Evaluation and production of bacosides from selected clones of *Bacopa monnieri* (L.) Wettst.

A Thesis
Submitted in fulfilment of the requirement
for the award of degree of

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

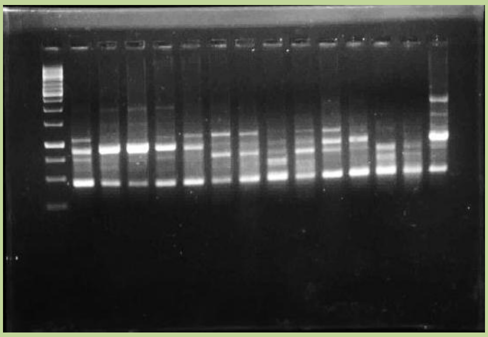
By

Mahima Bansal
(Reg. No. 900900006)

Department of Biotechnology

Thapar University, Patiala –147004

Punjab (India)



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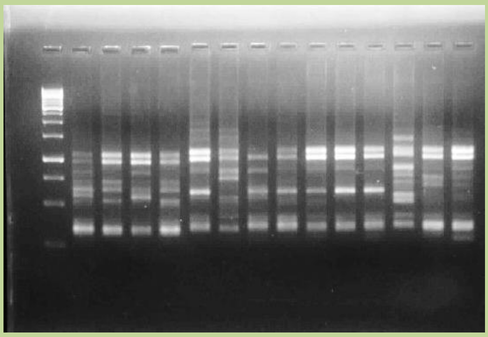

Thapar University

CERTIFICATE

Certified that the thesis **"Evaluation and production of bacosides from selected clones of *Bacopa monnieri* (L.) Wettst."** which is submitted by **Ms. Mahima Bansal**, in fulfilment of the requirement for the award of the degree of **Doctor of Philosophy** in the **Department of Biotechnology**, Thapar University, Patiala, is a record of the candidate's own independent and original research work carried out by her under our supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

(Dr. Anil Kumar)
Associate Professor
Department of Biotechnology,
Thapar University,
Patiala – 147004
Punjab

(Dr. M. Sudhakara Reddy)
Professor
Department of Biotechnology
Thapar University,
Patiala – 147004
Punjab

(Dr. Dinesh Goyal)
Head & Professor
Department of Biotechnology,
Thapar University,
Patiala – 147004
Punjab

Thapar University

DECLARATION

I hereby declare that the work which is being presented in this thesis **"Evaluation and production of Bacosides from selected clones of *Bacopa monnieri* (L.) Wettst."** submitted by me for the award of the degree of *Doctor of Philosophy* in the Department of Biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervisions of **Dr. Anil Kumar**, Associate Professor and **Dr. M. Sudhakara Reddy**, Professor, Department of Biotechnology, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

(Mahima Bansal)

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This thesis is the end of my journey in obtaining my Ph.D, probably the most challenging activity of my life yet. It was a long journey that I finished, not alone!! One of the joys of completion is to look over the past and remember all people who supported me along this long, but fulfilling road. In pursuit of this academic endeavour, I feel that I have been singularly fortunate because inspiration, guidance, direction, cooperation, love and care - all came in my way in abundance and it seems almost an impossible task for me to acknowledge the same in adequate term.

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I dedicate this thesis to

My parents for their constant support and unconditional love!!

The following publications are the outcome of the present research work:

1. **Bansal M**, Kumar A, Reddy MS (2014). Diversity among wild accessions of *Bacopa monnieri* (L.) Wettst. and their morphogenetic potential. Acta Physiologiae Plantarum. 36: 1177-1186.

2. **Bansal M**, Kumar A, Reddy MS (2014). Influence of *Agrobacterium rhizogenes* strain on hairy roots induction and 'bacoside A' production from *Bacopa monnieri* (L.) Wettst. Acta Physiologiae Plantarum. DOI 10.1007/s11738-014-1650-5.

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Conference presentations

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ABSTRACT

Plant derived natural products represent some of the most important pharmaceuticals available today. However, uncertainty regarding the commercial supply due to the limited availability of many plants in nature has resulted in a dramatic reduction in the use of natural products. Plant cell suspension culture capable of large scale industrial production of such pharmaceutically important molecules is the alternative which promises sustained and assured supply of these important molecules. Hairy roots induced following infection with *Agrobacterium rhizogenes* are capable of unlimited growth in culture in the absence of plant growth regulator and exhibit higher potential for the production of secondary metabolite production. The objectives of the present study were to select elite clones of *B. monnieri,* investigate the production of 'bacoside A' using cell suspension cultures and hairy root cultures and finally enhancing the production of 'bacoside A' of cell suspension cultures and hairy root cultures.

Fourteen accessions of *B. monnieri* (BM1- BM14) collected from different locations across India were maintained in nursery at Thapar University. Variation in the content of 'bacoside A' and biomass per plant in fourteen accessions of *B. monnieri* were studied during the different seasons of the year. Maximum biomass accumulation and 'bacoside A' contents were recorded in the samples processed in summer (June) in all the accessions and minimum biomass and 'bacoside A' content was recorded in winter (December). Amongst accessions, BM1 and BM7 recorded higher biomass accumulation, 'bacoside A' content, Relative growth rate (RGR) and Harvest Index (HI). These parameters showed minimum values in accession BM14. Molecular diversity was then investigated amongst these accessions using random amplified polymorphic DNA (RAPD) and Inter simple sequence repeats (ISSR). About, 35 % variations were detected in these populations based on combined data of RAPD and ISSR.

Clustering based on molecular marker data grouped these accessions into two major groups and placed accession BM14 as an out group. Maximum shoot organogenic potential was observed in accession BM6 and maximum rooting potential was observed in accessions BM1, BM2, BM7, BM10 and BM14. Based on *in vitro* morphogenetic response, ‘bacoside A’ content and growth, accession BM6 was selected for the studies of cell suspension and hairy root cultures. Cell suspension cultures established on MS medium supplemented with α-Napthaleneacetic acid (NAA) at 5.0 µM and Kinetin (KIN) at 1.15 µM showed maximum cell growth and levels of ‘bacoside A’. Hairy roots induced by strain MTCC 2364 showed higher biomass accumulation and 'bacoside A' content by five-folds. Further, attempts were made to optimize growth and production of ‘bacoside A’ using cell suspension and hairy root cultures for commercial supply. Optimization of conditions for ‘bacoside A’ production was explored using conventional method (one-variable-at-a-time approach) and statistical method (Response surface methodology, RSM). Optimization of medium components using RSM leads to around two-fold increment in biomass accumulation and levels of ‘bacoside A’. The current study uncovered several aspects of the strategies for the conservation of this medicinally important herb and increased the ‘bacoside A’ production using cell suspension and hairy root cultures. These have the potential of up-scaling in the bioreactors for the production of ‘bacoside A’. Thus can be helpful to reduce the pressure on the wild populations.

TABLE OF CONTENTS

List of Figures

List of Tables

Abbreviations

Abbreviations	Word (s)
%	Percent
2,4-D	2,4-Dichlorophenoxy acetic acid
ºC	Degree Celsius
Abs	Absorbance
BA	6-Benzylaminopurine
bp	Base pair
cm	Centimeter
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotide Triphosphates
EDTA	Ethylene Diamine Tetraacetic Acid
g	Gram
h	Hour
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid

ISSR	Inter-simple sequence repeat
kb	Kilobase
KIN	Kinetin (N6-furfuryladenine)
M	Molar
m	Meter
mg	Milligram
min	Minute
ml	Mililitre
mM	Milli molar
µM	Micro molar
MS	Murashige and Skoog medium 1962
NAA	1-Naphthaleneacetic acid
OD	Optical density
PCR	Polymerase chain reaction
PGRs	Plant growth regulator (s)
rpm	Rotation per minute
PCA	Principal component analysis
RAPD	Random amplified polymorphic DNA

s	Second
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
Tris	Tris-(hydroxymethyl)- aminomethane
U	Unit
UV	Ultra Violet
V	Volt
v/v	volume by volume
w/v	weight by volume`
YMB	Yeast Mannitol Broth
µg	Microgram
µl	Microliter
µm	Micrometre
µmol	Micromole
µM	Micromolar

Chapter 1

Introduction

1.1. General Introduction

"Eat leeks in March and wild garlic in May, and all the year after the physicians may play" (Tyler, 1987). Finding healing powers in plants is an ancient idea since the Vedic period (Rigveda- 4500-1600 B.C.) (Hussain et al., 2012). Initially, these were the main part of the folk or enthomedicine, practised in India and other parts of the world. Later a considerable part of this indigenous knowledge was documented and eventually passed into organised system of medicines such as Ayurveda, Yunani, Sidha and other systems (Arjariya and Chaurasia, 2009).

Medicinal plant preparation enjoyed the maximum representation in the indigenous system of medicine (Rates, 2001). In spite of advances in development of synthetic drugs and antibiotics, these are major sources of drugs in modern as well as traditional system of medicine (Newman et al., 2000; 2003). Plant based drugs are considered safe and more effective in comparison to synthetic drugs, which have higher risk of side effects (Butler, 2004). This has led to an increased interest in screening of plant species and reviewing of traditional remedies for development of new and effective drugs. Amongst 25 best-selling pharmaceutical medicines, 12 are of plant origin ('O' Neill and Lewis, 1993).

India harbours rich diversity of more than 1, 32, 000 species of plants and occupies top most position in use of herbal drugs, utilizing nearly 540 plant species in different formulations (Pandey et al., 1993). The global market of medicinal plants and herbal medicines is estimated to be US$ 80 billion per year (Purohit and Vyas, 2004). About 32, 600 Tonnes of medicinal plants are exported from India every year (Rawat and Garg, 2005) contributes to the foreign exchange in the Indian economy. The demand for medicinal plants has increased globally due to resurgence of interest in standardized plant based extracts, culinary herbs, natural therapeutic essential oils and phytopharmaceuticals (Shawl and Qazi, 2004). This has resulted in an increased rate of plant extraction from natural habitat. Therefore, populations of high value medicinal plant species are decreasing at an alarming rate and status of many such plants is now threatened to critically endangered (Sang et al., 2011).

