagents – 1. 80 ml of concentrated ethanol was added to 20 ml of distilled water for 80% ethanol.
2. 10 ml distilled water was added to 90 ml of phenol solution (90% w/v).
3. Dinitrosalicylic acid (DNSA) was prepared by dissolving 2.5 gm of DNSA in 50 ml distilled water containing 4 gm NaOH, 75 gm sodium tartarate. Total volume was made to 250 ml with distilled water.
Sample was prepared by drying 2 gm of tissue at 80°C in oven. After it was dried, samples were crushed to fine powder with pestle and mortar. 50 mg of powdered sample was taken in eppendorf and mixed with 80% ethanol. The extract was incubated at room temperature for 60 min. Centrifugation was carried out at (10,000xg, 60 min) at 4°C. Supernatant was collected and the pellet was re-extracted with fresh solvent. Pooled the supernatants and dry to 25 ml for the analysis of total soluble sugar, total reducing sugar and non reducing sugar.

Total soluble sugar (Dubois et al., 1956) – In a test tube, 100 µl of enzyme extract, 900 µl of distilled water was added along with 50 µl of phenol reagent. 5 ml of concentrated sulphuric acid was rapidly added and the mixture was incubated at room temperature for 30 min. Absorbance was measured at 485 nm. Standard curve was obtained using D-Glucose concentrations (20-200µg).

Total reducing sugar (Sumner, 1935)– In a test tube, 500 µl of extract, 500 µl of distilled water and 1 ml of dinitrosalicylic acid (DNSA) reagent was added. The mixture was boiled for 10 min in water bath and cooled at room temperature. Absorbance was recorded at 560 nm.

Enzymes :- extracts for enzymatic assays were prepared in manner similar to protein extraction

Ascorbate peroxidase assay (Nakano and Asada, 1981)

Reagents – 1. 100 mM ascorbate was prepared by dissolving 2 mg ascorbate per ml distilled water.

2. 100 mM of hydrogen peroxide was prepared by adding 102 µl of 30% H_2O_2 in 10 ml of MQ water.

Determination of ascorbate peroxidase activity was carried out by following the oxidation rate of ascorbate, leading to decrease in absorbance at 290 nm, observed spectrophotometrically at 25°C. The reaction mixture (2 ml) comprises of 20 µl of 10 mM EDTA, 100 µl of 100 mM ascorbate ,2 µl of 100 mM H_2O_2 and 1880 µl of phosphate buffer. Reaction was started in quartz cuvette by adding 20µl of extract. One unit of enzyme activity was calculated as the amount of enzymes required to oxidize 1µmole of ascorbate min^{-1} mg $protein^{-1}$.

Catalase (Aebi, 1983)

Reagents – 10 mM hydrogen peroxide and 50 mM phosphate buffer (pH-7) were prepared.

Reaction mixture contained 10mM hydrogen peroxide prepared in 50 mM phosphate buffer (pH-7) and 0.1 ml hydrogen peroxide was prepared and reaction was started by adding 0.02 ml of enzyme extract. Activity of catalase was estimated by measuring the decrease in absorbance at 530 nm and was expressed in terms of µmoles of hydrogen peroxide decomposed per minute (molar extinction coefficient 39.4 $mM^{-1}cm^{-1}$).

Histological analysis

For histological examination, nodal segments were cultured in basal MS media and MS media supplemented with 1µl of IBA. Samples (4-5 mm basal portion of shoots) were taken and collected daily from Day 0 to Day 7 after the experiment was started. 4 samples, two from each basal and IBA containing media were collected daily. Samples were fixed

in a mixture of FAA (formalin, glacial acetic acid and 50% ethanol in a ratio of 5:5:90 v/v/v). Dehydration was carried out through a series of tertiary butyl alcohol (TBA) series. Samples were then embedded in paraffin wax in oven maintained at 60°C and blocks were made. Transverse sections (10 µm) were cut with the help of rotary microtome. They were fixed to the slides and dewaxing was carried out with xylene for 3-4 h. The dewaxed slides were stained by passing through the series as mentioned in following tables.

Table 1 : The compostion of the TBA series used for dehydration.

