5. DISCUSSION

Plants are an important source of medicines and play a key role in world health (Constable, 1990; Kala, 2005). In almost all regions and cultures of the world, from ancient times till today, plants have been used as medicines. Today’s medicinal plants are important to the global economy, as approximately 80% of traditional medicine preparations involve the use of plants or plant extracts (Viera and Skorupa, 1993; Dhyani and Kala, 2005; Banerjee and Shrivastava, 2008).

The increasing demand for herbal medicines in recent years due to their fewer side effects in comparison to synthetic drugs and antibiotics has highlighted the need for conservation and propagation of medicinal plants. An efficient and most suited alternative solution to the problems faced by the phytopharmaceutical industry is development of \textit{in vitro} systems for the production of medicinal plants and their extracts.

The \textit{in vitro} propagated medicinal plants furnish a ready source of uniform, sterile and compatible plant material for biochemical characterization and identification of active constituents (Banerjee and Shrivastava, 2006; 2008). Recently in a report by the National Medicinal Plant Board (NMPB), Government of India and Technology Information Forecasting and Assessment Council (TIFAC) has recommended immediate attention to few medicinal plants, among which \textit{B. Monnieri} prominently features, which makes this plant in the category of highly endangered plants in India (http://www.nmpb.nic.in/prioritised medicinal plants.htm).
B. monneri is one of such important medicinal plants, belonging to the family Scrophulariaceae, an amphibious plant of the tropics, which possesses active principles such as bacosides A, B, C and D which are active triterpenoid principles and known as “memory chemicals” (Rastogi et al., 1994; Sivarama Krishnan et al., 2005). Two new dammarane-type jujubogenin bisdesmosides, bacosaponins E and F of biological interest have also been isolated from this herb (Mahato et al., 2000; Chakravarty et al., 2003). The present investigation was planned to report a simple and rapid but novel method for in vitro multiplication of B. monnieri by using different bioinocullants at hardening stage and also deals with the phytochemical and antibacterial activities of in vitro plantlet extracts against human pathogenic bacteria.

5.1. Micropropagation

Tissue culture techniques have been successfully employed to produce a large number of difficult to propagate plants. Tissue culture technology is a powerful tool for the conservation and rapid multiplication of many threatened plant species (Fay, 1992). It has been particularly useful for the conservation and rapid propagation of valuable, rare and endangered medicinal species. Large-scale, unrestricted exploitation of this natural resources to meet the ever increasing demand for its, by the Indian pharmaceutical industry coupled with limited cultivation and insufficient attempts for its replenishment, the availability of this medicinally important and endangered plant species is being dwindling day by day (Pandey et al., 1993).
5.1.1. Direct organogenesis

Present study on micropropagation of *B. monnieri* was based on research studies reported by Mathur and Kumar (1998); Tiwari *et al.*, (1998) and Tiwari *et al.*, (2000); Banerjee and Shrivastava (2008). The few earlier reports available on *B. monnieri* demonstrated plant regeneration through axillary nodes, internodes and young leaves on media with very high concentrations of cytokinin (Shrivastava and Rajani, 1999). In this study, the effect of different concentrations and specifically lower concentrations of cytokinin on shoot induction of *B. monnieri* for rapid and large-scale multiplication at a cost effective level. Explants remained green and fresh but failed to grow any further in growth-regulator-free medium i.e, control.

To initiate the study, nodal, leaf and shoot tip explants were taken from field established *B. monnieri* plants. The sterilization procedure includes soaking of explants in an aqueous solution containing 0.1% Bavistin and 0.03% streptomycin followed by treatment with 0.1% mercuric chloride aqueous solution with an intermediate step of sterile water wash.

Shrivastava and Rajani, (1999) has described sterilization treatment of *Bacopa*, which includes use of 0.1% mercuric chloride(W/V) for two minutes followed by rinsing thoroughly with sterile distilled water, Mathur and Kumar (1998) reported different sterilization treatment in which leaves and stem explants were shaken for 10 minutes in Tween-20 and Savlon (0.3%) V/V chlorohexidine gluconate and 0.6% W/V cetrimide in water for 10 minutes, rinsed in running water for 30 minutes, treated with 0.1% mercuric chloride for 3-
4 minutes and washed several times with sterile water. However, it was also found that duration of treatment for mercuric chloride is very critical due to soft and herbaceous nature of explants. During surface sterilization treatment it was found that treatment with 0.1% mercuric chloride as referred by Shrivastava and Rajani, (1999) and Mathur and Kumar, (1998) leads to blackening of the explants. Hence limited treatment of 0.01% mercuric chloride was given to the large number of explants were found contaminated. Contamination was controlled after the addition of antibiotic, 0.03% Streptomycin and antifungal agent, 0.1% Bavistin and there was no adverse effect on bud sprouting and shoot multiplication.