1.2. *Bacopa monnieri*

Bacopa monnieri (L.) Wettst., (Family Scrophulariaceae) also referred to as *Herpestis monniera*, water hyssop is locally known as 'Brahmi'. The name 'Brahmi' is derived from the word ''Brahma'', the ''creator'' in the Hindu pantheon. Because brain is the centre for creative activity, anything that improves the brain health is called Brahmi (Russo and Borrelli, 2005). The plant is small creeper with numerous branches, small oblong leaves and light purple or white colour flowers (Fig. 1.1). It is found in wetlands throughout the Indian subcontinents in damp and marshy areas up to an elevations of 1,320 m amsl (National Medicinal Plants Board, NMPB 2008), and is easily cultivated if adequate water is available. The genus *Bacopa* includes over 100 species of aquatic herbs distributed throughout the warmer regions of the world. Amongst these, *B. monnieri* has gained considerable importance because of the presence of many bioactive molecules (Bammidi et al., 2011). The most important one are 'bacosides'- triterpenoid saponin that possess memory enhancing properties (Chatterji et al., 1965). *B. monnieri* has been traditionaly used in Ayurvedic medicines for improving memory (Russo and Borrelli, 2005). The entire plant is reported to contribute to its medicinal property (Satyavati et al., 1976).

Fig. 1.1 Plants of *B. monnieri* growing at Thapar University Patiala (Punjab, India)

Traditional system of Indian medicine classifies “Brahmi” as *‘medhyarasayana’* i.e. an herb that is known to counter the effects of mental stress and improve intelligence and memory function. The earliest mention *B. monnieri* in several ancient Ayurvedic sculptures including the Charak Samhita (6th century A.D.) and the Bravprakash Var-Prakarana (16th century A.D.) in which it is recommended in formulations for the management of a range of mental conditions including anxiety, poor cognition and lack of concentration, (Govindrajan et al., 2005; Prasad et al., 2008). The herb constitutes an important ingredient of several Ayurvedic formulations such as ‘Brahmirasayanam’, ‘Sarasvataristam’, and ‘Brahmighritam’ (Shrivastava and Rajani, 1999; Govindrajan et al., 2005; Prasad et al., 2008). Brahmi based herbal preprations like ‘Mentat’, ‘Memory Plus’ and ‘Memory Perfect’s, that are rich in ‘bacosides’ are gaining popularity in both developed and developing countries (Pase et al., 2012).

1.2.1. Medicinal properties

Bacopa is an ancient medicinal plant with legendary reputation as a memory vitalizer (Anonymous, 1988). It is reported to contain a variety of other medicinally active compounds such as alkaloids, saponins, hersaponin, beutilinic acid, stigma sterol, β-sitosterol, nicotine and numerous bacosides (Chakravarty et al., 2003; Hou et al., 2002). The important compounds, of *B. monnieri* responsible for the nervine activity are 'bacoside A' and 'bacoside B' (Fig. 1.2), which are reported to be active constituents of this plant responsible for influencing memory (Singh et al., 1988; Singh and Dhawan, 1982) and are known as 'memory chemicals' (Bhandari et al., 2006). The alcoholic extracts of *B. monnieri* have also been shown to be antioxidant, free radical scavenger and anti-lipid peroxidative (Tripathi et al., 1996; Bhattacharya et al., 2000). *B. monnieri* is reported to directly inhibit superoxide radicle formation in a dose dependent manner (Russo et al., 2003). Apart from this, it possess hepatoprotective (Sumathy et al., 2001), bronchovasodilatory (Channa et al., 2003), anti-inflammatory (Channa et al., 2006), anti-helicopylori (Goel et al., 2003) and anti-cancer (Elangovan et al., 1995; Rohini et al., 2004) properties. *B. monnieri* is also reported to show cytotoxic activity (D'souza et al., 2002), shows inhibitory effects on carcinogen induced hepatotoxicity (Janani et al., 2009).

B. monnieri extract is shown to modulate the expression of certain enzymes involved in scavenging of reactive oxygen species in the brain (Govindarajan et al., 2005). The adaptogenic properties of *B. monnieri* are reported to be beneficial in stress management (Chowdhuri et al., 2002). It is also known to have a protective and curative effect on gastric ulcers (Dharmani and Patil, 2006; Sairam et al., 2001). The last two decades has witnessed remarkable progress in the area of medicinal plants, as most of the industries that utilize medicinal plants for the production of pharmaceuticals now adopt various biotechnological techniques for the production of such compounds.

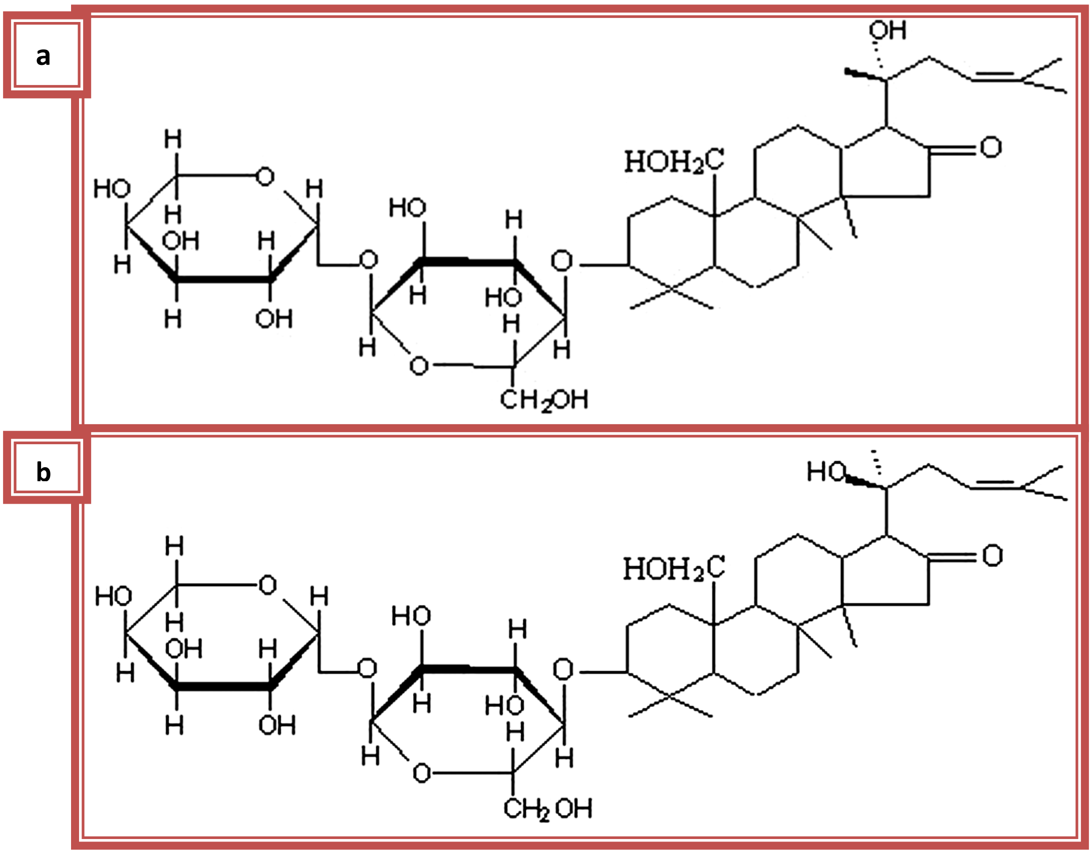


Fig. 1.2 Chemical structures of saponins from *Bacopa monnieri* a) 'Bacoside A' (levorotatory); b) 'Bacoside B' (dextrorotatory) (Gohil and Patel, 2010)

A world-wide shift towards herbal drugs has posed severe pressure on the wild populations of medicinal plants including *B. monnieri*. Therefore, there is a felt need to take immediate step for the development of efficient propagation protocol for generating uniform quality propagules and conservation of such plants (Leonti and Casu, 2013). The entire demand of the raw material of *B. monnieri* is met out from the wild population. This has contributed enormously towards the depletion of *B. monnieri* populations from natural habitat and put this herb in the list of endangered plants species compiled by International Union for Conservation of Nature and National Resources (Pandey et al., 1993). The gradual decline of population of *B. monnieri* from natural habitats requires immediate conservational steps for continuous and sustained supply through balanced cycle of harvest and renewal (National Medicinal Plants Board, 2004). Such efforts may also help bridging a gap of demand and supply of this important herb for the pharmaceutical industry (Jain et al., 2012; Ashraf et al., 2012). In view of the importance of *B. monnieri*, conservation activities using both conventional and biotechnological tools need to be taken up (Benson, 1999; Kaur et al., 2008).

Assessment of genetic diversity of rare and endangered species is an important step towards conservation and improvement programme (Khan et al., 2012). A number of biotechnological approaches are being used for studying genetic diversity (Kaur et al., 2013). Some important techniques that have great potential in assessing genetic diversity are morphological, biochemical and DNA based markers (Rao and Hodgkin, 2002).

Characterization of plants using morphological markers is the primary and most accessible approach to study the diversity amongst natural populations (Toklu et al., 2009). The use of morphological markers in plants for determining the diversity is a primary tool. Mendel followed visible phenotypic traits in progeny of sexual crosses, and use of morphological markers has contributed substantially in the present day knowledge (Lee et al., 2013). Morphological features include height, stem diameter, yield etc. are usually used as index to estimate species diversity (Mc Adam et al., 2014). Plant growth analysis approach i.e. biomass and relative growth rate has been used to estimate the performance of different ecotypes of species (Evans, 1972; Causton and Venus, 1981, Chandra, 2004). Because whole plant of *B. monnieri* is reported to contain active principle, therefore harvestable biomass is important for determining the productivity. Therefore, harvest index is considered an important parameter of study (Donald and Hamblin, 1976). It is an estimate of yield potential and also relatively easier to measure (Chandler, 1969). An investigation for the selection of genotype with higher growth potential will be useful in identifying elite accession. Raw material of medicinal plants has been reported to show considerable variations in the levels of active principle, which is affected by the genotype and season of harvest (Walker et al., 2001). Thus, identification of appropriate season will help in developing harvest strategy.

