Chapter 2

Review of Literature

2.1. *Bacopa monnieri*

Bacopa monnieri (L) Wettst., an herbaceous perennial herb (family Scrophulariaceae), commonly known as 'brahmi', also called 'the thinking person's herb' (Anonymous, 1988). Extracts of *B. monneri* has long been in use as a source of the herbal preparation which are prescribed in the Ayurvedic system and other ancient Indian system of medicine (Govindrajan et al., 2005; Prasad et al., 2008). It has gained considerable importance because plant contains several triterpenoid saponins (Singh and Dhawan, 1997), and among all 'bacosides' has came into prominence due to their memory enhancing property (Chatterji et al., 1965). Based on the traditional claims of memory enhancer, many classical and proprietors medicines are available in the market. In addition to memory enhancing activity, it is also used for the treatment of cardiac, respiratory, neuropharmacological disorder like insomnia, insanity, depression, psychosis, epilepsy and stress (Russo and Borrelli, 2005; Nadkarni, 1976).

2.1.1. Chemical Constituents

In view of increasing importance of *B. monnieri* in the indigenous system of medicine, several group of researchers carried out its chemical examination (Table 2.1). Chemical structures of some of the saponins isolated and characterized from *B. monnieri* are shown in Fig. 2.1. In 1931, Bose and Bose were the first to report isolation of alkaloid ''brahmine'' from *B. monnieri*. Later Chopra et al. (1956) reported the other alkaloids like nicotine and

herpestine from *B. monnieri*. Sastri et al. (1959) reported the isolation of D-mannitol, and a saponin, hersaponin providing further details of the chemical components of *B. monnieri*.

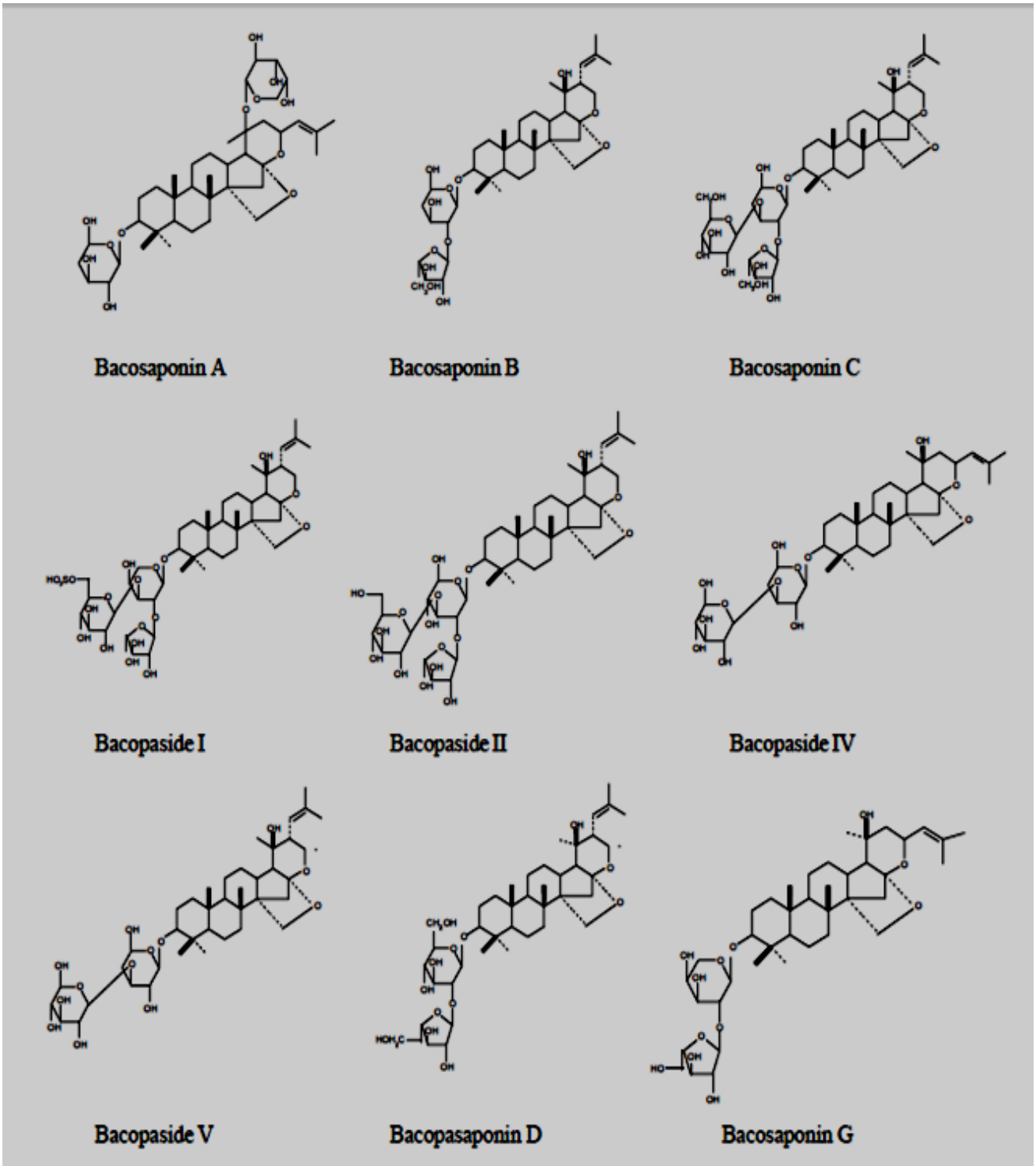


Fig. 2.1 Chemical structures of various saponins isolated from *B. monnieri* (Russo and Borreli, 2005)