Shoot initiation and establishment from *B. monnieri* nodal, leaf and shoot tip explants cultured on MS medium supplemented with various combinations of growth regulators i.e, BAP in combination with NAA and KN is described in Tables 3, 4 & 5. Most of the other research studies for other medicinal plant species have shown the use of cytokinin alone or in combination with other in different concentration. For example, in *Paederia foetida* and *Centella asiatica* multiple shoots were obtained on MS medium supplemented with BAP 1.0 mg/l (Singh *et al.*, 1999) and in *Rauwolfia serpentina* on MS medium supplemented with benzyladenine and NAA (Sehrawat *et al.*, 2001) whereas for *Bacopa*, optimum shoot proliferation was achieved in different combination of hormones in different concentrations.

Efficient regeneration protocol is a prerequisite for the mass propagation and application of bioinoculants on medicinally and economically important plants.
5.1.1.1. Leaf explant

An efficient method of direct plant regeneration from in vitro leaf explant was standardized. Response of the leaf explants cultured on MS medium with various concentrations of BAP, KN and NAA is presented in the Table – 3. Initially the adventitious buds originated 2 weeks after inoculation and developed shoots from these explants after 4 weeks. Among the different growth regulators tested, BAP (1.0 mg/l) in combination with NAA (0.5 mg/l) induced more number of shoot buds (18.2) per explant and yielded maximum percentage of response (93%). When kinetin was supplemented in the medium, the number of shoots increased with an increase in the concentration. However, only about 85% of shoot induction was observed in the medium containing KN (1.5 mg/l). Similar observations were also made in B. monnieri by Vaibhara et al., (2001). Formation of shoot buds occurred either on adaxial or in the abaxial surface, whichever was in contact with the medium. Although the shoot bud formation was observed in all the concentrations of BAP and NAA, the number of buds produced per explant was varied in each treatment. Thus the number of shoot buds produced per explant could greatly be increased by manipulating the balance of the growth regulators in the basal medium (Thorpe, 1980). Kinetin was less effective than BAP in inducing shoot multiplication.

Similar results have already been made with other species such as apple, Arocarpus heterophyllum (Lundergan and Janick, 1980; Rahaman and Blake, 1988) Phyllanthus niruri, (Karthikeyan et al., 2007) Centella asiatica, (Karthikeyan et al., 2008) Ocimum sanctum (Karthikeyan et al., 2009). The pattern of shoot organogenesis from leaf explants of Africa violet plants and Sesame leaf explant by the supplementation of
BAP (10 mg/l) has been reported (Start and Cumming, 1976; Manjusharma and Pareek, 1998). James et al., (1990) also reported direct shoot organogenesis and plantlet regeneration from leaves of grape when cultured on MS medium fortified with BAP (4 mg/l).

Among the various growth regulators, the regeneration response of the leaf explants on the basal medium containing BAP/KN and NAA was maximum. On the other hand Banerjee et al. (1999) developed a simple and rapid protocol for the in vitro multiplication of *Centella asiatica* from leaf explants on MS medium supplemented with BAP and IBA.

**5.1.1.2. Nodal explants**

High frequency of multiple shoot formation from nodal segments was observed in the present investigation when the medium was supplemented with BAP at 1.0 mg/l and NAA 0.5 mg/l. Nearly 26.9 shoots were developed from a single node.

Nodal explants produced more number of adventitious shoot than shoot tip. Similar observation was already made in *Emblica officinalis* (Verma and Kant, 1996); *Wedelia chinensis* (Kamalam and Jagadeesan, 1999).

Nodal explants cultured on basal medium without growth hormones, showed growth of single shoot only for few days. But later shriveled off. Similar observation was already made in *Dolichos biflorus* (Soundar Raj et al., 1989).
Among the different explants, nodal segments were found suitable for micropropagation in the case of medicinal plants such as *Ocimum sanctum* (Nirmal et al., 1999) and *Solanum surattense* (Govindaraju et al., 2003; Seetharam et al., 2003). In the present study, nodal segments were observed to have higher response for shoot multiplication.

In recent years nodal explants preferred over meristem to produce large number of genetically identical clones. Use of direct and large sized explants have higher survival and growth rates than the smaller one (Hu and Wang, 1983; Sharon and Marie, 2000).