Due to an increased interest of people for herbal drugs, researchers are focusing in the area of phytochemistry of medicinal plants (Briskin, 2000). Isolation and characterization of bioactive molecules from wild plant species is important for their sustainable use (Lata et al., 2010; Khan et al., 2012). Researchers are also focussing to study the variation in the levels of active principle in the wild populations (Nadeem et al., 2002; 2007). Quality of the raw material collected from wild populations has been reported to show considerable variation with respect to the level of active principle (Nadeem et al., 2007). The accession with higher levels of active principle will have an impact on the pharmacological activity of extracts (Bergonzi et al., 2001). Variation in the active principle among accessions is reported to be not only influenced by genotype but also by environmental factors (Bombardelli and Morazzoni, 1995; Upton et al., 1997; Kurth and Spreeman, 1998; Sirvent, 2001). Therefore, identification of appropriate season when the levels of the active principle are higher will be useful for developing harvesting strategies.

Assessment of genetic diversity of wild populations using molecular markers has been taken up by many researchers (Bussel, 1999; Archak et al., 2003; Alvarez and Martin, 2006; Chandra et al., 2011). Molecular markers are more useful than the morphological and biochemical markers as later are influenced by environmental factors (Williams et al., 1990). Therefore, it has been thought important to study the polymorphism at DNA level using various molecular markers (Welsch and Mc Clelland, 1990). At, molecular level, even seemingly identical accessions display considerable variations (Laurentin, 2009). Various DNA profiling techniques can be used to investigate polymorphism and to score information on genetic differences among individuals of populations and between accessions (Welsch and Mc Clelland, 1990; William et al., 1990). Although a lot of work has been done on application of molecular techniques in studying genetic diversity of many plant species (Khan et al., 2012), yet there is only a few reports on the genetic diversity of *B. monnieri* (Tripathi et al., 2012).

Micropropagation is an efficient method of multiplication of the endangered species thus helpful in conservation of plant biodiversity (Cruz-Cruz et al., 2013; Lata et al., 2014). It enables the propagation of the endangered species at a faster pace. Various *in vitro* propagation techniques, such as adventitious shoot multiplication, shoot organogenesis and somatic embryogenesis are currently employed for the large scale propagation of important species (Vengadesan and Pijut, 2009; Haines and Martin, 1997). Micropropagation exploits the regeneration potential of the selected tissue and is preferred choice for the multiplication of important plants. It has been successfully used for mass multiplication of many plants (Yasodha et al., 2004) including *B. monnieri* (Shrivastava and Rajani, 1999; Tiwari et al., 2001). In spite of progress made in development of micropropagation protocol, application of micropropagation protocol for improvement of elite accessions using various biotechnological tools needs to be assessed.

The potential of plant cell, tissue and organ cultures to produce bioactive molecules has been well recognized (Hussian et al., 2012). The strong and growing demand of natural plant products has shifted the focus on *in vitro* production of secondary metabolites (Ravishankar et al., 1999). There are many advantages of plant cell culture technology for the production of bioactive molecules.

These include:

- Sustainable and assured production of secondary metabolites
- Isolation and purification of secondary metabolites from cell culture is rapid and easy, as compared to extraction from complex whole plants.
- Interfering compounds that occur in the field-grown plant are lower in cell/organ cultures.

Production of secondary metabolites by plant cell cultures attracts an increased interest since 1950s. Plant cell cultures have emerged as an attractive alternative for the production of high-value secondary metabolites (Ravishankar et al., 1999; Dornenburg and Knorr, 1997; Di Cosmo and Misawa, 1995). Plant cells are biosynthetically totipotent, which means that each cell in culture retains complete potential and is capable of producing a range of phytochemicals that are produced by the parent plant (Stockigt et al., 1995; Endress, 1994; Ravishankar and Venkataraman, 1990).

Hairy roots have also been explored as an alternative to cell cultures for the production of plant secondary metabolites (Shanks and Morgan, 1999). These roots are induced by *Agrobacterium rhizogenes*, a gram negative soil resident plant pathogenic bacterium and are found to be suitable for the production of secondary metabolite and thereby hold immense potential for the production of pharmaceutical compounds (Flores et al., 1999). These are capable of growing on plant growth regulator-free (PGR-free) medium at a faster rate. Such roots are genetically stable, often exhibit about the same or greater efficiency for the biosynthesis and accumulation of secondary metabolite as compared to parent plants (Kim et al., 2002). Further, short doubling time, ease of maintenance and ability to synthesize range of phytochemicals offers additional advantage of using such roots in bioreactors for the production of secondary metabolites (Flores et al., 1999).

The application of plant cell/organ culture for the production of bacosides from *B. monnieri* needs to be assessed, therefore, in the present study; 'bacoside A' production using cell suspension cultures and hairy root cultures has been studied.

Production of secondary metabolite by plant cell/organ culture is also influenced by the composition of culture media and other conditions (Rao and Ravishankar, 2002). Constituents of culture media are important determinants of growth and accumulation of bioactive molecules (Stafford et al., 1986; Misawa, 1985). The secondary metabolite production by cultures is reported to be influenced by carbon and nitrogen sources, minerals and physical factors such as temperature, pH, agitation and inoculum density (Naik et al., 2010). A chemically defined medium permits the determination of specific requirements for growth and product formation by systematically adding or eliminating constituents from the media formulation (Zhang and Greasham, 1999). To achieve higher product yields, it is a prerequisite to develop a balanced production medium. Conventional practice of studying single factor optimization (OFAT) by maintaining other factors constant does not depict combined effect of all the factors involved (Davis, 1993). This method is also a time consuming and requires many experiments to be conducted to determine optimum levels. These limitations of a single factor optimization process can be eliminated by optimizing all the affecting parameters collectively by statistical experimental design using response surface methodology (RSM). RSM is a common practice for optimization of media components and culture conditions (Rao et al., 1993; Chen, 1996). Statistical optimization not only allows quick screening of a large experimental domain, but also reflects the role of each of the components (Rahulan et al., 2009). Therefore, in the present study an attempt has also been made to optimize the medium components for the enhancement of biomass accumulation and 'bacoside A' production using RSM.

With all this background, the present work was taken to assess and develop biotechnology tools for efficient production of 'bacoside A' with the following specific objectives.

1.3. The specific objectives of this thesis are:

1. Collection and characterization of the *Bacopa monnieri* from different locations of India.
2. Establishment of aseptic cultures from selected elite clones and selection of cell lines for the production of bacosides.
3. To establish hairy root cultures and asses the possibility of bacoside production using these cultures.

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*.

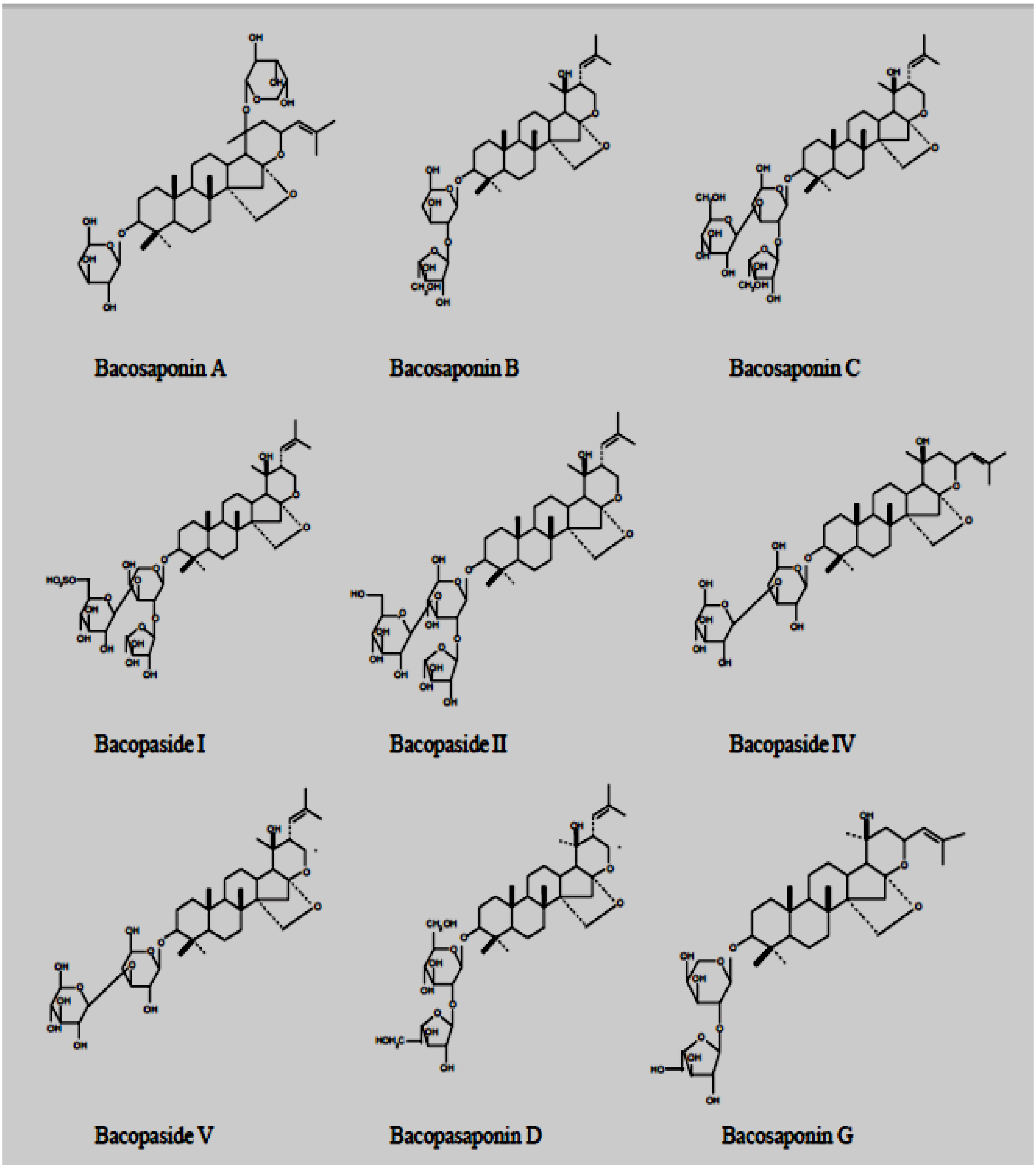


Fig. 2.1 Chemical structures of various saponins isolated from *B. monnieri* (Russo and Borreli, 2005)