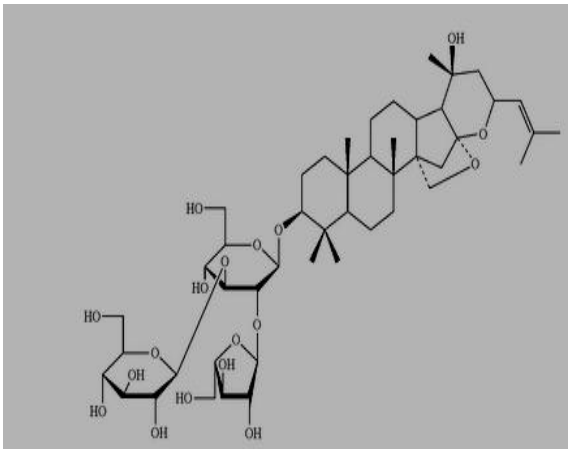
Chatterji et al. (1965) isolated and characterized the major chemical compound responsible for the memory-facilitating action of *B. monnieri*. This compound was identified as 'bacoside A', elucidated as 3-(α-L-arabinopyranosyl)-*O*-β-D-glucopyranoside-10, 20-dihydroxy-16-keto-dammar-24-ene. 'Bacoside A' thus isolated is reported to co-occurs with 'bacoside B', an artefact produced during the process of isolation of 'bacoside A' (Rastogi, 1990). However, Deepak et al. (2005) reported that 'bacoside A' is not a single chemical entity but is a mixture of four triglycosidic saponins viz., 'bacoside A3', 'bacopaside II', 'bacopasaponin C' and the jujobogenin isomer of the latter ('bacopaside X') (Fig. 2.2). The bacosides has been characterized on the basis of chemical and physical degradation studies. On acid hydrolysis, bacosides yield a mixture of aglycones (Kulshreshtha and Rastogi, 1973, 1974) bacogenin A_1, A_2, A_3 (Chandel et al., 1977). In 1994, Rastogi et al., reported the isolation and characterization of 'bacogenin A_4' a pseudojujubogenin, a minor saponin of 'bacoside A_1' and was characterized as 3-*O*-[α-L-arabinofuranoyl (1-3)-β-L-arabinopyranosyl] jujubogenin and a triperpenoid saponin 'bacoside A_3', assigned as 3-β-[*O*-β-D glucopyranosyl (1-3)-*O*-[α-L-arabinofuranosy (1-2)]-*O*-β-D-glucopyranosyl) oxy]. Most of chemical compounds were characterized by chemical and spectral analysis.

Table 2.1 Saponins (characterized using spectroscopic data) reported from *B. monnieri*

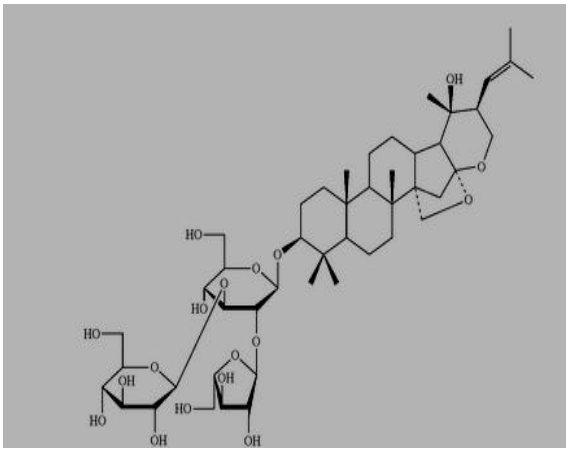
Name	Derivative	Reference
Jujubogenin derivatives		
Bacoside A1	3-O-α-L-arabinofuranosyl(1-3)]-α-L-arabinopyranoside	Jain and Kulshreshtha,1993
Bacoside A3	3-O-α-L-arabinofuranosyl-(1-2)-β-D-glucopyranosyl-(1-3)]-β-D-glucopyranoside	Rastogi et al.,1994
Bacopasaponin A	3,20-di-*O*-α-L-arabinopyranoside	Garai et al., 1996a
Bacopasaponin E	3-*O*-α-L-arabinofuranosyl-(1-2)-β-D-glucopyranosyl-(1-3)]-α-L-arabinopyranoside, 20-*O*-α-L-arabinopyranoside	Mahato et al., 2000
Bacopasaponin F	3-*O*-α-L-arabinofuranosyl-(1-2)-β-D-glucopyranosyl-(1-3)]-β-D-glucopyranoside, 20-*O*-α-L-arabinopyranoside	Mahato et al., 2000
Bacopasaponin G	3-*O*-α-L-arabinofuranosyl-(1-2)]-α-L-arabinopyranoside	Hou et al., 2002
Bacopaside X	[α-L-arabinofuranosyl-(1-2)-{β-D-glucopyranoside-(1-3)}-α-L-arabinopyranosyl]	Murthy et al., 2006
Bacopaside N1	[β-D-glucopyranosyl-(1-3)-β-D- glucopyranosyl]	Murthy et al., 2006
Bacopaside IV	[β-D-glucopyranoside-(1-3)-α-L-arabinopyranosyl]	Murthy et al., 2006
Pseudojujubogenin derivatives		
Bacoside A2	3-*O*-α-L-arabinopyranosyl-(1-5)-α-L-arabinofuranosyl-(1-6)]-α-D-glucofuranoside	Rastogi and Kulshreshtha, 1999
Bacopasaponin B	3-*O*-α-L-arabinofuranosyl-(1-2)]-α-L-arabinopyranoside	Garai et al., 1996a
Bacopasaponin C	3-*O*-α-L-arabinofuranosyl-(1-2)-β-D-glucopyranosyl-(1-3)]-α-L-arabinopyranoside	Garai et al., 1996a
Bacopasaponin D	3-*O*-α-L-arabinofuranosyl-(1-2)]-β-D-glucopyranoside	Garai et al., 1996b
Bacopaside I	3-*O*-α-L-arabinofuranosyl-(1-2)-[6-*O*-sulfonyl-β-D-glucopyranosyl-(1-3)]-α-L-arabinopyranoside	Chakravarty et al., 2001
Bacopaside II	3-*O*-α-L-arabinofuranosyl-(1-2)-β-D-glucopyranosyl-(1-3)]-β-D-glucopyranoside	Chakravarty et al., 2001
Bacopaside III	3-*O*-[6-*O*-sulfonyl-β-D-glucopyranosyl-(1-3)]-α-L-arabinopyranoside	Hou et al., 2002
Bacopaside N2	[β-D-glucopyranosyl-(1-3)-β-D-glucopyranosyl]	Murthy et al., 2006
Bacopaside V	[β-D-glucopyranoside-(1-3)-α-L-arabinopyranosyl]	Murthy et al., 2006

Garai et al. (1996a) reported the isolation of three new dammarane-type triterpenoid saponins, 'bacopasaponins A' identified as 3-*O*-α-L-arabinopyranosyl-20-*O*-α-L-arabinopyranosyl jujubogenin, 'bacopasaponins B' identified as 3-*O*-[α-L-arabinofuranosyl (1-2)-α-L-arabinopyranosyl] pseudojujubogenin and 'bacopasaponins C' identified as 3-*O*-[β-D-glucopyranosyl (1-3) {α-L-arabinofuranosyl(1-2)-α-Larabinopyranosyl] pseudojujubogenin. These three triterpenoid saponins were characterized using spectroscopic technique. These authors (Garai et al., 1996b) also successively isolated and characterized another new dammarane-type pseudojujubogenin glycoside, bacopasaponin D, identified as 3-*O*-[α-L arabinofuranosyl (1-2)-β-D-glucopiranosyl].