As far as the influence of growth regulator concerned with reference to *B. monnieri*, such as individual cytokinins like BAP and TDZ favoured for maximum shoot multiplication from pre existing or *de novo* buds (Anjali et al., 2000). Combination of cytokinins, BAP and KN showed a positive effect on the multiplication of axillary buds (Ashutosh et al., 2004). In the present investigation, nodal segment produced multiple shoots on MS medium containing individual or combination of cytokinins (BAP and KN) along with or without low auxin (NAA). Thus the finding of present experiment confirms the earlier observations and suggest that the nodal explants and the combination of BAP and NAA could be effectively used for direct regeneration.

In the present investigation BAP favoured the induction of solitary shoot from nodal explants. KN was less efficient in shoot multiplication of meristem containing explants. In the present
experiment, lower level of KN (0.5 and 1.0 mg/l) favoured the growth of solitary shoot; where as KN at higher level (2.0 mg/l) suppressed the growth of explant. In *Pisonia alba*, axillary bud explants maintained on MS medium containing KN showed only the enlargement of the axillary buds.

BAP was proved to be an ideal hormone for shoot multiplication of shoot tip and nodal cultures in herbaceous plants. In the present attempt maximum number of shoot multiplication observed in BAP supplemented medium.

Of the two cytokinins tested, BAP was more effective than KN, in inducing shoot development and as well as multiple shoots from nodal explants. BAP was found to be superior to the other cytokinins for shoot proliferation in several medicinal plants (Pattanaik and Chand. 1996; Ramanujam et al., 1999). Thus the present report confirms earlier findings.

### 5.1.1.3. Shoot tip explant

In recent years, shoot tip and nodal explants preferred over meristem tissues to produce large number of genetically identical clones, as suggested. Use of direct and large sized explants has higher survival and growth rates than the smaller one (Hu and Wang, 1983; Sharon and Marie, 2000). For higher frequency of multiple shoot induction, shoot tip and nodal explants were selected from *in vitro* regenerated plants (Chandran *et al*., 2007). In the present study supplementation of 1.0 mg/l
BAP and 0.5 mg/l NAA showed 10.3 mean shoots per explant. Supplementation of KN with NAA showed a maximum of 9.6 mean shoots per explant. So, BAP and NAA were found suitable for the development of more number of shoots than in the KN supplemented explants.

For higher frequency of multiple shoot induction, shoot tip and nodal explants were selected from several in vitro regenerated plants (Dipak et al., 1985).

In the direct shoot multiplication of shoot tip explants are shows like as in the earlier findings made in Lavandula angustifolia (Oliphant, 1988); Yucca aloifolia (Kulkarni and Rao, 1999). Holarrhena pubescens (Sumana et al., 1999)

Multiple shoot formation in in vitro culture is more advantageous over a single shoot formation for rapid clonal multiplication as well as for its conservation. Many scientists have analyzed the resident meristem of juvenile origin for rapid clonal propagation of medicinal plant taxa such as Gymnema sylvestris (Komalavalli and Rao, 2000), Vigna unguiculata (Kulothungan et al., 2008) and Gomophrena officianlis (Mercier et al., 1992). MS medium supplemented with BAP was found to be the best as compared with other growth regulators showing maximum percentage of shoot induction from apical shoot explants of B. monnieri. The percentage of shoot induction in B. monnieri was higher in BAP and NAA as compared to KN and NAA. Thus in the present study a simple and efficient protocol was developed
for the establishment of direct plant regeneration from the apical shoot explants of *B. monnieri*.

### 5.1.2. Indirect organogenesis

Indirect shoot organogenesis through callus using leaf, node, and shoot tip explants supplemented with various concentrations of auxins and cytokinins in MS medium was studied.

#### 5.1.2.1. Leaf explant

*In vitro* regeneration of plants through callus is a prerequisite for the applications of genetic improvement. Standardization of protocols for callus mediated regeneration is of immense application in clonal propagation and crop improvement programme, such as, raising somoclonal variants and transgenic plants. Leaf explant is reported as a potential explant for the regeneration of shoot bud in several plants species such as *Aristolochia bracteolate*.

In *Ocimum sanctum*, high concentration of cytokinins favoured for bud regeneration from leaves (Ahuja *et al.*, 1982; Pattnaik and Chand, 1996; Sahoo and Chand, 1998). The leaf segments of *Murraya koenigii* showed different responses on MS medium containing different cytokinins *B. monnieri* although, a small quantity of cytokinins may synthesized by shoots *in vitro* the role of cytokinins in organogenesis is well established. However as the concentration of cytokinins was increased to 5 mg/l and above no regeneration could be observed from any culture (Tejavathi and Shailaja, 1999). In accordance with earlier
reports, in the present experiment, leaf culture on BAP containing medium showed regeneration of shoot buds from all over the surface of explant.