Sr. no.	Xylol (%)	Alcohol (%)	Water (%)
1	75	25	0
2	50	50	0
3	25	75	0
4	0	100	0
5	0	90	10
6	0	75	25
7	0	50	50
8	0	25	75

Table 2 : The compostion of alcohol : clove oil series used for washing

Sr. no.	Alcohol (%)	Clove oil (%)	Water (%)
9	25	0	75
10	50	0	50
11	75	0	25
12	95	0	05
13	Absolute-1	0	0
14	Absolute-2	0	0
15	75	25	0
16	50	50	0

Samples were dipped in fast green (0.1-0.5 % w/v) in 50% ethanol and 50% clove oil (a dip).

Table 3 : The compostion of fast green series.

Sr. no.	Xylol (%)	Clove oil (%)
17	50	50
18	75	25
19	100	0
20	100	0

Sections were mounted in DPX after dewaxing and observed under microscope.

Chapter 4: Results

Process of rooting includes numerous biochemical changes such as increase in carbohydrates, proteins and decrease in activity of certain enzymes responsible to counteract stress conditions. Plant growth regulators, specifically IBA is known as a root inducer. Therefore, present study was focused on evaluation of above mentioned factors in relation to rooting.

Effect of IBA on rooting of *Bacopa monnieri*

It was interesting to note that although cent percent explant showed rooting on MS medium supplemented with (0 - 2.5) µM IBA, but, significant decrease in rooting frequency was observed when IBA concentration was further increased. Number of roots per explant and length of roots was also found to decrease with increasing IBA concentration from 1 to 5 µM (Fig 1)

Table 1: Effect of IBA on rooting of *Bacopa monnieri*.

IBA Concentrations (µM)	Percent explants showing rooting	Number of roots per expant (mean)	Mean length of roots (cm)
0	100 ± 0.0	3.9 ± 0.216	1.42 ± 0.083
1	100 ± 0.0	6.4 ± 0.373	0.55 ± 0.050
2.5	100 ± 0.0	4 ± 0.448	0.25 ± 0.0
5	66.66 ± 14.29	3 ± 0.552	0.26 ±0.073

Nodal explants were cultured on MS medium supplemented with different IBA concentration. Rooting parameters were observed after 10 days of culture

Fig 1: The effect of IBA concentration in MS medium on rooting of nodal exlants taken from microshoots of *Bacopa monneiri.*

Table 2 : The effect of IBA pulse treatment on rooting of nodal explants of *Bacopa monneiri.*

Pulse treatment (h) with 25 µM IBA	Medium	% explants showing rooting	No. of roots per explant (mean)	Mean Length of roots (cm)
12 hours	Solid	96 ± 0.29	3.55 ± 0.29	0.856 ± 0.085
	liquid	96 ± 4	3.86 ± 0.36	0.822 ± 0.126
24 hours	Solid	76 ± 4	3.68 ± 0.42	0.702 ± 0.089
	liquid	96 ± 4	3.97 ± 0.31	1.028 ± 0.207

Footnote: Nodal explants were cultured on liquid and solid MS medium supplemented with 25 µM IBA concentration. Rooting parameters were observed after 10 days of culture.

Effect of IBA pulse treatment on rooting was also studied. Nodal explants cultured on MS medium supplemented with 25 µM IBA for 12 and 24 h and then transferred to basal MS medium were observed for rooting. Maximum rooting frequency was observed when pulse treatment was given in liquid MS medium supplemented with 25 µM IBA , whereas number of roots per explant was found to decrease when pulse treatment time was increased from 12 to 24h. It was also observed that pulse treatment on solidified medium lead to decrease in rooting frequency with no change in number of roots per explant.

Histological analysis

Histological studies revealed different phases of root formation. As root primordia was formed after 9 days in case of nodal explants cultured on MS medium supplemented with IBA. Thus, undeveloped vascular bundle was observed after 3rd day of culture (Fig 2A), whereas fully developed vascular bundles were observed in nodal explants cultured on basal MS medium (Fig 2B). Further, initiation of root primordia was observed after 5 days of culture (Fig 2D). Cells of tissue was found to show immense division which is initial step of root formation (Fig 2C).