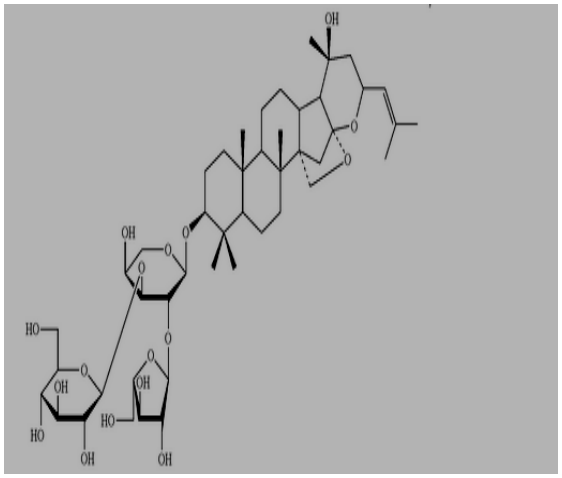
In view of increased interest towards herbal drugs, Chakravarty et al. (2001) undertook a thorough chemical reinvestigation of methanol extract of *B. monnieri* and isolated two new pseudojujubogenin glycosides designated as 'bacopaside I' and 'bacopaside II'. Their structures were elucidated as 3-*O*-α-L arabinofuranosyl- (1-2)-[6-*O*-sulphonyl-β-D-glucopyranosil-(1-3)]-α-L-arabinopyranosyl pseudojujubogenin and 3-*O*-α-L-arabinofuranosyl-(1-2)-[β-D-glucopyranosil-(1-3)]-β-D-glucopyranosyl pseudojujubogenin respectively, using two dimensional (2D) NMR and other spectroscopic techniques. The three more saponins were subsequently isolated from *B. monnieri* by the same group (Chakravarty et al., 2003), these were designated as 'bacopaside III', 'bacopaside IV' and 'bacopaside V', with structures 3-*O*-α-L-arabinofuranosyl-(1-2)-β-D-glucopyranosyl jujubogenin, 3-*O*-β-D-glucopyranosyl-(1-3)-α-L-arabinopyranosyl jujubogenin,3-*O*-β-D-glucopyranosyl-(1-3)-α-L-arabinofuranosyl pseudojujubogenin. The characterizations of these saponins were done on the basis of three dimensional (3D) NMR and other spectral analyses.



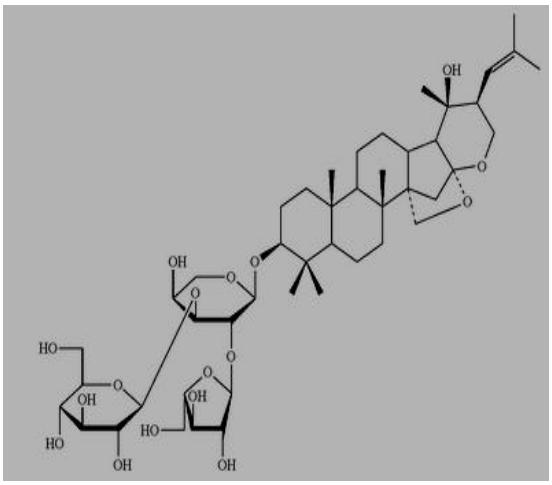
Bacoside A3



Bacopaside II



Bacopaside X



Bacopasaponin C

Fig. 2.2 Chemical structures of various components of 'bacoside A'

In addition, Hou et al. (2002) isolated a new saponin, 3-*O*-[α-L-arabinofuranosyl-(1-2)]-α-L-arabinopyranosyl jujubogenin, named bacopasaponin G, a new alcohol derivative, (3R)-1-octane-3yl-(6-*O*-sulfonyl)-β-D-glucopyranoside, a new phenylethanoid glycoside,3,4-dihydroxy phenyl ethyl alcohol (2-*O*-feruloyl)-β-D-glucopyranoside, and a new glycoside, phenyl ethyl alcohol [5-*O*-p-hydroxybenzoyl-β-D-apiofuranosyl-(1-2)]-β-D-glucopyranoside. Moreover, three new phenyl ethanoid glycosides, viz. monnierasides I–III along with the known analogue plantainoside B have been isolated from the glycosidic fraction of *B. monnieri* (Chakravarty et al., 2002).

Murthy et al. (2006) isolated three new jujubogenin derivatives bacopaside X characterized as [α-L-arabinofuranosyl-(1-2)-{β-D-glucopyranoside-(1-3)}-α-L-arabinopyranosyl], bacopaside N1 characterized as [β-D-glucopyranosyl-(1-3)- β-D - glucopyranosyl] and bacopaside IV characterized as [β-D-glucopyranoside-(1-3)-α-L-arabinopyranosyl]. These authors also isolated two new pseudojujubogenin derivatives, [β-D-glucopyranosyl-(1-3)-β-D-glucopyranosyl], named as bacopaside N2 and [β-D-glucopyranoside-(1-3)-α-L-arabinopyranosyl], named as bacopaside V. These saponins were also characterized using spectroscopic and chromatographic technique.

2.1.2. Biological activity

Extracts of *B. monnieri* have been extensively investigated for their biological activity in traditional as well as scientific literature (Table 2.2). The most important effects of *B. monnieri* are on cognition and memory functions. The alcoholic extracts of *B. monnieri* have been reported to enhance learning ability in normal rats (Malhotra and Das, 1959) and also in human beings (Singh and Dhawan, 1992; Dhawan and Singh, 1996). The alcoholic extracts have also been reported to show effect against amnesic by inhibiting the scopolamine,

electroshock and immobilization of stress (Dhawan and Singh, 1996). The alcoholic extract induces membrane dephosphorylation, with a concomitant increase in protein and RNA turnover in specific brain areas (Singh et al., 1990). Further, *B. monnieri* extracts have been reported to show nootropic activity, by enhancing protein kinase activity in the hippocampus (Singh and Dhawan, 1997).