KN was less efficient in shoot multiplication than BAP. In the present experiment, individual KN or combination of KN with NAA (0.5 mg/l) supported for the regeneration of lesser number of shoots. Improved performance of NAA and BAP in the regeneration of shoots from the leaf observed, Shahzad et al., (1999). In accordance with earlier reports, present experiment reveals that the combinations of NAA and BAP favoured for higher percentage (63.2%) response and induction of maximum number (36.4) of shoots and higher mean shoot length 7.4 cm was observed. However on lower concentrations of BAP and NAA the frequency of shoot regeneration was decreased.

An efficient method in which maximum number of indirect plant regeneration from \textit{in vitro} leaf explants through callus (86.2%) was achieved when the medium was supplemented with 1.0 mg/l of BAP and 2, 4-D. However, this response was less (80.2%) in the combination of 2, 4-D and NAA (each 1.0 mg/l). Supplement of 2, 4-D alone gave a good result for leaf callusing (73.6% in 1.0 mg/l).

MS medium containing KN and 2, 4-D (each 1.0 mg/l) showed a response of 65.7 per cent of callus induction. No callus induction was observed on MS medium without plant growth promoters. These results are in conformity with some of the earlier findings such as \textit{Solanum}
lanciniatum (Chandler et al., 1982), S. sarrachoides (Banerjee et al., 1985), S. dulcamora (Emke and Eilert, 1986), S. melongina (Filippone and Lurquin, 1989), S. nigrum (Shahzad et al., 1999) and Nicotiana tabacum (Rathore and Goldsworthy, 1985).

Increased concentration of BAP and constant level of NAA increased the callus formation, while the combination of IAA and BAP did not exhibit any callus formation from W. somnifera explants (Kannan et al., 2005). BAP in combination with NAA or IAA results in the regeneration of multiple shoots in Rauwolfia tetraphylla (Patil and Jayanthi, 1997; Ghosh et al., 2001 and Ghosh and Banerjee, 2003). Thus, the present experimental result are in accordance with earlier finding and suggests that suitable combination of BAP and NAA could be used for regeneration of multiple shoots in the case of B. monnieri.

The differences in the number and height of the shoots recorded with different concentrations of PGR’s were found to be statistically significant at the 5 per cent level. In the present experiment BAP (1 mg/l) with NAA (0.5 mg/l) was found to be most effective for callus mediated shoot regeneration, which is in agreement with earlier results showing that BAP is the most effective cytokinin for indirect shoot regeneration in many other plants such as Trifolium pratense (Campbell and Tomes, 1984), Dipterocarpus intricatus (Linington and Kew, 1989), Morus alba (Sharma and Thorpe, 1990), Aegle marmelos (Varghese et al., 1993), Bacopa monnieri (Tiwari et al., 1998) and Holarrhena pubescens (Sumana et al., 1999).
The classical findings of Skoog and Miller (1957) revealed that organogenesis in tissue culture is governed by the balance of auxins and cytokinins. In the present study also balanced combination of BAP and NAA (5:1) showed similar response in B. monnieri. Ignacimuthu et al. (1996) reported that the highest frequency of callusing in Eryngium foetidum was found when the combination of BAP and 2, 4-D was used. According to Seetharam et al. (2003), callus proliferation from leaf explants of Solanum surratense was high with BAP and NAA. On the other hand, a contradictory observation was made by Muthukumar et al. (2000) in Datura metel where BAP individually induced high frequency of callusing.

5.1.2.2. Nodal explants

Nodal explants cultured on BAP / KN supplemented media showed highest frequency of callus initiation (94.4%) after 12 days of inoculation on the medium containing BAP and 2, 4-D (1.0 mg/l) (Table - 6). Gita Rani and Grover (1999) observed the best callusing (92%) of W. sominifera on the medium supplemented with 2, 4-D and KN. They also found that the compact green calli, transferred to regeneration medium showed initiation of shoot buds after three weeks of culture. The high shoot differentiation was recorded (95%) with BAP 1.0 mg/l and 0.5 mg/l NAA after 20 days. In the present investigation also observed that BAP and 2, 4-D produced maximum callus. But the combination of BAP and NAA produced lower amount of callus than the combination of NAA and 2, 4-D. Gita and Grover (1999) used KN with BAP for 84 per cent frequency of callus induction.
In *W. somnifera*, significant improvement in shoot formation over control has previously been achieved with the addition of cytokinins such as BAP and KN (Conchou *et al.*, 1992; Fauconnier *et al.*, 1996; Wildi *et al.*, 1998). Thus in accordance with earlier reports, in the present investigation also the combination of BAP and KN with different concentrations of NAA resulted in higher per cent of shoot regeneration.