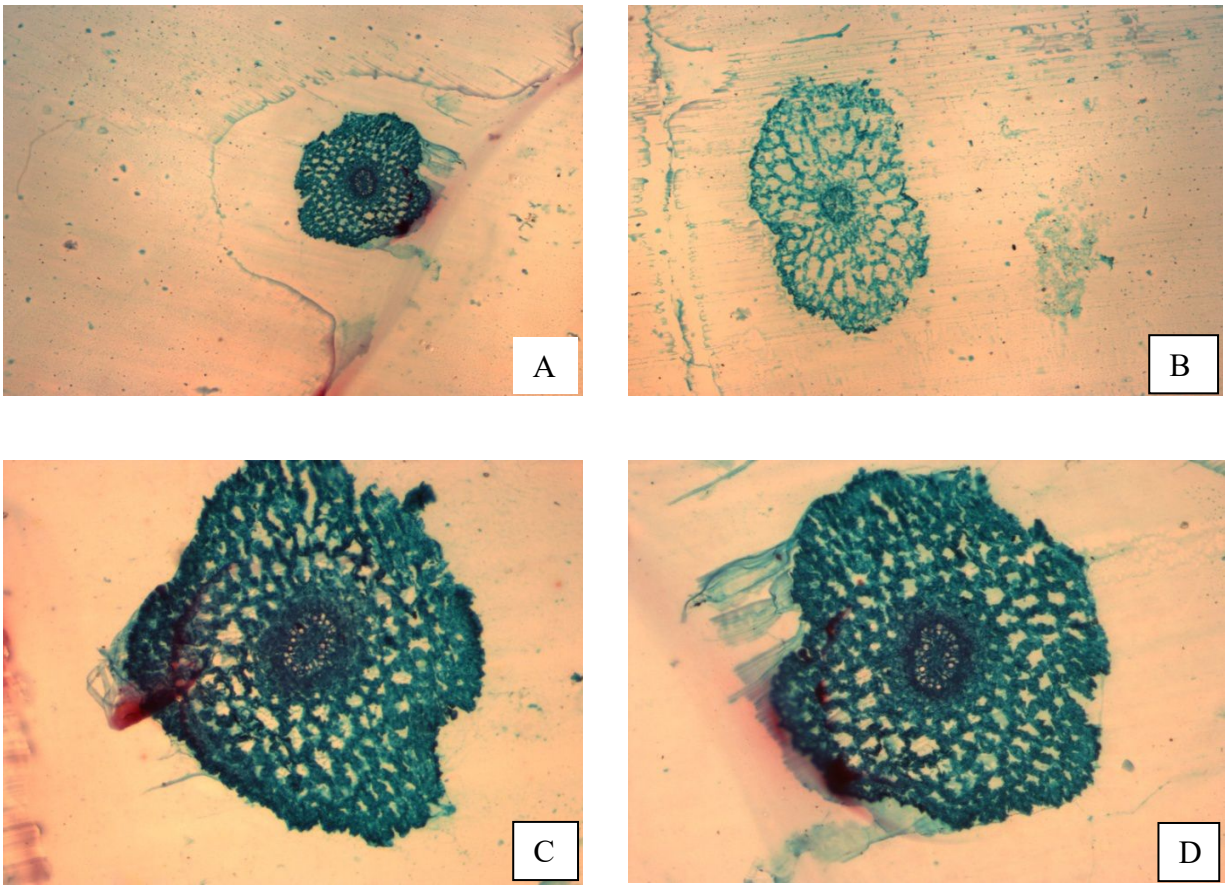

Fig 2: Transverse sections of nodal explants of *Bacopa monnieri* during root formation. cells undergo rapid division on 3rd day of culture as initial step of rooting on **A** basal MS medium**. B** supplemented with 1 µM IBA. **C-D** Root primordial formation was observed after 5 days of culture on basal MS medium.

Biochemical changes during rooting of nodal explants

Biochemical changes in nodal explants during different phases of rooting was studied. The adventitious rooting occurred in three phases- induction (1-3 day), initiation (4-6 day) and emergence (7 day) in MS media whereas in IBA supplemented media emergence started on 6th day.