B. monnieri extracts have been reported to show amnesic action comparable to benzodiazepine anxiolytics (Bhattacharya and Ghosal, 1998). The *B. monnieri* extract were reported to show higher anxiolytic properties and did not induce amnesia but has instead a memory-promoting action in animals and man (Singh and Dhawan, 1992; Dhawan and Singh, 1996). Methanolic extract of *B. monnieri* has been reported to show antidepressant activity in rodent models of depression (Sairam et al., 2002). The effect of *B. monnieri* extract was compared with standard antidepressant drug imipramine. Another important use of *B. monnieri* in traditional medicine is due to its anticonvulsive action. The crude water extract of *B. monnieri* has been reported to control epilepsy (Shanmugasundaram et al., 1991). It offer protection against electroshock seizures and has an involvement of the GABA-ergic system in the mediation of central nervous system (Singh et al., 1996). Substances which stimulate GABA are known to possess anticonvulsant, pain relieving and sedative effects (Shanker and Singh, 2000).

The alcoholic and hexane extract of *B. monnieri* have been reported to show antioxidant activity (Tripathi et al., 1996). Pawar et al. (2001) demonstrated that the hydroalcoholic extracts of the plant exhibited an inhibitory effect on superoxide released from polymorphonuclear cells in nitroblue tetrazolium assay. Sumathy et al. (2001) investigated the hepatoprotective activity of *B. monnieri* alcoholic extract.

Table 2.2 Data of *B. monnieri* extracts used in pharmacological and clinical studies

Extracts	Chemical activity	Reference
Extracts with 95 % ethanol	No severe effects on cognitive function of healthy human	Elangovan et al., 1995
90 % ethanol	Anti-depressant activity in helplessness and forced swim rat models	Singh and Dhawan, 1997
Extract with 90 % ethanol	Significant effect in mice models against cholinesterase and dementia properties	Das et al., 2002
50 % ethanol extract	Curative and Prophylactic effect against the gastric ulcers in rat models	Bhattacharya and Ghosal, 1998
Extract enriched with 50 % ethanol	Significant antioxidant effect against striatum, hippocampus and frontal cortex in rat models	Bhattacharya et al., 2000
50 % ethanol extract	Modulates the central cholinergic function of memory, tested in two rat models of Alzheimer's disease	Bhattacharya et al., 2000
100 % methanol	Significant anti-cancer activity by inhibiting cell growth of Sarcoma 180	Sairam et al., 2001
50 % enriched ethanol	12 week prolonged administration of extract, significantly improved verbal learning, memory consolidation and information processing in healthy humans.	Nathan et al., 2001
Methanolic extracts	Anti-anxiolytic activity in rats comparable to lorazepam	Sairam et al., 2002
50 % *B. monnieri* ethanol extract combined with 50 % extract of *Gingko biloba*	Does have acute effect on normal human on their cognitive functions.	Maher et al., 2002

B. monnieri extracts are known to effectively suppress experimentally induced inflammatory reactions, by inhibiting prostaglandin synthesis and partly by stabilizing lysosomal membranes, and did not cause gastric irritation (Jain et al., 1994). The mast cell stabilising activity of the methanolic extacts of *B. monnieri* has also been reported (Samiulla et al., 2001). It has been found comparable to that of disodium cromoglycate and thus has the potential of application in allergic reactions. The *B. monnieri* were reported to show cytotoxic activity against Sarcoma-180 cell lines in culture (Elangovan et al., 1995; Mathur and Kumar, 1998). The extract of *B. monnieri* has also known to be effective against acute (AS) and chronic stress (CS) (Rai et al., 2003). Thus *B. monnieri* has gained immense importance as antistress agent and thus has been reported to show adaptogenic activity.

2.1.3. Morphological characterization

Assessment of the genetic diversity is important for characterization and conservation of genetic resource (Rao and Hodgkin, 2002). Variations among the populations are characterized by range of methods. Traditionally, morphological characteristics were used for such studies. The conventional way to characterize plants is to study their morphological features such as colour, texture, shape, height etc. (Kuss et al., 1986; Sun and Liddle, 1993). Though, this characterization is carried widely but it has several limitations. It does not serve as a basis of sound characterization and reliable identification because environmental factors are also known to influence morphological trait (Grime, 1979; Hutchings and de Kroon, 1994; Jonsdottir and Watson, 1997).

Mathur et al. (2000) studied the various growth characteristics of fifteen accessions of *B. monnieri* collected from different geographical locations of India. These authors documented considerable variations amongst these accessions. The accessions responded differently to the

environmental conditions, and maximum growth was found in the monsoon season. Subsequently, Mathur et al. (2003) also studied the diversity among twenty seven accessions of *B. monnieri* collected from distinct geographically locations in India. The accessions were assessed based on 13 qualitative and 24 quantitative characters. The variation in the accessions was further investigated by principal component analysis, which accounted for about 46 % variation. The metroglyph analysis using quantitative traits, cluster analysis and dendrogram drawn using D2 data, separated the accessions into seven clusters.

Naik et al. (2012) assessed phenotypic characters in twenty two accessions of *B. monnieri* collected from Karnataka, India. These accessions varied in the phenotypic characters significantly and also showed variations according to different agro-climatic environment of the accessions.

Most of the above mentioned reports were on the study of the morphological features and no report on the biomass, harvest index and growth from the selected plants of *B. monnieri* is available. However, it is important to study the growth parameters, which will be helpful in selection of elite clones with high growth rate and identification of season will be helpful in developing the harvest strategy.

2.1.4. Biochemical characterization

Plants are the rich source of bioactive molecules, which are used for treatments of many human diseases (Gupta and Chadha, 1995). The wild accessions of medicinal plants have been reported to show considerable variations in the levels of active principle (Nadeem et al., 2007). Biochemical characterization helps in identifying accession with high secondary metabolite content. However, the concentration and composition of these secondary metabolites varies in nature amongst the members of same species (Bagdonaite et al., 2010).