### 5.1.2.3. Shoot tip explant

The shoot tip explants were formed swellings after 5 days of inoculation and then callus was induced from the cut ends (Plate – VII). Higher percentage of callusing was observed in 1.0 mg/l of BAP and 2, 4-D supplemented medium (80.6%). Very meager difference in callus induction was recorded in the medium supplemented with combination of NAA and 2, 4-D or 2, 4-D alone.

The compact green callus was transferred to medium with different concentrations of cytokinins (BAP and KN) and NAA (0.5 mg/l) for shoot differentiation. Maximum of 85 % response was achieved in the BAP (1.0 mg/l) and NAA (0.5 mg/l) supplemented medium (Table-9). The shoot number and shoot length were increased in this combination. Induction of shoots by BAP in combination with NAA as observed in the present investigation is in agreement with the reports of Patra *et al.* (1998), Banerjee *et al.*, (1985) and Hossain *et al.* (2000). Combination of KN and NAA showed 75 per cent of shoot differentiation. This is compatible to earlier reports in *Citrullus lanatus* (Janosi *et al.*, 1991), *Centella asiatica* (Hossain *et al.*, 2000) and *Sorghum bicolor* (Abubacker and Murugesan, 1999). In the studies of
regeneration with *Cymbopogon martini* (Anjana and Borodoloi, 1991), BAP was found better than KN for shoot formation. It has been reported that the higher concentration of BAP produced hyperhydric shoots, while malformed shoot with basal callus was developed in the medium supplemented with higher concentration of KN (2 mg/l) (Kukreja *et al.*, 1997).

5.2. Shoot elongation

The aim of this step was to get healthy, normal and elongated shoots for the induction of rooting and survival. It was achieved by transforming the shoot clumps to the elongation medium containing GA$_3$ (1.5 mg/l). These results were confirmed earlier reports.

Shoot elongation was achieved by the synergistic influence of GA$_3$ and BAP. In the present study healthy shoots were transferred to the medium containing 0.25 to 2.5 mg/l GA$_3$ and constant concentration of BAP (1.0 mg/l) and KN (1.0 mg/l).

Optimum growth was noticed on 1.5 mg/l of GA$_3$, BAP (1.0 mg/l) and KN (1.0 mg/l) containing medium. Maximum shoot length was also noticed with this concentration. It was confirmed that the lower level of BAP and KN and higher level of GA$_3$ positively influenced the growth of shoots on the elongation medium. These results were analogous to the findings of Mohamed *et al.* (1992); Kathiravan and Ignacimuthu, (1999). On the contrary GA$_3$ alone at low concentration had a promising effect on shoot regeneration and also shoot elongation (Mc Comb and Bennet, 1986).
The promotive effect of GA$_3$ in combination with BAP on shoot bud induction as well as internode elongation in culture was reported earlier in other perennial medicinal herbs including *Saussurea lappa* (Arora and Bhojwani, 1989), *Ocimum americanum*, *O. Sanctum* and *O. basilicum* (Sahoo *et al.*, 1997). In the present study it was observed that increase in the concentration of GA$_3$ decreased the elongation of shoots. A similar observation was also made in the case of *Vigna radiata* (Amutha *et al.*, 2003), where as GA$_3$ alone supplemented at low concentrations had a promoting effect on shoot regeneration and also shoot elongation in *V. sublebala* (Bhadra *et al.*, 1994) and *Phaseolus vulgaris* (Martins and Sondoahl, 1989; Franklin *et al.*, 1991). About 12 cm of shoot length was observed at 0.3 mg/l of GA$_3$ by Dronne *et al.* (1996) in *Lavendula intermedia*. Thus shoot regeneration and elongation are promoted by the medium supplemented with BAP (1.0 mg/l), KN (1.0 mg/l) and GA$_3$ (1.5 mg/l).

In the present experiment combination of BAP (1.0 mg/l) & KN (1.0 mg/l) with GA$_3$ favoured for shoot elongation and vigorous growth as previously observed in medicinal plants. BAP (1.0 mg/l) and KN (1.0 mg/l) along with GA$_3$ (1.5 mg/l) promoted shoot elongation. Based on the previous reports observed (Kathiravan, K. and Ignacimuthu, 1999).