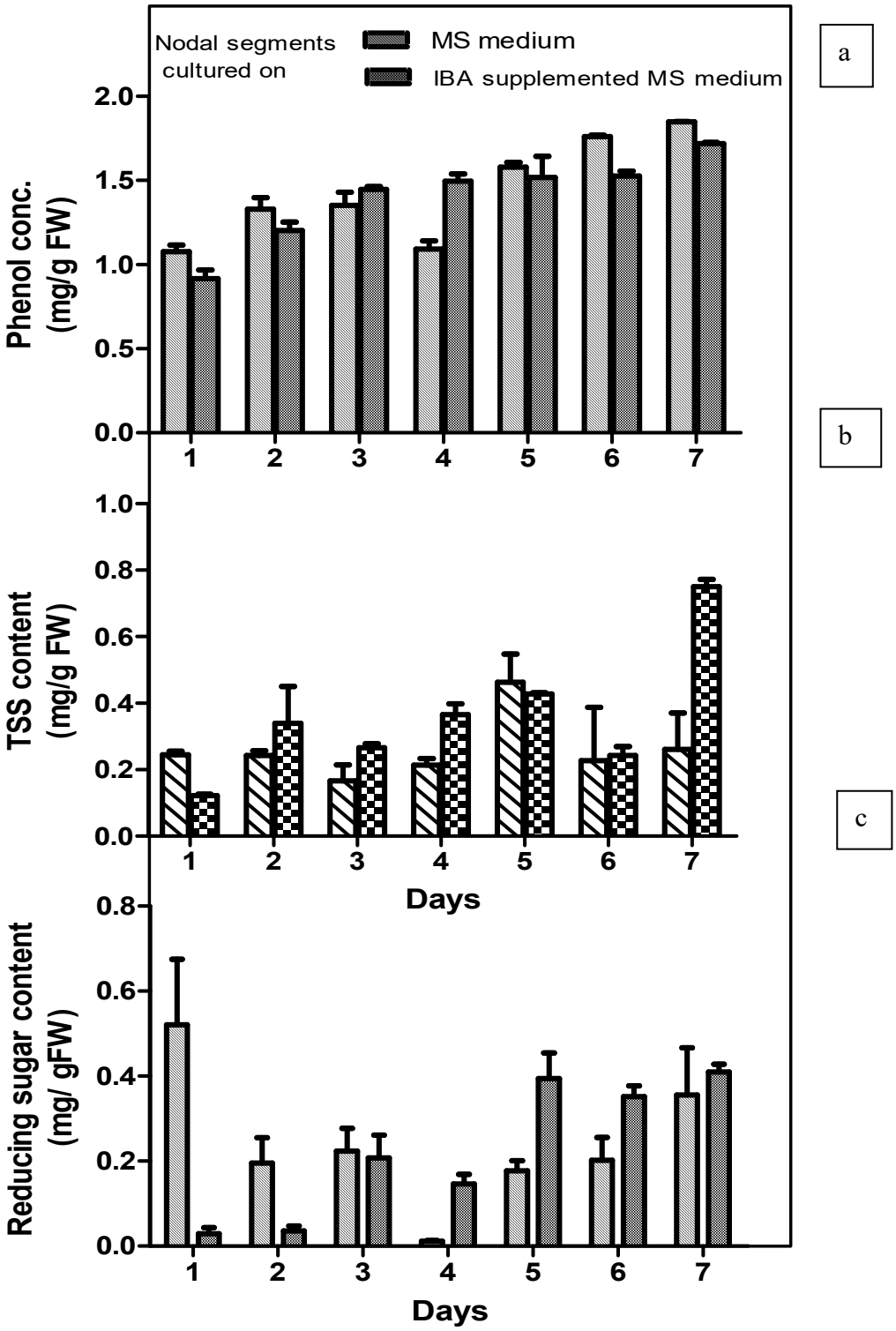

Fig 3 : Changes in levels of (a) total phenols (b) total soluble sugar (c) reducing sugar content during different rooting phases and effect of IBA on rooting behaviour in *Bacopa monneiri*.