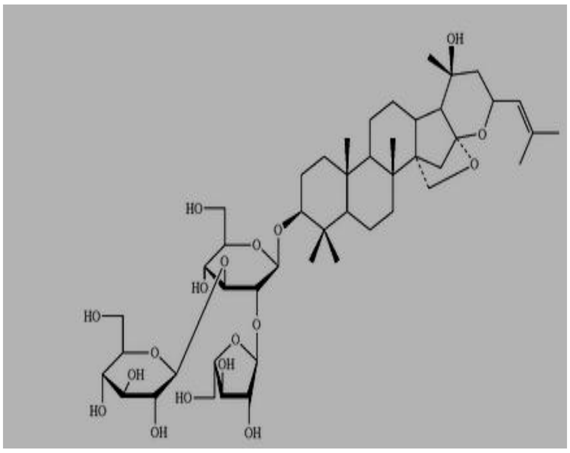
Chatterji et al. (1965) isolated and characterized the major chemical compound responsible for the memory-facilitating action of *B. monnieri*. This compound was identified as 'bacoside A', elucidated as 3-(α-L-arabinopyranosyl)-*O*-β-D-glucopyranoside-10, 20-dihydroxy-16-keto-dammar-24-ene. 'Bacoside 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*

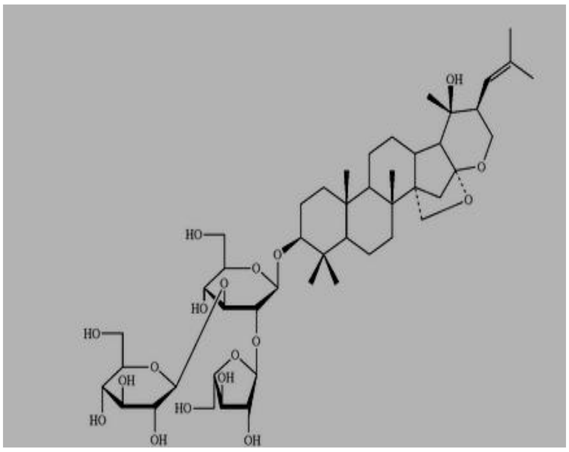
Name	Derivative	Reference
Jujubogenin derivatives		
Bacoside A1	3-O-α-L-arabinofuranosyl(1-3)]-α-L-arabinopyranoside	Jain and Kulshreshtha,1993
Bacoside A3	3-O-α-L-arabinofuranosyl-(1-2)-β-D-glucopyranosyl-(1-3)]-β-D-glucopyranoside	Rastogi et al.,1994
Bacopasaponin A	3,20-di-*O*-α-L-arabinopyranoside	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].

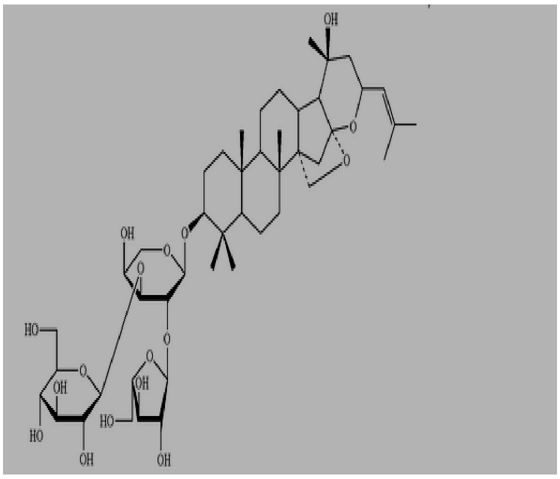
In view of increased interest towards herbal drugs, Chakravarty et al. (2001) undertook a thorough chemical reinvestigation of methanol extract of *B. monnieri* and isolated two new pseudojujubogenin glycosides designated as ‘bacopaside I’ and ‘bacopaside II’. Their structures were elucidated as 3-*O*-α-L 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.



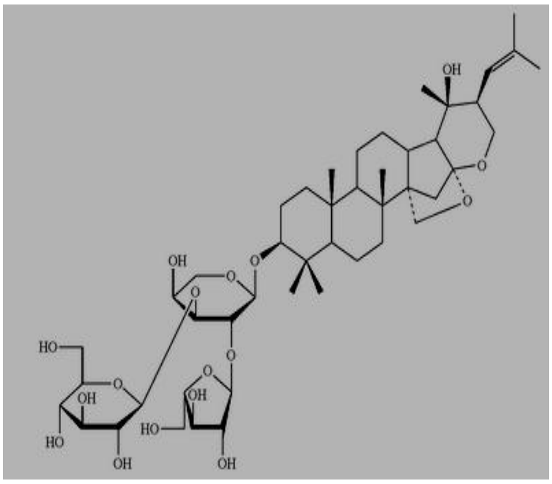
Bacoside A3



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*.

Chapter 3

Materials and methods

3.1. Plant material

A total of fourteen accessions of *Bacopa monnieri* were collected from different locations across India (Table 3.1; Fig. 3.1). These were multiplied by vegetative propagation and were maintained in the nursery at Thapar University, Patiala (30^0 35' N, 76^0 36' E). The herbaria vouchers of these accessions were submitted at the Herbarium of the Department of Botany, Punjabi University Patiala and were provided with the accession numbers mentioned in Table 3.1.

Table 3.1 Accessions of *B. monnieri* collected from various locations

Accession Name	Accession no.	Places of Collection
BM1	58597	Kolkata
BM2	58598	Solan
BM3	58599	New Delhi
BM4	58600	Yamunanagar
BM5	58601	Chandigarh
BM6	58602	Haridwar
BM7	58603	Dehradun I
BM8	58604	Dehradun II
BM9	58605	Manakpur
BM10	58606	Ambala
BM11	58607	Varanasi
BM12	58608	Saharanpur
BM13	58609	Rohtak
BM14	58610	Joginder Nagar

Fig. 3.1 Map of India showing locations of collection of accessions of *B. monnieri*. Accession has been marked as numbers and there herbaria accession numbers has been mentioned in parenthesis

1: BM-Kolkata (58597); 2: BM2-Solan (58598); 3: BM3-Delhi (58599); 4: BM4-Yamunanagar (58600); 5: BM5-Chandigarh (58601); 6: BM6-Haridwar (58602); 7: BM7-Dehradun I (58603); 8: BM8-Dehradun II (58604); 9: BM9-Manakpur (58605); 10: BM10-Ambala (58606); 11: BM11-Varanasi (58607); 12: BM12-Shaaranpur (58608); 13: BM13-Rohtak (58609); 14: BM14-Jogindernagar (58610)

3.2. Chemicals, glassware and plasticware

All routinely used chemicals (AR Grade) were purchased from HiMedia Laboratories, Mumbai, India. Growth regulators, antibiotics and other fine chemicals were procured from Sigma Chemical Co. (St Louis, MO, USA). Taq DNA Polymerase was procured from Larova (Teltow, Germany). Plasticware and sterile disposable filter sterilization units were purchased from Tarsons Products Pvt. Ltd. (Kolkata, India). Glassware such as conical flask, measuring cylinders etc were procured from Borosil Glass Works Ltd. (Mumbai, India). Glass culture bottles of 300 ml capacity were procured from Kasablanka Corporation, (Mumbai, India).

3.3. Morphological Characterization

The collected accessions were planted in the experimental field at Thapar University Patiala in 2 X 2 m plots in Random Block Design (RBD) and maintained for one year before sampling. The plants of each accession (3 plants per accession selected randomly) were uprooted, washed under running tap water to remove soil particles and wiped dry with tissue paper to record the morphological characters i.e. plant height, stem diameter, no. of branches per plant and biomass. Plant height and stem diameter were measured using a ruler and digital vernier calipers (Mitutoyo, Japan), respectively. After determining the fresh weight of the entire plant, these were placed inside perforated paper envelopes and dried in an oven (30 °C, 72 h).

3.4. Relative growth rate

Samples (aerial parts of plants) were harvested from all the accessions at the end of every season i.e. spring (March), summer (June), autumn (September) and winter (December). The fresh weight per plant (FW per plant) was recorded. These were then dried at 80 ^{0}C till the constant weight and the dry weights (DW per plant) were recorded.

The relative growth rate (RGR) was calculated using following equation (Evan, 1972)

RGR = *(ln W2 - ln W1)/(t2-t1)*

where W_2 *and* W_1 represents mean dry weights at harvest (T_2) and initial (T_1) times respectively.

3.5. Biochemical Characterization

3.5.1. Sample preparation

Samples (aerial parts of plants) were harvested from all the accessions at the end of every season (spring, summer, autumn and winter) and were dried in shade. These were ground to fine powder using blender and stored in sealed polypropylene bags till use.

3.5.2. Extraction of 'bacoside A'

The samples were extracted according to Phrompittayarat et al. (2007) with minor modification. Samples (1.0 g dried powder in triplicate) were soaked in 10.0 ml water for 24 h. These were filtered through glass wool and filtrates were discarded. Residues were extracted with 20.0 ml of aqueous ethanol (95 %, v/v) for 3 days. The extraction from the residues was repeated three times (X 20 ml) and filtrate were pooled and dried *in vacuo* at 30 °C. Dried residues were dissolved in 1.0 ml HPLC grade methanol and filtered through 0.45 μm filters (Millipore-Carrigtwohill, Ireland) prior to quantification using high performance liquid chromatography (HPLC).

3.5.3. Quantification of 'bacoside A'

'Bacoside A' content in the extracts was estimated using reverse phase HPLC (Waters Corporation, USA) equipped with high pressure binary pump system (515), Photo diode array

(PDA) detector (2998) and Rheodyne injector with 20 μl sample loop. Samples (20 μl) were injected through injector into Sunfire^{TM} C18 column (250 mm X 4.6 mm i.d. particle size 5.0 μm, Waters, Ireland) and elution was carried out in an isocratic mode with a mobile phase consisted of aqueous acetonitrile (35 %; v/v) containing phosphoric acid (0.2 %, v/v; pH 3.0) at a flow rate of 1.0 ml/min. Column eluates were monitored with online PDA detector set at 205 nm. Quantifications were carried out using external standard curves plotted by taking known quantities of standard compounds (individually 'bacoside A3', 'bacopaside II', 'bacopaside X' and 'bacosaponin C') (Sigma Chemical Co., St Louis, MO) (Fig. 3.2).