### 5.3. Rooting and Hardening

The regenerated shoots were excised and transferred on rooting medium supplemented with different concentrations of IBA or IAA (0.25 to 2.0 mg/l). The present observations clearly indicate that auxins were found to induce and enhance rooting from the basal cut ends of the shoots. Highest rate of rooting frequency was observed in the medium
containing 1.0 mg/l of IBA (58.2%) followed by IAA at 1.0 mg/l (52.6%). Similar observations were already made by Hunault (1981), Banerjee (1999) and Hossain et al. (2000). Comparatively IAA/IBA was found to be the most effective rhizogenic agents for *B. monnieri.* (Tiwari *et al.*, 1998). The highest percentage of rooting (100%) and root length were recorded by Manickam *et al.* (2000) in Indian Ginseng plantlets. In accordance with earlier observations in the present study, maximum percentage 58.2 and 50.4 half rooting was recorded IBA and IAA supplemented media respectively.

For further growth and establishment of root, the rooted plantlets were transferred from culture tube to plastic cups containing vermiculite along with supply of MS half strength nutrient solution. Minimum supply of nutrients kept the plantlets under starved condition and induced their growth of roots to scavenge the nutrients available in the substrate. Rooting was best in half strength MS medium supplemented with different auxins in plants like *Plectranthus vetiveroides* (Sivasubramanian *et al.*, 2002) and *Tridax procumbens* (Sahoo and Chand, 1998).

Use of vermiculite gave a maximum of 85% survival of micropropagated plantlets followed by the transfer of them to pot containing sand: FYM: Soil (2:1:1) (Hadid *et al.*, 1995). In accordance with earlier reports, rooted plantlets were found higher survival rate, when transferred to pot containing mixture of sand: FYM: Soil.

Thus during hardening of rooted plantlets, the results of the present investigation are in accordance with similar earlier findings in
some other species such as *Eucalyptus sideroxylon* (Burger, 1987) *Quercus suber* (Romano et al., 1992) *Elaeagnus angustifolia* (Iriondo et al., 1995) *Capparis decidua* (Tyagi and Kothari, 1997) *Melia azadirachta* (Thakur et al., 1998) and *Eucalyptus tereticornis* (Sharma and Ramamurthy, 2000).

**5.4. Hardening with Bioinoculants**

During hardening, various bioinoculants were supplied to plantlets in different combinations. All the bioinoculant treatments influenced the growth and yield of the herbs under pot culture experiment. It may be due to the improved fertility and the soil ecoclimate. Similarly, synergistic influences of organic and inorganic fertilizers on growth and yield of medicinal plants have been reported by Verma (1996).

In the various treatments, chlorophyll content, growth of shoot and root, percentage of alkaloid and phenol content were found significantly increased. Among the different treatments, combination of *Azotobacter*, *Pseudomonas striata* and *Glomus aggregatum* (T8) showed a maximum growth of root and shoot. The chlorophyll content was also increased in this treatment. This result is supported by the earlier observations made by Srivastava et al. (1999). demonstrated that *Azotobacter* inoculation enhanced the root elongation, root branching, root surface area and dry weight of root in cereals crops. In the present attempt inoculation of *Azotobacter* alone showed improved shoot length (74.5 cm), root length (18.15 cm) total alkaloid (1.12 per cent) and phenol content (0.32 μg/g dry wt.).
Combined inoculation of *Azotobacter* and Phosphobacteria (T5) recorded maximum total alkaloid content (1.20%) of the roots of *B. monnieri*. In the present investigation, inoculation of rhizobacteria enhanced the growth and yield parameters of *B. monnieri*. Inoculation of *Azotobacter*, Phosphobacteria and AM Fungi (Ac + Ps + Ga) (T8) enhanced the shoot and root elongation and proliferation. This might be due to the production of growth regulators like IAA and GA3 by these microorganisms. Inoculation of PGPRs strains usually have been found to increase the shoot, root length and shoot root biomass (Yan *et al*., 2003, Chakraborthy *et al*., 2003 and Khalid *et al*., 2004) and this better developed root system may increase the mineral uptake in plants. Organic farming has great relevance with the cultivation of this crop with regard to quality of the products.