Also, biochemical diversity resulted from the plasticity of plant secondary metabolism which has evolved to respond to stress and interactions with continuously changing environment (West-Eberhard, 1989).

Mathur et al. (2000) studied variation in 'bacoside A' content during different seasons among the fifteen accessions of *B. monnieri* collected from different geographical locations of India. It was found that in most of the accessions 'bacoside A' content was higher during monsoon season. Ganjewala et al. (2001) also reported that maximum 'bacoside A' content in samples collected during monsoon period. Mathur et al. (2003) also evaluated twenty seven accessions of Indian germplasm using multivariate approaches. All the accessions were examined for their genetic variability. The accessions were grown in earthen pans, arranged in completely random block design, and were observed for bacoside content of plant yield. On the basis of principal component analysis and canonical variable analyses, accessions were grouped into cluster representing wide geographical origin. This study reported the interaction between the gross agroclimatic environments of a region with the microenvironment of the *B. monnieri*, where genotype occurred.

Phrompittayarat et al. (2011) studied the variation in the 'bacoside A' content during different seasons of year. The highest 'bacoside A' content in shoots of *B. monnieri* was observed during rainy seasons. The high 'bacoside A' content in rainy season can be due to the high temperature and moisture content which are favouring production of saponins in Brahmi.

Naik et al. (2012) evaluated 'bacoside A' content in different accessions of *B. monnieri* collected from different locations of Karnataka. These accessions showed significant variation in the content of 'bacoside A', showing genetic variability amongst accession. The

author reported that variation in the ‘bacoside A’ content amongst accessions is due to the variability in agro-climatic environment of the plant origin.

Sharma et al. (2013) evaluated the bacoside production on monthly basis for two consecutive years in the accession collected from Jammu. Significant variation in bacoside content was observed during the course of whole year. Maximum bacoside content was obtained during July to October (monsoon), whereas minimum bacoside content was found in samples collected during March-June (summer).

Although, variation in active principle among accessions is reported to be influenced by both genotype and environmental factors (Cirak et al., 2007), yet there is no report on the variations in the levels of ‘bacoside’ contents amongst different populations collected from different places after growing at a common location during different times of the year. An investigation for the selection of genotypes capable of accumulating higher levels of active principle will be useful. Further, this study will help in identification of season, when ‘bacoside A’ levels are higher, which will help in developing management and conservation strategy for this important medicinal herb.

2.1.5. Molecular characterization

The genetic diversity within species is crucial for breeding and improvement programme. PCR-based molecular markers are widely used in many plant species for identification, phylogenetic analyses, population studies and genetic linkage mapping (Williams et al., 1990). Molecular markers highlight genetic differences (polymorphisms) between different individuals and are stable markers. These markers do not, however give encompass on the activity of specific genes. These markers are not influenced by environmental factors and generate reliable, reproducible results (Li et al., 2011). DNA-based markers that are most

frequently in use include: restriction fragment length polymorphism (RFLP, Singh et al., 1999), amplified fragment length polymorphism (AFLP, Sharma et al., 2007a), random amplified polymorphic DNA (RAPD, Williams et al., 1990), inter simple sequence repeats (ISSR, Zietjiewicz et al., 1994; Sood et al., 2002) and single nucleotide polymorphism (SNP's, Gupta et al., 2001).

The RAPD and ISSR markers have proven to be efficient in detecting genetic variation within and between populations (Welsh and Mc Celland, 1990). Both RAPD and ISSR markers have been successfully used to detect the genetic variability in plants (Martin et al., 2006; Carvalho et al., 2004; Martins et al., 2004; Ramage et al., 2004; Sanchez et al., 2003; Al-Qurainy et al., 2013). There are many reports highlighting the use of combination of two markers amplifying different regions of genome to study the genetic diversity in plants (Lattoo et al., 2006; Martin et al., 2006; Ray et al., 2006; Dhiman and Singh, 2003; Palombi and Damiano, 2002; Nag et al., 2012).

In 2001, Darokar et al. first investigated genetic diversity amongst twenty four accessions of *B. monnieri* collected from different agroclimatic regions of India and Malaysia using RAPD based markers. During this study a total of forty RAPD primers were used, out which thirty seven primers generated polymorphic bands. These primers produced a total of 222 clear, distinct and reproducible amplicons. The size of bands produced by these primers ranged from 100 to 2500 bp. Similarity matrices generated from the RAPD data on the basis of Nei's estimates showed 0.8-1.0 similarity level among all accessions.

Karthikeyan et al. (2011) investigated genetic diversity amongst accessions of *B. monnieri* collected from four South Indian states, along with *in vitro* micropropagated samples maintained in the laboratory for 5 years. RAPD fingerprinting approach was applied to assess genetic diversity in accessions using 10 primers. These primers generated 110 clear, distinct

and reproducible bands. The size of bands produced by these primers ranged from 250 to 870 bp. Primer produced fragments showed 12.72 % polymorphic bands. Cluster analysis generated on the basis of similarity co-efficient, indicated genetic similarity within the accessions varied from 0.24 to 0.80, the matrix ranged from 0.36 to 0.80.

Tripathi et al. (2012) studied genetic diversity amongst the fifteen accession of *B. monnieri* collected from various location of Central India using RAPD and ISSR markers. During the study, twenty two RAPD primers generated 197 clear, distinct and reproducible bands, of which 187 were polymorphic. Twenty five ISSR primers produced 284 clear, distinct and reproducible bands, of which 270 bands were polymorphic. The amplified products varied in size from 240 to 2800 bp. Similarity index values ranged from 0.16 to 0.95 (RAPD), 0.18 to 0.98 (ISSR) and 0.179 to 0.945 for combined ISSR and RAPD data. The results indicated high level of genetic diversity amongst the accessions of Central India.

2.1.6. Micropropagation

Micropropagation is carried out in a controlled and artificial environment, and includes induction of adventitious buds, and somatic embryogenesis. This technique is in fact the outcome of work by Haberlandt (1902) who attempted the first plant cell culture on a nutrient medium and laid the foundation of plant tissue culture. This technique offers a rapid means of multiplication, and thus useful in conservation of elite and endangered germplasm (Bajaj, 1986; Karp, 1994; Roja and Rao, 1998). Successful regeneration of plants is considered a prerequisite for application of modern genetic and biotechnological approaches for crop improvement (Litz and Gray, 1992; Rai et al., 2010). In recent years, several reports have been published on regeneration of *B. monnieri* through organogenesis and somatic embryogenesis.