3.5.4. Principal component analysis

Plants from different populations were grouped based on bacosides content by principal component analysis (PCA) using loading plots (SPSS 16, IBM, Chicago, USA) to reveal pattern of relatedness within a matrix coordinates in two dimensions for each accession.

Average level of 'bacoside A' components during different seasons was also grouped by principal component analysis using loading plots (SPSS 16, IBM, Chicago, USA).

3.5.5. Hierarchical cluster analysis

The cluster analysis (CA) was carried out to group accessions on the basis of total 'bacoside A' content (SPSS 16, IBM, Chicago, USA) to reveal pattern of relatedness within a matrix coordinates in two dimensions for each accession.

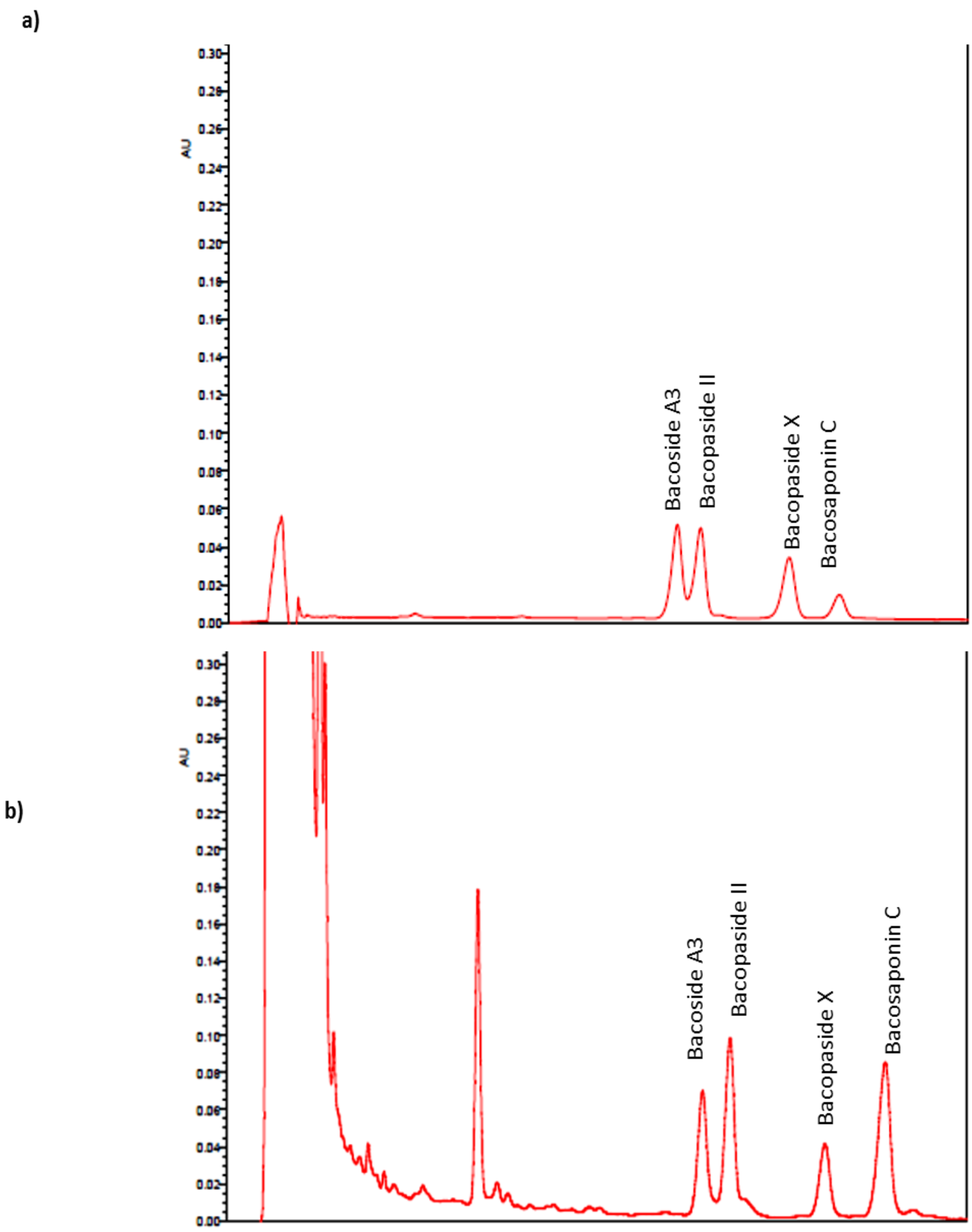


Fig. 3.2 HPLC chromatographs showing elution of 'bacoside A' components i.e. 'bacoside A3', 'bacopaside II', 'bacopaside X' and 'bacopasaponin C' in (a) standard (b) plant extract

3.6. Determination of Harvest Index

Samples (aerial parts of plants) were harvested from all the accessions at the end of every season (spring, summer, autumn and winter). The fresh weight per plant (FW per plant) was recorded and these were then dried at 80 ^{0}C till the constant weight and then dry weight (DW per plant) were recorded. The ‘bacoside A’ content in these plants was calculated and harvest index (HI) was calculated by dividing the total ‘bacoside A’ content per plant with total dry weight of plant.

3.7. Molecular Characterization

PCR-based molecular markers are widely used in many plant species for identification, phylogenetic relation among the population and genetic linkage mapping (Williams et al., 1990). Both RAPD and ISSR markers have proved to be a reliable, easy to generate, inexpensive and versatile set of markers that rely on reproducible amplification of DNA sequence using single primer.

3.7.1. Isolation of genomic DNA

Genomic DNA was isolated from actively growing shoots using the modified CTAB method (Doyle and Doyle, 1990). Fresh tissue 2.0 g of each sample was washed with distilled water, dried and grounded in liquid nitrogen to fine powder, followed by immediate transfer to 50 ml centrifuge tube. To the samples pre-warmed CTAB extraction buffer (Appendix I) (10.0 ml) was added to make slurry and incubated at 60 °C for 1 h in water bath. Equal volume of Chloroform and isoamylalcohol (24:1 v/v) was added to the above slurry and mixed for about 3 min, followed by centrifugation (5000 X g; 10 min). Aqueous phase was removed with the help of wide-bore pipette and transferred to clean tube. Chloroform extraction step was repeated again in case extracts were coloured. DNA was precipitated with 0.66 volume of cold isopropanol followed by incubation for 1 h at -20 °C. After centrifugation (10,000 X g; 15 min) the supernatant was discarded and the pellet was dissolved in 1 ml TE buffer (Appendix I) and transferred to microfuge tube. To the above solution 2 µl of pre heated RNase solution (10 mg/ml stock) was added and incubated at 37 °C for 1 h. To the samples equal volume of phenol-chloroform (1:1 v/v) was added followed by gentle shaking and centrifuged (10000 X g; 10 min). Aqueous layer was retained. To this aqueous solution 0.3 volume of 3M sodium acetate (Appendix I) and 0.6 volume of chilled isopropanol was added and incubated for 1 h at -20 °C. Following incubation, samples were centrifuged (10000 X g; 10 min). The pellet was retained, dried and dissolved in TE Buffer and stored at -20 °C.

3.7.2. Electrophoresis of DNA on agarose gel

Quality of DNA was checked on 0.8 % agarose gel (w/v). Gel was prepared by adding 0.32 g of agarose (Life Technologies India Pvt. Ltd.) in 40ml of 0.5 X TAE (Tris–Acetate-EDTA) buffer (Appendix I). The agarose was melted in microwave oven until dissolved completely. The molten agarose was cooled to about 40 °C and 1.0 µl of ethidium bromide (10 mg/ml) was added and poured into casting trey inserted with combs and allowed to solidify at room temperature. 5.0 µl of DNA sample were mixed with 1.0 µl of 6 X gel loading dye (Appendix I) and loaded into well. The gel was electrophoresed on horizontal electrophoresis apparatus (Amersham Bioscience, U.S.A) in TAE running buffer at 50 volts for one hour and visualized on a U.V. transilluminator (Vilber Loumart, France).

3.7.3. Quantification of DNA

The concentration of extracted DNA in solution was calculated by spectrophotometric measurement using NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) at A_{260}. The quality of DNA was also evaluated by taking the ratio of absorbance at

260 nm and 280 nm. Ideally, the A_{260}/ A_{280} ratio should be 1.8-2.0, if it is less than 1.8 indicate the contamination of proteins, while ratios greater than 2.0 indicate the contamination of RNA.

3.7.4. PCR based markers (RAPD and ISSR)

PCR amplification was performed in 20 µl volume using 27 inter simple sequence repeat (ISSR) (16-20 nucleotide) primers (Table 3.2) and 40 random amplified polymorphic DNA (RAPD) decamer primers (OPD1–OPD 20, OPA 1- OPA 20; Operon Technologies, Alameda, CA**,** Table 3.3) for RAPD analysis. The reaction mixture consisted of 40 ng of genomic DNA, 1.0 U Taq DNA polymerase (Larova, Teltow, Germany), 100 µm dNTPs mixture, 2.0 µl reaction buffer (10X), and 10 nmol primer, Mill-Q water (Millipore India, Bangalore, India) was added to make up the final volume to 20 µl. Amplifications were carried out in thermal cycler model Gene Amp 9700 (Applied Biosystem, San Francisco, USA). Amplification conditions were initial denaturation at 94 ºC for 5 min; 41 cycles of : 94 °C for 60 sec, 36 °C (55 °C in case of ISSR) for 90 sec and 72 °C for 90 sec; with final extinction at 72 °C for 5 min. The amplified products were separated on a 1.2 % (w/v) agarose gel at 50 volts on horizontal midi gel electrophoresis system (Life technologies, USA).

Amplified products were separated with 1.2 % (w/v) agarose gel containing ethidium bromide using 0.5 X TBE buffer. A constant voltage of 55V was provided for 4 - 5 h. DNA fragments were visualized under UV light. The patterns were photographed using Geldoc system (BioRad) and stored as digital pictures. The reproducibility of the amplification was confirmed by repeating each experiment three times.