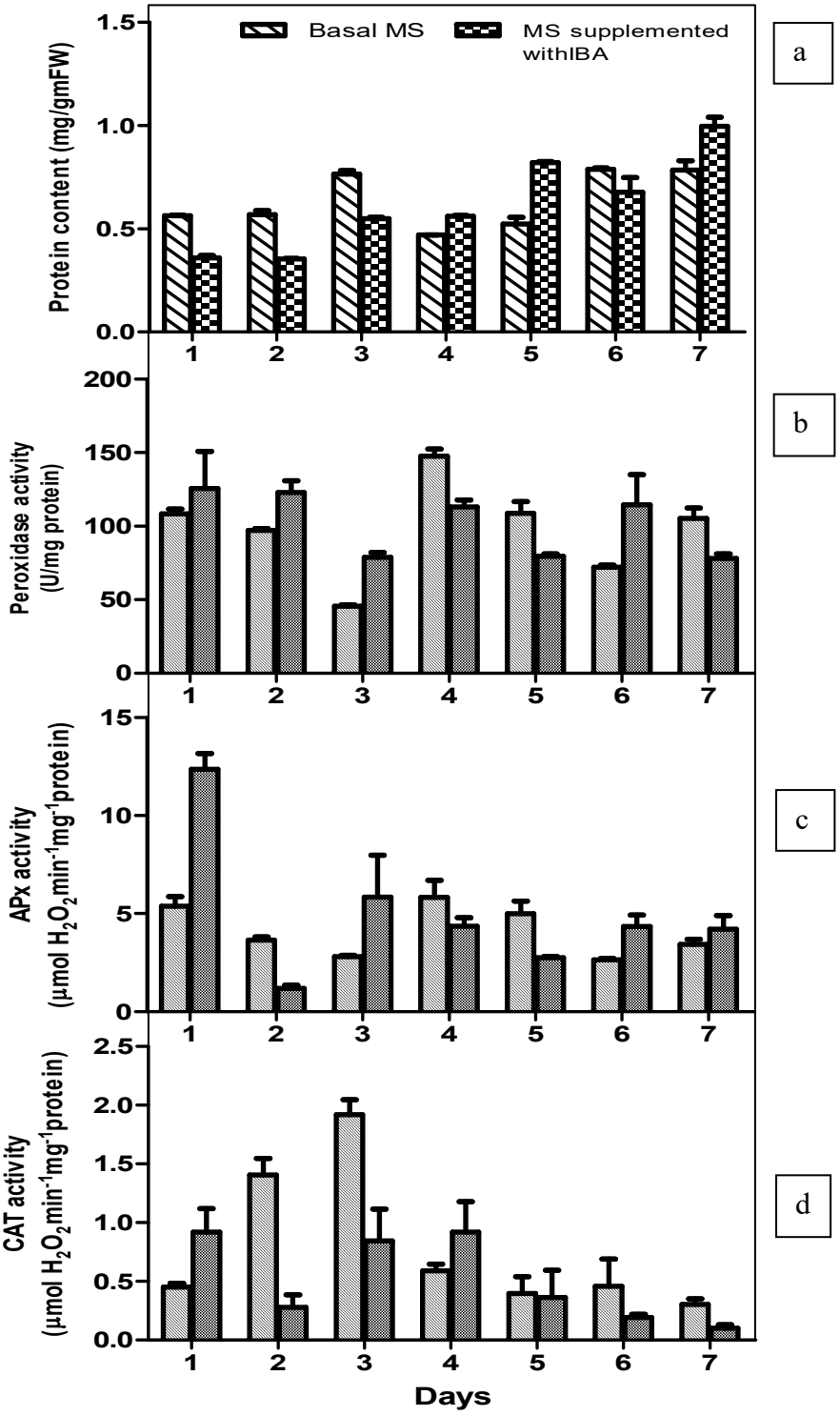

Fig 4 : Changes in levels of (a) Total protein content, (b) Peroxidase activity, (c) APx activity and (d) - Catalase activity during different rooting phases and effect of IBA on rooting behaviour in *Bacopa monneiri*.

Changes in total phenolic content (Fig 3a) indicates that phenols increased in initial phase of root induction and then declined during initiation phase, followed by gradual increase till 7th day, which corresponds to elongation and emergence phase, however, on IBA supplemented medium, a gradual increase was noticed from 1st to 7th day and have more phenolic content as compared to basal MS media.

Total soluble sugar (TSS) (Fig 3b) content in the tissue remained constant on basal MS medium. It declined further followed by decrease on 7th day. However, a steady increase in TSS was observed in explants during rooting on MS medium supplemented with IBA. Highest total soluble sugar content was observed in IBA supplemented medium after 7 days when emergence and elongation of roots were observed.

Reducing sugars (Fig 3c) content decrease upto 4 days in explants on basal MS medium and then a steady increase was recorded upto 7th day. On IBA supplemented medium, a lower level of reducing sugars were recorded upto day 3 which then increased upto day 7. After day 5, the levels of reducing sugars were higher in IBA supplemented medium as compared to basal medium.