Tiwari et al. (1998) reported *in vitro* propagation of *B. monnieri* using different explants. Maximum frequency of adventitious shoot buds were induced on MS medium supplemented with α-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BA). Mathur and Kumar (1998) reported shoot bud induction from internodal explant of *B. monnieri*. Higher frequency of shoot buds were induced on PGR-free MS medium.

Shrivastava and Rajani (1999) achieved adventitious shoot bud regeneration from leaf and internode explant of *B. monnieri*. Both explant type and gelling agent used in the medium was found to influence shoot bud induction. Highest percent of explants showed shoot bud induction from leaf explant taken from micro-shoots growing on MS medium supplemented with BA.

Tiwari et al. (2000) reported micropropagation of *B. monnieri* using nodal segment. Shoot bud induction and rooting of microshoots at higher frequency were obtained in liquid MS medium in a shake culture. Tiwari et al. (2001) also reported *in vitro* propagation using node, internode and leaf as explant. These explants were cultured on MS medium supplemented with different concentrations of BA, thidiazuron (TDZ), Kinetin (KIN) and 2-isopentenyladenine (iP). Maximum shoot bud induction was achieved on MS medium supplemented with TDZ. Rooting of these micro-shoots was obtained on MS medium supplemented with indole-3-butyric acid (IBA).

Tejavathi et al. (2001) achieved reported shoot multiplication from shoot tip and node on MS medium supplemented with KIN. Regenerated plantlets following rooting was transferred to soil after a period of hardening.

Binita et al. (2005) reported shoot organogenesis in *B. monnieri* using different explant. Higher percent of explants regenerated shoots from leaf explant cultured on MS medium

supplemented with BA (1.1 μM). Maximum shoot bud proliferation was achieved on agar gelled medium when leaf was used as explant, whereas liquid medium was more effective for nodes and internode explant.

Sharma et al. (2007b) reported shoot multiplication from nodal explant of *B. monnieri* cultured on MS medium supplemented with BA. Rooting was also achieved at higher frequency from these shoots on the same MS medium. Banerjee and Shrivastava (2008) reported *in vitro* propagation of *B. monnieri* using inter-nodal segment. These explants were cultured on MS medium supplemented with BA and KIN. The combined effect of BA and KIN resulted in maximum shoot proliferation with 6.33-fold increase in shoot length over the control. Maximum percent of shoots induced to roots on MS medium supplemented with NAA.

Vijayakumar et al. (2010) achieved *in vitro* propagation of *B. monnieri* on MS medium supplemented with BA + KIN + NAA. Rooting of these microshoots was achieved on MS medium supplemented with TDZ + indole-3-acetic acid (IAA). Callus from these microshoots was induced on MS medium supplemented with NAA + 2, 4-dichlorophenoxy acetic acid (2, 4-D) + TDZ. Thakur et al. (1978) also reported shoot organogenesis in *B. monnieri* using internode segment. Shoot regeneration was achieved at a maximum frequency when cultured on PGR-free medium.

In 1998, Tiwari et al. reported shoot regeneration and somatic embryogenesis from the different explants of *B. monnieri.* These explants were cultured on MS medium supplemented with BA and KIN. Maximum number of shoot buds per explant differentiated from leaf explant when transferred to MS medium supplemented with 6.6 μM- 8.87 μM BA. Shoot elongation was achieved on MS medium supplemented with BA and IAA. Rooting of these

shoots was achieved on full and half strength basal MS medium. Callus derived from nodal explant when cultured on MS supplemented BA and KIN, differentiated somatic embryos. Same authors in 2006 achieved shoot bud regeneration on antibiotic such as trimethoprim (TMP) or fungicide bavistin (BVN). Maximum shoot buds were differentiated from the internode explant cultured on MS medium supplemented with BVN.

Ceasar et al. (2010) achieved shoot regeneration from leaf and internode explant of *B. monnieri*. Maximum soot buds were obtained on MS medium supplemented with TDZ and NAA. Shoot multiplication was achieved at higher frequency on MS medium supplemented with BA. Maximum of root induction was achieved in half strength MS medium supplemented with IBA and phloroglucinol.

Joshi et al. (2010) also reported shoot organogenesis from leaf explant of *B. monnieri* cultured on MS medium supplemented with BA. Rooting of these microshoots was achieved on half strength basal MS medium supplemented with IBA.

Tiwari et al. (2012) reported synergitic effect of TMP and BVN on *in vitro* propagation of *B. monnieri* using node, internode and leaf as explant. Organogenesis without callus formation from leaf explant was achieved on PGR free MS medium. Shoot multiplication at higher frequency was obtained when cultured on MS medium supplemented with TMP and BVN.

2.1.7. Production of secondary metabolite

Biotechnological approaches, specifically plant cell tissue and organ culture play a vital role in developing alternatives methods for the production of desirable active principle from plants (Rao and Ravishankar, 2002). The capacity of plant cell, tissue, and organ cultures to produce and accumulate many of the valuable chemical compounds similar to parent plant in

nature has been well recognized (Shimomura et al., 1986; Payne et al., 1991; Buitelaar and Tramper, 1992; Fowler and Stafford, 1992; Benjamin et al., 1994; Sevon et al., 1998). Plant-produced secondary compounds have been used in wide range of commercial and industrial applications. Plant tissue culture techniques offer the rare opportunity to tailor the chemical profile of a phytochemical product, by manipulation of the chemical or physical microenvironment, to produce a compound of potentially more value for human use (Misawa, 1985; Stafford et al., 1986).

Application of cell culture and hairy root culture leads to decrease in the variability of secondary metabolite content due to genetic stability of such cultures (Rao and Ravishankar, 2002). The prospective of cell and hairy root cultures in increasing the biomass and secondary metabolite production has been investigated by many researchers (Zehra et al., 1999; Zhao et al., 2001; Lian et al., 2002; Prakash et al., 2005; Piatczak et al., 2012; Nagella and Murthy, 2010; Nagella et al., 2013; Sudha et al., 2013).