Table 3.2 Sequence of various ISSR primers used in the study

PRIMER NO. (ISSR)	PRIMER SEQUENCE (5'-3')
ISSR-1	$(CA)_8CG$
ISSR-2	$(GA)_8CG$
ISSR-3	$(GA)_8TC$
ISSR-4	$(AC)_8GCGC$
ISSR-5	$(AC)_8$
ISSR-6	$(CA)_8TG$
ISSR-7	$(CA)_8GC$
ISSR-8	$(GA)_8TA$
ISSR-9	$(GC)_8T$
ISSR-10	$(GC)_8A$
ISSR-11	$(GC)_8AT$
ISSR-12	$(CT)_8G$
ISSR-13	$(CT)_8A$
ISSR-14	$(CT)_8AG$
ISSR-15	$(GT)_8A$
ISSR-16	$(GT)_8C$
ISSR-17	$(AT)_8C$
ISSR-18	$(AT)_8G$
ISSR-19	$(AT)_8GC$
ISSR-20	$(AT)_8$
ISSR-21	$(GA)_8TG$
ISSR-22	$(GA)_8C$
ISSR-23	$(GA)_8CT$
ISSR-24	$(GA)_8CA$
ISSR-25	$(GA)_8CC$
ISSR-26	$(GA)_8T$
ISSR-27	$(CT)_8T$

Table 3.3 Sequence of various RAPD primers used in the study

PRIMER NO. (RAPD)	PRIMER SEQUENCE (5'-3')
OPD-1	ACC GCG AAG G
OPD -2	GGA CCC AAC C
OPD -3	GTC GCC GTC A
OPD -4	TCT GGT GAG G
OPD -5	TGA GCG GAC A
OPD -6	ACC TGA ACG G
OPD -7	TTG GCA CGG G
OPD -8	GTG TGC CCC A
OPD -9	CTC TGG AGA C
OPD -10	GGT CTA CAC C
OPD -11	AGC GCC ATT G
OPD -12	CAC CGT ATC C
OPD -13	CTT CCC CAA G
OPD -14	CAT CCG TGC T
OPD -15	AGG GCG TAA G
OPD -16	TTT CCC ACG G
OPD -17	GAG AGC CAA C
OPD -18	CTG GGG ACT T
OPD -19	CTG CGG TCA G
OPD -20	ACC CGG TCA C
OPA-01	CAG GCC CTT C
OPA-02	TGC CGA GCT G
OPA-03	AGT CAG CCA C
OPA-04	AAT CGG GCT G
OPA-05	AGG GGT CTT G
OPA-06	GGT CCC TGA C
OPA-07	GAA ACG GGT G
OPA-08	GTG ACG TAG G

OPA-09	GGG TAA CGC C
OPA-10	GTG ATC GCA G
OPA-11	CAA TCG CCG T
OPA-12	TCG GCG ATA G
OPA-13	CAG CAC CCA C
OPA-14	TCT GTG CTG G
OPA-15	TTC CGA ACC C
OPA-16	AGC CAG CGA A
OPA-17	GAC CGC TTG T
OPA-18	AGG TGA CCG T
OPA-19	CAA ACG TCG G
OPA-20	GTT GCG ATC C

3.7.5. Phylogenetic analysis

The size of the amplicons was determined from gel photographs by comparing with molecular weight markers. Each band of amplified DNA fragment was transformed in to discrete variables or binary characters matrix, ‘1’ (to mark presence) and ‘0’ (to mark absence). The binary data matrixes were used to estimate the level of polymorphism by dividing the number of polymorphic bands (not present in all samples) by the total number of scored bands. Amplified fragments in the size range of 250-3000 bp, were included in the analyses. Data were subjected to analysis by Jaccard’s coefficient to generate matrix and the values were used to construct dendrograms of unweighted pair-group method with arithmetic means (UPGMA) using Multivariate Statistical Package 3.2.1 (MVSP; Kovach Computing Services, Anglesey, Wales).

A scatter plot of these accessions was drawn by PCA using the RAPD and ISSR data (SPSS 16) to reveal pattern of relatedness within a matrix coordinates in two dimensions for each accession.

3.8. Establishment of aseptic cultures

3.8.1 *Media and culture conditions*

Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 58 mM sucrose and gelled with 0.7 % agar (w/v) (basal MS medium) was used for all tissue culture experiments (Table 3.4). Various plant growth regulators (PGR's) like BA and 2, 4-D were added to the medium in different concentrations and combinations as specified with each experiment. The concentrated stock solutions of all the ingredients (macronutrients, micronutrients, vitamins) were prepared individually, which are then used to prepare the medium (Table 3.4). Stock solutions of all plant growth regulators (PGR's) in concentration of 2.5 mM were prepared by dissolving them in respective solvents (1N HCL, 1N KOH/ NaOH, 70 % ethanol or DMSO etc.) and finally volume was made up using Milli Q water. All the stock solutions were kept under refrigeration (~ 4 °C). The pH of medium was adjusted to 5.8 with 1N KOH or 1N HCl using pH meter (Cyberscan 510, Eutech Instruments, Singapore). After preparation, medium was dispensed (50 ml) into 300 ml glass culture bottles (Kasablanka, Mumbai) and agar (0.7 %; w/v) was added to the individual culture bottle. The medium was then sterilized in an autoclave (121 °C; 15 psi; 20 min, Equitron, Mumbai, India). Stock solution of antibiotics such as ampicillin etc. were prepared in required concentration and filter sterilized using disposable sterile filters of 0.22 μm pore size (Merck Millipore, India) and were stored at -20 °C in a freezer (Vest frost, India).

3.8.2. Culture/ growth conditions

Unless otherwise mentioned, all cultures were incubated at 25 ± 1 °C under cool white fluorescent lights (CFL) (Philips India Ltd, Mumbai) with the light intensity of 42 μmol m^{-2} s^{-1} inside the culture vessel in 16 h light/8 h dark cycle.

Table 3.4 Composition of MS medium (Murashige and Skoog)

Sr. No.	Components	Concentrations (mg/l)
1.	KNO_3	1900.0
2.	NH_4NO_3	1650.0
3.	$MgSO_4.7H_20$	370.0
4.	$CaCl_2.2H_2O$	440.0
5.	KH_2PO_4	170.0
6.	H_3BO_4	6.2
7.	$MnSO_4.4H_2O$	16.90
8.	$ZnSO_4.7H_2O$	8.6
9.	$Na_2MoO_4.2H_2O$	0.25
10.	$CuSO_4.5H_2O$	0.025
11.	$CoCl_2.6H_2O$	0.025
12.	KI	0.83
13.	Nicotinic Acid	0.5
14.	Pyridoxine HCl	0.5
15.	Thiamine HCl	0.1
16.	Glycine	2.0
17.	Myo-Inositol	100.0
18.	FeEDTA. $2H_20$ (sodium salt)	30.0
19.	Sucrose	$3 X 10^4$

(pH of the final medium was adjusted at 5.8 with 0.1N HCl or 0.1N KOH, pH was adjusted after addition of PGR's)

3.8.3. Preparation of explants and establishment of aseptic cultures

Cultures were established using terminal portions of actively growing shoots. First of all young actively growing freshly shoots were collected and leaves were removed from the shoots. Shoots were then excised into smaller segments (each piece with 2 nodes) to facilitate proper cleaning during the disinfection. The explants were then washed thoroughly under running tap water for 20 min followed by washing with liquid detergent (Rankleen, Ranbaxy Lab. Ltd., India) for 10 min. The explants were again washed thoroughly under running water to remove traces of detergent. The washed explants were treated with bavistin (50 %; w/w, Carbendazim WP, BASF India Limited) solution (0.2 % w/v) for 30 min. These were again washed thoroughly with distilled water. Subsequent operations were carried out in a laminar flow cabinet under aseptic conditions. These were then taken for surface disinfection, which was carried out by treating explants with an aqueous solution of mercuric chloride (0.1 %, w/v) containing few drops of Tween-20 (0.2 %, v/v) for 5-6 min. Explants were then washed with sterile distilled water (4 times equal volume) till the traces of disinfectant were removed. Following disinfection, the exposed ends of nodal segments were trimmed with the help of sterile forceps and scalpel fitted with sterile surgical blade on cool sterile glass plate (autoclaved and flamed with rectified spirit prior to use) and then these were planted vertically on MS medium supplemented with 2.5 µM BA. Initially cultures were sub-cultured on fresh medium at every seven days interval for three subculture cycles and subsequently these were subcultured on same medium at 14 days interval. The shoots sprouted from the axillary buds were excised and cultured on same medium and further used for experiments.

3.8.4. Shoot organogenesis

Shoot organogenic potential of leaf segments taken from microshoots of various accessions was attempted. Young, expanded leaves (1.5–2 cm) from microshoots maintained on MS medium supplemented with 2.5 µM BA were taken as explants. Leaves were cut transversely along the midrib and cultured on the basal MS medium supplemented with 12.5 µM BA and 1.0 µM 2, 4-D. The explants were sub-cultured on the same medium at 4 week interval. Data for the shoot organogenesis was recorded after 8 weeks of culture.

3.8.5. Rooting of microshoots

Microshoots (3-4 cms; 15 shoots per culture vessel) from each accession were excised and inoculated on basal MS medium. Data for percent rooting of microshoots, number of roots per shoot and root length were recorded after two weeks of culture.

3.9. Establishment of callus and cell lines

3.9.1. Callus culture

Based on the potential of *in vitro* morphogenetic response, biomass accumulation and 'bacoside A' content; accession BM6 was selected for the experimental work on callus culture, cell culture and hairy root culture establishment. The expanded leaves from micro-shoots were used as explants. The leaves were cultured on MS medium variously supplemented with NAA (2.5-7.5 µM), KIN (1.15- 4.6 µM) and casein hydrolysate (1 g/l). The pH of the medium was adjusted to 5.8 before autoclaving at 121 ºC for 15 min. All the cultures were examined periodically and visual observations were recorded. The growth of callus was determined at the end of 3 weeks.

3.9.2. Cell Suspension Culture

Callus cultures established above from leaf explants were selected for cell suspension study. The 2 g/l DCW of friable callus tissue was inoculated into 250 ml Erlenmeyer flask (Borosil Glass Works Ltd. Mumbai, India) containing 50 ml of MS medium supplemented with NAA (2.5, 5.0 and 7.5 µM) in combination with KIN (1.15, 2.3 and 3.5 µM) and sucrose (20 g/l). The cultures were kept on gyratory shaker at 125 rpm and in complete darkness at 25 ± 2 ºC. The growth of cell and 'bacoside A' content was determined at the end of 3 weeks.