Use of organic manures was found essential to boost up yield through low cost farm inputs of *Coleus forskohlii* (Patra *et al*., 1997). In the treatment (T4), *Glomus aggregatum* alone improved the root length, shoot length, phytochemicals like alkaloid and phenol content of the leaf. Several reports have indicated that VAM colonization can greatly improve the growth and nutrition of host plant (Mosse, 1973) and can also suppress soil borne plant pathogens effectively (Rosendahl, 1985, Jalali and Chand, 1988 and Linderaman, 1994). Colonization of mycorrhizae in roots was also known to enhance lignification and increase the amount of vasculature, which serve as barrier for many soil borne root pathogens (Dehne, 1982). The enzyme, chitinase activity in older roots due to the presence of VAM also confines the growth of pathogen (Dehne *et al*., 1978). The inoculation of arbuscualr mycorrhizal fungi i.e., *Glomus aggregatum* have been enhanced the
ability of roots to take up nutrients, especially phosphorous from the soil, and to increase the yield and protein content of the crop (Gerdeman, 1968; Mosse, 1973; Tinker, 1975; Mathur and Vyas, 1990; Sivaprasad et al., 1990). Thus the various treatments favoured for the increased growth and accumulation of biochemical in the experimental plants. The bioinoculants also accelerated the process of hardening and made the regenerated plantlets suitable for field transfer.

5.5. Phytochemical Analysis

India is one of the richest floristic regions of the world and has been a source of plants and their products, since antiquity and man used them in different ways according to his needs, particularly as food and medicine. Despite the availability of different approaches for the discovery of therapeuticals, natural products still remain one of the best reservoirs of new structural types.

The preliminary phytochemical screening of the in vitro leaf samples of B. monnieri showed the presence of alkaloids, flavonoids, phenols, tannins, saponins, phytosterols and carbohydrates, and interestingly these did not show proteins (Table-13).

Presence of secondary metabolites and carbohydrates in B. monnieri suggests that this plant is one of the potential sources of drugs, which could be used for the preparation, formulations and delivery of medicines of various cures, and thus it forms one of the important medicinal plant as suggested by Kapoor (2001) and Gupta and Rana (2007).
Gupta et al., 1996 reported the presence of highest alkaloid content in the leaves of *B. monnieri* in Indian chemotypes. Leaves with tannins and flavonoids (Johri et al., 2005). Thus the leaves of the plant from essential part, as they possess rich chemical constituents and could yield relatively high amount of chemical constituents of medicinal importance.

The qualitative phytochemical analysis of *B. monnieri* indicated the presence of alkaloids, flavonoids, phytosterols, saponins, carbohydrates and tannins in the crude methanolic extracts (Table – 13). The chemical analysis of the previous studies revealed that there are about 25 chemical constituents identified, extracted and isolated from *B. monnieri*. Among them the biologically active chemical constituents include alkaloids, flavonoids and saponins (Nigam and Kandalkar, 1995; Mishra et al., 2000).

GC-MS analysis of *in vitro* leaf samples of *B. monnieri* indicated the presence of various alkaloids, flavonoids and steroidal compounds. Especially in the leaf samples there are 6 compounds recorded by their peak values. They are alkaloids, terpenoids and saturated aliphatic compounds (Table – 14).

In the earlier reports, the chemistry has been extensively studied and over 25 chemical constituents have been identified, extracted and isolated. The biologically active chemical constituents identified were alkaloids (brahmine, herpestine and a mixture of 3 alkaloids), steroidal medicinally important and endangered plant species is being dwindling day by day (Pandey et al., 1993).
It is well known that the plant polyphenols are widely distributed in the plant kingdoms which are sometimes present in surprisingly high concentrations in some specific plant growing under particular environmental conditions.

5.5.1. HPLC Analysis of callus and in vitro plantlets of B. monnieri

HPLC Analysis of callus of B. monnieri:
In the present experiment of HPLC analysis, flavonoids were estimated. Already Watoo Phrompittayarat et al., (2007); Rehni et al., (2007) were reported that the estimation of flavonoids by using HPLC analysis in B. monnieri. In this study Quercitin, Rutin and Gallic acid were determined and only the Quercitin was present slightly high amount. These flavonoids were active constituent of this highly valuable medicinal plant and involved in cognitive and intelectual effects.