Protein content (Fig 4a) increased upto 3rd day on basal MS medium followed by decrease during initiation phase and gradual increase during elongation phase. On IBA supplemented medium, protein content increase during initiation and elongation phase. Protein content was found to be highest on IBA supplemented medium during emergence phase in comparison to basal medium.

Enzyme activities

Peroxidase (POX) activity (Fig 4b) in nodal explant cultured on basal medium initially decreased in the induction phase and was followed by an increased during initiation phase and finally during elongation phase gradual decrease was observed in POX activity. On the other hand, nodal explants cultured on medium supplemented with 1 µM IBA observed increase in peroxidase activity during elongation phase. Highest peroxidase activity was found during onset of initiation phase.

Ascorbate peroxidase APx activity (Fig 4c) follows a pattern similar to POX activity for nodal explants cultured on basal medium and medium supplemented with IBA. Maximum APx activity was observed during induction phase in contrast to POX activity.

In contrast to POX and APx, Catalase (CAT) activity (Fig 4d) was observed to increase during induction phase and then decrease in initiation and elongation phase when nodal explants were cultured on basal MS medium. On IBA supplemented media, CAT activity was decreased during induction phase followed by increase during root initiation and then a gradual decrease in elongation phase.

Discussion

The present work was focused on the study of rooting and its related biochemical and morphological changes. *Bacopa monnieri* microshoots were used to conduct experiments. Further, the effect of IBA on rooting was also studied.

In *Bacopa monnieri,* efficient rooting was observed on MS medium incorporated with low concentration (Table 1) of IBA wheareas higher concentration of IBA result in reduced rooting. Efficiency of IBA in rooting has been previously reported (Han et al., 2009). Furthermore, IBA is a preferred auxin due to its greater stability (Smith and Thorpe, 1975). To understand the effect of auxin rooting, various biochemical factors such as phenols, proteins, carbohydrates and enzymes like peroxidase, ascorbate peroxidase and catalase were studied during different rooting phases and comparison was made with rooting behaviour observed on medium without incorporation of IBA.

Phenolics are heterogenous substances interacting with intra and inter cellular process. Phenolics are important substrate for peroxidase and carry out its oxidation and promotes lignifications (Sato et al., 1993). They may also stimulate and influence the root emergence from nodal cuttings of *Bacopa monnieri*. Phenolic content is seen to increase in root initiation phase and then a sudden decline and gradual increase thereafter. The increase at the end of induction phase is associated with phenylalanineammonia-lyase (PAL) activity which plays key role in synthesis of phenols (Bisbis et al., 2003). The

increased accumulation of phenols at end of induction phase accounts for increased PAL activity. Phenolic compound are precursors for lignin formation, thus decreased phenolic content can be due to its utilization for same (Bisbis et al., 2003). In IBA supplemented media, continuous elevated level of phenols were observed till end of initiation phase and slight decrease at start of elongation phase. At initial phase of rooting, higher phenolic content rapidly promotes rooting (Satish et al., 2008).

Total soluble sugar level follows same pattern on MS medium and IBA supplemented medium except for the fact that its level increases during induction phase on IBA supplemented medium unlike MS medium where it decreases. This may be due to the fact that sugar content and metabolism increases with the application of exogenous auxin (Haissig, 1974). Increased level of sugars during initiation phase accounts for its higher requirement of energy for rooting (Kumar, 1996). This can also be due to the interaction of carbohydrates with internal hormones which stimulates the rooting process (Weisman and Lavee, 1995). In IBA supplemented medium, there is a rapid increase in its level during elongation phase which is due to the ethylene production by sucrose which promotes rapid rooting by increasing auxin sensitivity to plants (Calamer and Klerk, 2002). It means higher level of sugars is present during cell differentiation. Sugars are the building blocks and serve as energy source. The decreased level of reducing sugars indicates that they play a major role in rooting during induction of rooting process. Sugars metabolise faster in presence of the auxin which accounts for low level of sugars during initial stages of rooting. During root elongation high energy is required and thus high reducing sugars are required which accounts for elevated level of sugars during elongation phase.