3.9.3. Determination of Dry Cell Weight

The cells were separated from the media by filtering the cultures through filter paper. Fresh cell weight (FCW) was determined after they were washed with distilled water and the excess surface water blotted. Dry cell weight (DCW) was recorded after the cells were dried at 60 ºC till constant weight was attained. Increase of dry cell weight was determined as the quotient of the dry cell weight of harvested biomass and the dry cell weight of the inoculum.

3.10. Establishment of hairy root cultures

3.10.1. Bacterial cultures

Five strains of *Agrobacterium rhizogenes*, viz., R1000, SA79, A4 (obtained from Professor A. K. Srivastava, Indian Institute of Technology Delhi, India) MTCC 532 and MTCC 2364 (obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India), were used in the present study. The bacterial strains were grown in yeast mannitol agar (YMA) medium (10 g/l glucose, 10 g/l yeast extract, 1 g/l ammonium sulphate, 0.25 g/l di-potassium ortho-phosphate, 15 g/l agar and pH 6.8) at 28 ºC overnight. Single colony from overnight grown cultures were picked and inoculated into yeast mannitol broth (YMB) (Appendix I) and incubated at 28 ± 2 ºC on orbital shaker (220 rpm) for 24 h. Bacterial cells were pelleted by centrifugation (4000 X g; 2 min) and re-suspended in YMB liquid medium with or without 100 µM acetosyringone to attain the desired OD_{600}.

3.10.2. Hairy roots

Leaf and internode explants obtained from the microshoots of *B. monnieri* were injured by gently wounding with a sterile needle dipped in bacterial suspension before submerging them completely in above mentioned suspension of *A. rhizogenes.* The infected explants were blotted on the sterile blotting paper to remove excess bacteria and were co-cultivated on basal MS medium. Following co-cultivation, explants were washed 4-5 times with sterile distilled water containing 500 mg/l ampicillin, blotted on sterile filter paper and transferred to MS basal medium containing 500 mg/l ampicillin and incubated at 25 ± 1 °C under complete darkness.

3.10.3. Factors effecting Agrobacterium rhizogenes mediated hairy root induction

3.10.3.1. Effect of bacterial concentration

Leaf segments were infected with suspension of *A. rhizogenes* with different OD_{600} values (0.2, 0.4, 0.6, 0.8 and 1.0) for time period of 5 min in Petri plates. Following infection, leaves were co-cultivated for two days and then washed. These were then cultured on basal MS medium containing ampicillin (500 mg/l).

3.10.3.2. Effect of infection time

Leaf segments were infected with suspension of *A. rhizogenes* for different time periods (10 ml, 0–20 min) in Petri plates. Following infection, leaves were co cultivated for two days and were then washed with distilled autoclaved water. These were then cultured on basal MS medium containing ampicillin (500 mg/l).

3.10.3.3. Effect of co-cultivation period

To find out the effect of co-cultivation period on root induction, leaf segment following infection with the suspension of *A. rhizogenes* ($O.D_{600}$ at 0.6) were blotted with sterile filter paper to remove the excess of bacterial cells and medium. These were then cultured on antibiotic-free MS medium for different time periods (0–4 days).

3.10.4. Molecular analysis

3.10.4.1. Isolation of plasmid DNA from the A. rhizogenes strain by rapid boiling method

The plasmid DNA was isolated by rapid boiling method (Holmes and Quigley, 1981). A single bacterial colony was transferred into 20 ml of Luria broth medium (Appendix I) in 250 ml Erlenmeyer flask and incubated the culture overnight at 28 °C with vigorous shaking. 1.5-2.0 ml of the above culture was poured into a microfuge tube and cells were harvested by centrifugation (8,000 X g; 1 min). Supernatant was discarded and the pellet was vortex for a few seconds to re-suspend the cells. Then to each tube 20 µl STET mix (Appendix I) was added. Immediately the tubes were placed in the open-bottom rack in the boiling water for exactly 45 s and were then centrifuged (12,000 X g; 10 min). A large, sticky, loose pellet was formed. The pellet was removed from each tube by "fishing" it out with a sterile wooden toothpick. 200 µl of isopropanol was added to each tube, and were again centrifuged (12,000 X g; 5 min). The supernatant was aspirated, and the pellet was washed with 50 µl of 70 % ethanol. Centrifuged the tube for 1 min to compact the pellet, and then the 70 % ethanol was aspirated. Finally, the pellets were resuspended in 40 µl TE buffer/ milliQ water and stored at 4 °C for further use.

***3.10.4.2. PCR amplification of rolB* gene**

Confirmation of T-DNA integration in the nuclear genome of hairy roots was carried out using polymerase chain reaction (PCR) to amplify DNA fragment specific to *rolB* gene (an important gene of T-DNA of Ri plasmid) from the genomic DNA isolated from root cultures. A fragment of 380 bp specific to *rolB* gene was amplified using gene specific primer pair (forward primer 5-GCTCTTGCAGTGCTAGATTT-3 and reverse primer 5-GAAGGTGCAAGCTACCTCTC-3). Genomic DNA from bacteria was used as positive control and untransformed roots of *B. monnieri* were the source of DNA for negative control. The amplified products were separated on 1.2 % (w/v) agarose gel and viewed under UV transilluminator (Biorad, CA, USA) following ethidium bromide staining.

3.10.4.3. PCR amplification of virD1 gene and 16S rDNA

To detect the presence of bacterial contamination in hairy roots; a fragment of about 440 bp specific to *virD1* gene was amplified using primer pair (forward primer 5-TGTCGCAAGGCAGTAAG-3 and reverse primer 5-CAAGGAGTCTTTCAGCATG-3). Similarly, amplification of 16S rDNA fragment of about 1,500 bp was also carried out using forward primer 5-AGAGTTTGATCCTGGCTCAG-3 and reverse primer 5-ACGGGCGGTGTGTTC-3 (Weisburg et al., 1991). Genomic DNA from bacteria was used as positive control. Amplification conditions were same as mentioned above. The amplified products were separated on 1.0 % (w/v) agarose gel and viewed using UV transilluminator (BioRad, CA, USA) following ethidium bromide staining.

3.10.5. Growth and morphological study of hairy root cultures

The growth characteristics of hairy roots generated from five different strains of *A. rhizogenes* were evaluated on basal MS medium. Roots from un-transformed microshoots were used as control. Four root tips (approx. 1 cm long) from roots induced by each bacterial strain, harvested from 3 week old root cultures were transferred to the same medium (20 ml) in 9-cm petridishes and cultured for 30 days. The experiment was conducted in triplicate. Total root elongation, number of lateral roots (number of roots per centimetre) on the primary roots induced by each strain was recorded and expressed as the lateral root density.

To determine the growth performance of roots, five lines of hairy root (one line induced by each strain) was taken up along with control (un-transformed) root line were taken up to study the growth kinetics. Fifty mg of actively growing hairy roots from 30-day old culture were transferred to 250 ml Erlenmeyer's flask containing 30 ml of basal MS medium with 58 mM sucrose. All cultures were incubated in dark at 25 ± 2 ºC on a gyratory shaker at 60 rpm. After 30 days of culture, the growth of hairy roots was assessed in terms of biomass. 'Bacoside A' content in these roots was also determined.

3.10.6. Determination of root biomass

The roots were separated from the medium. Their fresh weight (FW) was determined after they were washed with distilled water and the excess surface water blotted away. Dry weight (DW) was recorded after the roots were dried at 60 ºC till constant weight was attained. The growth ratio (GR) was determined as the quotient of the dry weight of harvested biomass and the dry weight of the inoculum.

3.11. Optimization for bacoside production using cell and hairy root cultures

3.11.1. Media optimization for cell growth and 'bacoside A' production in suspension cultures

The constituents of the growth media and process variables play a significant role in cell growth and secondary metabolite production. Among these, the major players are: carbon source, nitrogen source, temperature, growth kinetics, medium pH etc. which were studied by one factor at a time (OFAT) approach. This was followed by RSM to investigate the most significant parameters and their optimum concentration for cell growth and secondary metabolite production.

3.11.1.1. Optimization of production medium by OVAT method

Growth conditions and chemical components were varied one at a time to check their impact on cell growth and 'bacoside A' production. Various parameters such as illumination, Carbon sources (Glucose/Sucrose), Nitrogen sources (KNO_3/NH_4NO_3), medium pH, agitation speed and medium strength were optimized.

3.11.1.1.1. Effect of carbon source (glucose/sucrose) on cell suspension cultures

Cell growth and 'bacoside A' production was studied with sucrose and glucose (taken separately) as carbon source at 20 g/l concentration levels (with rest of the constituents same as that of MS media). Experiments were performed by inoculating the cells of *B. monnieri* (2 g/l, DCW basis) into 50 ml MS medium supplemented with 5.0 µM NAA and 1.15 µM KIN in 250 ml flask. The cultures were incubated in gyratory shaker set at 125 rpm and maintained at 25 °C under dark conditions. The cells were harvested after 21 d to analyse growth and 'bacoside A' content.

3.11.1.1.2. Effect of different nitrogen source (NO_3^- / NH_4^+) on growth and 'bacoside A' production

Different NO_3^-/NH_4^+ ratios (60:0, 50:10, 40:20, 30:30, 20:40, 10:50, 0:60 mM) were implemented as nitrogen source (at 60 mM concentration) with rest of the constituents same as that of MS media. Experiments were performed by inoculating the cells of *B. monnieri* (2 g/l, DCW basis) into 50 ml MS medium supplemented with 5.0 µM NAA and 1.15 µM KIN in 250 ml flask. The cultures were incubated in gyratory shaker set at 125 rpm and maintained at 25 °C under dark conditions. The cells were harvested after 21 d to analyse cell growth and 'bacoside A' content.

3.11.1.1.3. Effect of agitation speed on biomass accumulation and 'bacoside A' production

To study the effect of agitation speed on growth and 'bacoside A' production, the cell suspension cultures were evaluated by incubating them in shaker set at 80, 100, 120, 140 and 160 rpm under total darkness for 21 d. The cultures were raised by inoculating the cells of *B. monnieri* (2 g/l, DCW) in 50 ml of MS medium containing casein hydrolysate (1 g/l), 5.0 µM NAA and 1.15 µM KIN and 2 % glucose in 250 ml flask. The MS medium with static condition was maintained as a control. After 21 d of culture, harvested cell's fresh weight, dry weight, and 'bacoside A' production were examined.