HPLC Analysis of in vitro plantlets of B. monnieri
This aspect becomes essential in the study of plants of medicinal importance, as their chemical constituents form the source of pharmaceutical industry. B. monnieri is one of such important medicinal plant, which possesses active principles such as bacosides A & B, brahmine, herpestine, leutioline, quercitine etc., which form the part of the medicinal preprations in the pharmaceutical industries for the cure of various diseases such as rheumatism, rejuvenating the reproductive organs, antiinflammatory, antitumour, antioxidant, anticonvulsive, immune suppressive, etc.,
According to scientists at the Central Drug Research Institute in Lucknow, India, certain “memory chemicals” in Bacopa, called bacosides A and B, help repair damaged neurons by enhancing proteins involved in the regeneration of neural-cell synapses (Rastogi et al, 1994). These are the relay stations of the brain that facilitate the transmission of neural impulses. Thus Bacopa can be viewed as a neural nourisher, restoring depleted synaptic activity and leading to enhanced memory function. In scientific studies, it has been shown to exert a remarkable and unique effect on neurotransmitters. In the present investigation of B. monnieri in in vitro plantlets we analysed the five major saponins viz., bacoside A3, bacopaside II, bacopasaponin C isomer, bacopasaponin C and bacopaside I. Bacopa also has important antioxidant properties and acts as a metal chelator, removing excess damaging metals from the blood, thus limiting the propagation of free radicals.

Dried plantlets of Brahmi were used as raw materials for the extraction as described in previous studies (Bhattacharya et al., 2001; Das et al., 2002). The advantage of using dried plant materials instead of fresh plant materials for herbal products is that the dried plant materials is easier to store and also has longer shelf life.

We mainly used ethanol as an extraction solvent as it has been shown in many studies that the biological activity of Brahmi was found in ethanol extracts of the plant (Bhattacharya et al., 2001; Das et al.,
2002). Also, ethanol is relatively safe and cheap for herbal medicine preparation compared to the toxic methanol or chloroform.

However, the amounts of total saponins in both extracts were not significantly different (6.60±0.12% and 5.89±0.4%) (Table – 16). In the results showed that the yield of the crude extract increased to 26.08±1.25% while the level of the total saponins was higher (8.00±0.67). In Method 5, the plant material was defatted with hexane prior to maceration with ethanol. Both % yield of the extract and the total saponins (16.63±0.87 and 5.64±0.43, respectively) were not different to that without defatting.

Therefore, in Method 9, a percolation method was used in stead of maceration in Method 7. The yield of the extract obtained was not high (10.09±0.07%), but the total amount of saponins was in the same level as that from Method 7 which was the highest among all other methods (19.28±0.12). Moreover, in comparison to other Method 7, the time used for extraction was shorter and high temperature was not needed. Thus, we conclude that among the nine methods studied, Method 9 was the most practical and efficient extraction method for Brahmi. It could be easily up-scaled for commercial purpose.

5.6. Antibacterial activity

Plants have been used for centuries as remedy for human diseases because they contain components of therapeutic values (Kaushik, 1985). They are natural sources of antimicrobial agents primarily because of the large biodiversity of such organisms and the relatively large quantity of metabolites that can be extracted from them. The acceptance of these
traditional medicines as an alternative form of health care has led researchers to investigate the antimicrobial activity of medicinal plants (Nostro et al., 2000).

Screening of plants for antimicrobial activity many plant extracts proves to be a potential source of novel antibiotic prototypes (Maurer-Grimes et al., 1996; Rabe and Van Staden, 1997; Karthikeyan et al., 2008). However, the antimicrobial effect of the plant extracts obtained from different parts was not uniform in the present investigation. The presence of antibacterial and antifungal substances in the higher plants has been well established (Javed Ali, 2002; Belboukhari and Cheriti, 2005).

In the present investigation the leaf extract of *B. monnieri* displayed a broad spectrum of antibacterial activity against Gram negative and Gram positive bacteria so, the *in vitro* leaves of *B. monnieri* was found to contain more antimicrobial compounds.

The antibacterial principles were either polar or non-polar and were extracted only through the organic solvent medium (John Britto, 2001). Earlier reports also clearly indicated that the antibacterial activity was due to different chemical constituents including flavonoids, terpenoids and other compounds which were classified as active antimicrobial compounds (Rojas et al., 1992). Methanolic extracts of leaves of *B. monnieri* were found to have potent antibacterial activity against *Pseudomonas aeruginosa* and *Escherichia coli* (Arora et al., 2004).
In addition, to these results, the present experiment confirmed the previous studies which reported that methanol is a better solvent for more consistent extraction of antimicrobial substances from medicinal plants compared to other solvents, such as water, ethanol and chloroform (Ahmad et al., 1998; Eloff, 1998).

Thus, from the above discussion, it can be concluded that nodal explants are suitable for clonal propagation, and they may be used for higher rate of shoot multiplication. The protocol standardized in the present study is reproducible and can be used in future biotechnological improvement programme of this medicinal plant.