Proteins are important nitrogen source for plants. It was observed that the protein content increases in the initial step. This may be due to the fact that during in vitro conditions, plants adapt to the environment and to avoid any enviromental stress, higher level of proteins are produced during induction phase. Decrease in protein level accounts for the cell development stage for rooting. In IBA supplemented medium there is a continuous increase in protein till the cell differentiation starts.

Peroxidases are involved in IAA catabolism and in cell wall lignifications. Peroxidase activity, in some species underwent a unique curve with an early gradual decrease followed by sudden increase and then a decline (Gasper et al., 1992). This variation is normally associated with inverse pattern of total phenol compounds which regulates peroxidase activity (Gasper et al., 1992). Peroxidase activity was found to be minimum at induction phase and maximum during initiation phase. The time between minimum and maximum peroxidase activity can be considered as initiation, itself preceding expression phase (Barthon et al., 1990). Decreased level of peroxidase activity after a peak can be due to the inhibited *de novo* synthesis of peroxidase isozymes (Chao et al., 2001). The decrease in peroxidase activity results in decreased rigidity of cell wall, thus allowing plant growth and cell expansion (Brownleader et al., 2000).

Ascorbate peroxidase uses ascorbic acid as a substrate. It breaks down the cell quiescent stage. Ascorbate regulates cell cycle progression (De Tullio et al., 1999). During induction and proliferation, there is an increase in endogenous level of hydrogen peroxide, due to metabolic processes. Availability of hydrogen peroxide for other antioxidant enzymes may account for the decreased level of a APX activity. High APX activity may also be due to accumulation of hydrogen peroxide which can be toxic to plant.

Catalase activity is seen to increase initially. When the nodal explants were cultured on medium, metabolic process starts, thus accumulating hydrogen peroxide. Catalase and peroxidase enzymes carries out hydrogen peroxide scavenging which resulted in their increased level. Decrease in catalase activity during initiation phase is because cells are not entering the stage of oxidative stress. It increases the level ROS which further led to elevated level of catalase during elongation phase

In addition to biochemical and enzymatic analysis, morphological changes during rooting were also studied. Histological studies revealed three stages of rooting, that is, induction phase, initiation phase and elongation phase. Variation in cells packing was also observed when comparison was made between nodes cultured on basal MS medium and medium

supplemented with 1 μM IBA. During initiation phase, cells were found to be in clusters and may be passing mitotic phase of dividing. Active cell division has already been reported as a key feature of initiation phase (Altamura, 1996). Furthermore, induction phase was marked with formation of root primordia. Our findings were in line with previous reports of Ballester et al., 2009.

In summary, effect of IBA on biochemical factors related with rooting behaviour of plant has been studied in addition to morphological studies during different rooting phases. Present study can be used in further studies of rooting.

Conclusion

Biochemical changes during adventitious root formation in Bacopa *monnieri* (L.) Wettst were studied.

- Protein content was found to be increased during induction and elongation phase but was low during initiation phase.
- Peroxidase activity and ascorbate peroxidase activity decreased during induction phase and elongation phase but was decreased during inititiation phase. Ascorbate peroxidise activity was maximum during induction phase.
- Catalase activity, in MS medium was found to be increased during induction phase and was decreased during further two phases but on IBA supplemented medium, catalase activity followed inverse pattern.
- Phenol content was found to be gradually increasing in nodal explants cultured on MS medium supplemented with IBA but was found to be decrease during initiation phase on MS medium
- Total soluble sugar content was constant on basal MS medium but decreased during elongation phase. On IBA supplemented MS medium, there was a steady increase throughout 3 phases of rooting.
- Reducing sugar content was found to be decreased during induction and initiation phase followed by steady increase during elongation phase on basal MS medium. It was found that on IBA supplemented MS medium, reducing sugar decreased during induction phase and increased during elongation phase.

Appendix 1

- **Murashige and Skoog (MS) media (1962)**

Components:

Macro nutrients	**Concentration (mg/l)**
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170

Micro nutrients	**Concentration (mg/l)**
$MnSO_4.H_2O$	16.9
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025

Vitamins	**Concentration (mg/ml)**
Nicotinic acid	0.5
Pyridoxine	0.5
Thiamine	0.1
Glycine	1

Freshly added components	**Concentration (g/l)**
Myoinositol	0.1
EDTA	0.03
Sucrose	30

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