2.1.7.1. Callus and cell suspension cultures

Cell suspension cultures are rapidly dividing homogenous suspension of cells that allow rapid and uniform access to nutrition and precursors (Mustafa et al., 2011). Cell suspension cultures are more advantageous for the large scale production of fine chemicals in bioreactors (Sivanandhan et al., 2013). The applicability of cell suspension cultures to improve the secondary metabolite content and biomass accumulation has been proved by several studies (Ravishankar and Venkataraman, 1993; Dixon, 1999; Ravishankar and Ramachandra Rao, 2000; Zhao et al., 2001)

Rahman et al. (2002) was the first to report the callus cultures of *B. monnieri*. The frabile callus was established on the MS medium supplemented with NAA (5.3 µM), KIN (2.3 µM),

casein hydrolysate (1 g/l) and sucrose (30 g/l). The callus cultures thus established accumulated higher levels of bacoside content than tissue culture raised plants.

Showkat et al. (2010) reported the establishment of callus cultures of *B. monnieri* from leaf explants on MS medium supplemented with 2, 4-D. A good friable callus was obtained on medium supplemented with 2.2 µM 2, 4-D. Mehta et al. (2012) reported the establishment of callus cultures using leaf as explant on a modified MS medium supplemented with 2, 4-D and KIN. The friable callus was obtained on MS medium supplemented with 1.1 µM 2, 4-D and 2.3 µM KIN.

Cheng et al. (2006) studied the effect of different concentration of 2, 4-D and BA on the biomass and production of dehydrocavidine and berbirine in the suspension cultures of *Corydalis saxicola*. Maximum growth and dehydrocavidine, berbirine production was observed in B5 medium. Maheshwari et al. (2007) investigated the effect of different concentration of NAA and BA on the establishment of callus and cell suspension cultures. Maximum cell growth and secondary metabolite production was obtained in the medium supplemented with 5.3 µM NAA and 2.2 µM BA. Nagella and Murthy (2010) observed the effect of different growth regulators on 'withanolide A' production and optimized medium formulations to increase cell growth and 'withanolide A' production.

Though work on callus culture of *B. monnieri* has been carried out, but there is need to understand the role of different growth regulator on establishment of cell suspension cultures. Its relationship with cell growth and 'bacoside A' production also needs to be evaluated.

2.1.7.2. Hairy root cultures

Hairy root obtained following infection with *A. rhizogenes* has received attention for the production of secondary compounds (Flores and Curtis, 1992). These can be maintained in

culture on PGR-free medium for long and also display an interested growth capabilities (Tepfer and Tempe, 1981; Hibino and Ushiyama, 1999). Hairy roots are more stable for the production of secondary metabolites as compared to other cultures (Sevon et al., 1998). Hairy roots have been studied for enhancing the biomass and active principle production by various researchers. Overview of secondary metabolite produced from hairy root culture in various plants is given Table (2.3).

Majumdar et al. (2011) attempted to establish hairy root from leaf explants of *B. monnieri* using two strains of *A. rhizogenes* (A4, LBA9402). Transformed roots induced by strain LBA9402 spontaneously differentiated callus (tortomas), whereas roots induced by strain A4 showed induction of shoot buds within 10 days. The integration of T-DNA was confirmed by amplification of *rolAB, rolA*, TR and *ags* gene using PCR and RT-PCR studies. Growth and biomass accumulation was significantly increased by two-fold in the transformed shoots and fourfold in roots than in the non-transformed plants.

Subsequently, Majumdar et al. (2012) reported the transformation of *B. monnieri* with a gene encoding cryptogein, a proteinaceous elicitor. They were able to obtain transformed tissue on kanamycin supplemented medium which was confirmed by amplification of *rol* and crypt gene using PCR and RT-PCR analysis. Ri-crypt transformed plants showed significantly enhanced accumulation of ‘bacoside A3’, ‘bacopasaponin D’, ‘bacopaside II’, ‘bacopaside III’ and ‘bacopaside IV’.

Table 2.3 Hairy root cultures derived from some of plant species used for the production of secondary metabolites

Plant	Secondary metabolite	Reference
Aconitum heterophyllum	Aconites	Giri et al., 1997
Atropa belladonna	Atropine	Christen, 1999
Azadirachta indica A. Juss	Azadirachtin	Allan et al., 1999
Beta vulgaris	Betalaine pigments	Thimmaraju et al., 2004
Brugmansia candida	Tropane alkaloids	Pitta-Alvarez et al., 2000
Brugmansia candida	Tropane alkaloids	Cardillo et al., 2013
Catharanthus roseus	Indole alkaloids, ajmalicine	Vitali and Ventrone, 2002
Centranthus rubber	Valepotriates	Christen, 1999
Cinchona ledgeriana	Quinine	Hamill et al., 1989
Datura candida	Scopolamine, Hyoscyamine	Christen et al., 1989
Datura stramonium	Hyoscyamine, Sesquiterpene	Payne et al., 1988
Daucus carota	Flavonoids, Anthocyanin	Bel-Rhlid et al., 1993
Fagra zanthoxyloids Lam.	Benzophenanthridine	Couilerot et al., 1999
Fragaria	Flavanol	Motomari et al., 1995
Geranium thubergee	Polyphenol	Ishimaru and Shimomura, 1991
Gmelina arborea	Verbascoside	Dhakulkar et al., 2005
Harpagophytum procumbens	Iridoid and phenylethanoid glycoside	Grabkowska et al., 2010
Hyoscyamus muticus	Hyoscyamine, lubumin	Singh et al., 1998
Lithospermum erythrorhizon	Sesquiterpenes, hernandulcin	Fukui et al., 1998
Lobelia cardinalis	Shikonin, Benzoquinone	Yamanaka et al., 1996
Lotus corniculatus	Lobeline, polyacetylene	Carron et al., 1994
Nicotiana rustica	Nicotine, Anatabine	Hamill et al., 1986
Nicotiana tabacum	Nicotine, Anatabine	Flores and Filner, 1985
Nicotiana tabacum	Alkaloid nicotine	Zhao et al., 2013
Ocimum basilicum	Rosmarinic acid	Bais et al., 2002
Panax ginseng	Ginsenosides	Washida et al., 1998

Papaver somniferum	Codeine	Arellano et al., 1996
Pogostemon Cablin	Coumarins	He- Ping et al., 2011
Rhamnus fallax	Arthaquinones	Rosic et al., 2006
Rauwolfia serpentine	Reserpine	Lodhi et al., 1996
Ruta graveolens	Furanocoumarins	Sidwa-Gorycka et al., 2003
Saussurea involucrata	Syringin	Chun-Xiang et al., 2005
Solanum lacinialum	Steroidal alkaloids	Yu et al., 1996
Withania somnifera	Withanoloides	Banerjee et al., 1994

A close survey of literature reveal that there is no report on the establishment of stable lines of hairy roots in *B. monnieri*. There is also a need to understand the effect of *Agrobacterium rhizogenes* strains on root induction.

2.1.8. Optimization of growth and secondary metabolite production

Production of various products on large scale often needs optimization and up-scaling for maximum production. Secondary metabolism is reported to be influenced by media composition and other physio-chemical factors such as temperature, pH, agitation etc. (Chattopadhyay et al., 2002). Physical factor and chemical constituent of the medium helps in induction of pathways for the production of secondary metabolite (Ramakrishna and Ravishankar, 2011; Hussain et al., 2012). These chemical and physical factors are reported to vary plant to plant, thus need optimization for each plant (Ghaemi-Oskouie et al., 2008).

Several strategies have been used for the optimization of physical factors as well as medium components. The most commonly used method being the ‘one-variable-at-a-time (OVAT) technique, in which one factor is changed for determining optimal concentration while keeping the others at a constant level (Srivastava and Srivastava, 2012). The other method of optimization is statistical optimization using response surface methodology (RSM).

Response surface methodology (RSM) is a tool comprised of experimental strategies, mathematical models and statistical inference for constructing and exploring relation between a response variable and set of design variables (Placket and Burman, 1946; Khuri and Cornell, 1987; Myers and Montgomery, 1995; Murthy et al., 2000; Xin et al., 2005).

The multivariate approach of RSM helps in evaluation of interactions among different parameters involved in the process (Khuri and Cornell, 1987; Myers and Montgomery, 1995). In the first phase, this method simplifies the huge number of factors. It screens and selects the variables which have major positive effect on response (Plackett and Burman, 1946). This phase makes considerable use of the first-order model. Upon selection of relevant factors RSM is used to work out optimum level of each factor that has major effect on desired response. The true response surface usually exhibits curvature near the optimum and second-order model (or perhaps some higher-order polynomial) is then used. Once the required model has been obtained, it is analyzed to determine the optimum conditions for the process. In this, the response surface is expressed in the form of second degree polynomial equation as:

$$Yi=\beta 0+\Sigma\beta iXi+\Sigma\beta iiXi2+\Sigma\beta ijXiXj$$

where Yi is the response variable, XiXj are input variables which influence the response variable Y; βo is the interception coefficient; βi is the ith linear coefficient; βii is the ith quadratic coefficient and βij is the ijth interaction coefficient.

The significant terms in the model are assessed by analysis of variance (ANOVA) for each response. Level of significance is judged by determining the probability level of the F-statistic calculated from the data less than 5 %. Maximization and minimization of the

polynomials fitted is performed by desirability function method, and mapping of the fitted responses is achieved using computer software such as Design Expert 7.0.0.

2.1.8.1. One-variable-at-a-time (OVAT) optimization

The applicability of OVAT to improve the biomass accumulation and secondary metabolite production has been proved by several studies (Yu et al., 2001; Wang and Tan, 2002; Zhang et al., 2012; Singh and Chaturvedi, 2012). Naik et al. (2010) studied the effect of sucrose concentration and medium pH on biomass and 'bacoside A' in the shoot cultures of *B. monnieri*. They found that sucrose at 2 % concentration and medium pH of 4.5 was optimum for biomass accumulation and 'bacoside A' production. The same authors also investigated the effects of macro nutrients (NH_4NO_3, KNO_3, $CaCl_2$, $MgSO_4$ and KH_2PO_4) and nitrogen source [NH_4^+/NO_3^-] on adventitious shoot culture of *B. monnieri* for the production of biomass and 'bacoside A' (Naik et al., 2011). Optimum number of adventitious shoots and 'bacoside A' content was obtained on the medium with double strength of NH_4NO_3. Also number of adventitious shoot biomass and 'bacoside A' content were optimum when the NO_3^- concentration was higher than that of NH_4^+.

Pavlov et al. (2009) optimized growth of hairy roots and secondary metabolite content with OVAT technique. Sivanandhan et al. (2013) investigated the application of OVAT approach on cell suspension cultures of *Withania somnifera*. They found significant increase in the 'withanoide A' production and cell growth. Murthy et al. (2013) studied the effect of medium pH and sucrose concentration on the hairy root cultures of *Withania somnifera*. They found the increase in 'withanolide A' production and root biomass at a 2 % sucrose concentration and medium pH set at 5.8.

2.1.8.2. Optimization using response surface methodology

RSM has become a very popular technique for the optimizing the medium components. Many researchers have applied this process for optimization of medium components and other variables for the production of various secondary metabolites, enzymes and other fermentation products (Rao et al., 2000; Chattopadhyay et al., 2002; Chauhan et al., 2006; Rajendran et al., 2008; Parkash and Srivastava, 2008).

Parkash and Srivastava (2005) carried out optimization of cell growth and azadirachtin production in *Azadirachta indica* cell suspension cultures using RSM. Their investigation revealed that glucose, phosphate and inoculum level influenced the cell growth and azdirachtin production. Ryad et al. (2010) reported the *Datura stramonium* hairy roots medium optimization using RSM and found increase in the biomass by 51.2 % and production of hyoscyamine by 81 %. Srivastava and Srivastava (2012) used RSM to optimize production from hairy root culture of *Azadirachta indica*. They found 68 % enhancement of azadirachtin production than that obtained under non-optimized condition.

Many studies have highlighted the importance of RSM for the production of various fermentation products by optimizing various parameters and interactions involved in the final product. But, no report till date has been traced on the standardization of conditions using RSM on cell and hairy root cultures of *B. monnieri*. Therefore it was felt important to investigate and optimize the major factors involved in biomass production so as to enhance the production of 'bacoside A'. Along with RSM, optimization using OVAT technique also needs to be evaluated on cell and hairy root cultures of *B. monnieri*.