3.11.1.1.4. Effect of illumination on cell growth and 'bacoside A' production

B. monnieri cell suspension cultures were incubated under a 16/8-h light/dark regime and in complete darkness to assess the effect of light and dark on cell growth and 'bacoside A' production in MS medium supplemented with 5.0 µM NAA and 1.15 µM KIN. Fresh and dry cell weight and 'bacoside A' content was analysed after 21 d of culture.

3.11.1.1.5. Growth kinetics of B. monnieri suspension cultures

To study the growth kinetics of *B. monnieri* cells, 2 g/l (DCW) cells were inoculated in 50 ml media in 250 ml Erlenmeyer flasks. The cultures were kept on gyratory shakers set at 125 rpm and incubated at 25 ± 2 ºC in 24 h dark. Each individual flask was harvested at a regular interval (3 d) of time for analysis of cell growth and 'bacoside A' content.

3.11.1.1.6. Effect of medium pH on cell growth and 'bacoside A' production

B. monnieri cell suspension cultures 2 g/l (DCW) cells were inoculated in 50 ml of MS medium supplemented with 5.0 µM NAA and 1.15 µM KIN with varying pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) in 250 ml Erlenmeyer's flask. After 21 d, the fresh and dry weight of cells was recorded and 'bacoside A' content was determined.

3.11.1.1.7. Effect of medium strength on cell growth and 'bacoside A' production

To optimize the effect of medium strength, various strengths of basal MS medium (0.25, 0.50, 0.75, 1.0, 1.5 and 2.0) were used. *B. monnieri* cell suspension cultures 2 g/l (DCW) cells were inoculated in 50 ml of medium supplemented with 5.0 µM NAA and 1.15 µM KIN in 250 ml Erlenmeyer's flask. After 21 d, the fresh and dry weight of cells was recorded and 'bacoside A' content was determined.

3.11.1.2. Plackett – Burman design

Nutrients; glucose (A), nitrate (B), phosphate (C), $MgSO_4.7H_2O$ (D), $CaCl_2.4H_2O$ (E) and inoculum size (F) were selected for study using Plackett – Burman design. Each nutrient was tested at two concentrations (high and low) (Table 3.5).

Table 3.5 Concentration (high or low) range of variables selected for medium optimization

Sr.NO.	Variables	Low (-) level	High (-) level
1.	**Glucose (g/l)**	15.00	60.00
2.	**Potassium nitrate (mM)**	15.00	90.00
3.	**Potassium dihydrogen ortho-phosphate (g/l)**	0.50	2.5
4.	**Magnesium sulphate (g/l)**	0.18	0.74
5.	**Calcium chloride (g/l)**	0.22	0.88
6.	**Inoculum (g/l)**	2.0	8.0

Eight experiments were formulated using six nutrients and responses were measured in terms of FCW, DCW and 'bacoside A' content. The design was developed using Design-Expert software version 7.0.0.0 (Stat-Ease Corporation, USA). The experimental design is given in table (3.6). After 21 d of incubation at 25 °C under dark conditions and mixing at 120 rpm, FCW, DCW and 'bacoside A' were estimated.

Table 3.6 Plackett – Burman design matrix for screening variables influencing cell growth and 'bacoside A' production

Std	Run	Glucose (g/l)	Nitrate (mM)	K_2HPO_4 (g/l)	$MgSO_4.7H_2O$ (g/l)	$CaCl_2.2H_2O$ (g/l)
1.	6	15.00	90.00	0.50	0.18	0.88
2.	2	15.00	15.00	0.50	0.74	0.88
3.	5	60.00	15.00	0.50	0.18	0.22
4.	7	60.00	90.00	2.50	0.74	0.88
5.	4	15.00	15.00	2.50	0.18	0.22
6.	1	60.00	15.00	2.50	0.18	0.88
7.	3	60.00	90.00	0.50	0.74	0.22
8.	8	15.00	15.00	2.50	0.74	0.22

3.11.1.3. Response surface methodology

Once the effective nutrients were identified from the Plackett – Burman design, Response Surface Methodology was used to determine the optimum concentration of the various nutrients for growth of cell suspension and 'bacoside A' synthesis. A total of 30 sets of experiment were designed (Table 3.7) to optimize the glucose (A), nitrate (B), phosphate (C) and inoculum level (D). The experimental design protocol for Response Surface

Methodology was developed using Design-Expert Software (version 7.0.0.0) (Stat-Ease Corporation, USA) (Table 3.7).

Table 3.7 Combinations of independent variables of medium ingredients predicted by Central composite design

Std	Run	Glucose	Nitrate	Phosphate	Inoculum
1	10	60.00	15.00	3.00	8.00
2	6	15.00	15.00	3.00	2.00
3	15	37.50	52.50	2.00	5.00
4	19	60.00	90.00	3.00	2.00
5	22	15.00	15.00	1.00	2.00
6	29	37.50	52.50	2.00	5.00
7	16	15.00	15.00	3.00	8.00
8	9	60.00	90.00	1.00	2.00
9	23	37.50	52.50	2.00	5.00
10	3	15.00	90.00	1.00	2.00
11	4	60.00	15.00	3.00	2.00
12	12	15.00	90.00	1.00	8.00
13	14	15.00	90.00	3.00	8.00
14	21	37.50	52.50	2.00	5.00
15	20	15.00	15.00	1.00	8.00
16	13	60.00	90.00	1.00	8.00
17	7	15.00	90.00	3.00	2.00
18	26	60.00	15.00	1.00	8.00
19	25	60.00	15.00	1.00	2.00
20	28	60.00	90.00	3.00	8.00
21	24	37.50	52.50	2.00	11.00
22	2	37.50	52.50	2.00	1.00
23	8	37.50	52.50	2.00	5.00
24	11	37.50	22.50	2.00	5.00
25	5	82.50	52.50	2.00	5.00
26	1	37.50	52.50	2.00	5.00
27	17	37.50	52.50	2.00	5.00
28	30	37.50	52.50	1.00	5.00
29	18	37.50	127.50	2.00	5.00
30	27	37.50	52.50	4.00	5.00

Experiments were carried out in 250 ml Erlenmeyer flasks containing 50 ml of the medium (pH 6.0). The flasks were sterilized by autoclaving at 120 °C for 20 min, inoculated with the cell cultures under aseptic conditions, and incubated at 25 °C for 21 d, in an orbital shaker set at 120 rpm. At the end of the incubation period (21 d), the cell biomass (FCW/DCW) was recorded and 'bacoside A' level was estimated. All experiments were carried out in triplicate and mean were calculated.

Following second-order polynomial equation relationship between the dependent and independent variables was described. The model was statistically analyzed. Analysis of variance (ANOVA) involved Fischer's *FF* test to judge the model's overall significance, associated probability values, and coefficient of determination to measure the regression model's goodness of fit. The fitted polynomial equation was further expressed in the form of 3D and contour plots which depicted the interactions graphically.

In order to further validate and confirm the suitability of the model, verification experiments with numerically optimized levels of tested variables were performed.

3.11.2. Optimization of conditions for biomass and 'bacoside A' production in hairy root cultures

Production of 'bacoside A' was optimized by standardizing the media for maximum biomass production and 'bacoside A' synthesis. 'One factor at a time' approach was used to optimize the carbon source, nitrogen source, pH, media-to-flask volume etc. for biomass accumulation and 'bacoside A' production in *B. monnieri* hairy root cultures. Following this, RSM was used to optimize different variables and optimize their concentration to achieve maximum biomass accumulation and 'bacoside A' production in *B. monnieri* hairy roots.

3.11.2.1. Optimization of production medium by OVAT

Growth conditions and media components were varied one at a time to check their impact on biomass accumulation and 'bacoside A' production. Various parameters such as growth kinetics (No. of days), media-to-flask volume, optimal pH, carbon sources, nitrogen sources were optimized. *B. monnieri* roots were inoculated in different basal medium with varying carbon, nitrogen, Media-to-flask volume and optimal pH.

3.11.2.1.1. Optimization of medium-to-flask volume ratio

The effect of medium-to-flask volume ratio (in the range 0.06 to 0.18) on growth and 'bacoside A' production in the hairy root culture was investigated in shake flasks. Roots were inoculated into 250 ml Erlenmeyer flasks having different volumes of basal liquid MS medium. Cultures were inoculated in complete dark at 25 °C. The initial pH of the medium was 5.8. Roots were harvested after 40 d for the estimation of biomass (grams per liter) FW and 'bacoside A' production (micrograms per gram) DW.

3.11.2.1.2. Growth kinetics of the hairy root culture in liquid medium.

Knowledge of the growth and 'bacoside A' production by hairy root cultures in basal MS medium is a prerequisite for optimization of the various physical and chemical processes to achieve maximum 'bacoside A' production. Hairy roots were cultured in 250 ml Erlenmeyer flasks containing 30 ml of liquid MS medium and kept on a gyratory shaker at 60 rpm at 25 °C. The pH of the medium was 5.8 and cultures were incubated in complete dark. Flasks were harvested at 5-d interval for 40 d to generate the profiles for biomass accumulation and 'bacoside A' production in hairy roots.

3.11.2.1.3. Effect of medium pH

The effect of medium pH on growth and 'bacoside A' production in the hairy root culture of *B. monnieri* was investigated in shake flasks. Roots were inoculated into 250 ml Erlenmeyer flasks containing 30 ml of basal MS liquid medium with pH range of 4-7. The cultures were incubated in complete dark at 25 °C. Roots were harvested after 25 d for the estimation of biomass and 'bacoside A' production.

3.11.2.1.4. Effect of different carbon source (glucose/sucrose)

Growth and 'bacoside A' production was also studied using sucrose or glucose as carbon source (taken separately) as carbon source (30 g/l). Experiments were performed by inoculating the roots into 250 ml Erlenmeyer flasks having basal MS liquid medium. The cultures were incubated under complete darkness at 25 °C. The initial pH of the medium was set at 6.0. Roots were harvested after 25 d for the estimation of biomass and 'bacoside A' content.

3.11.2.1.5. Effect of different nitrogen source (NO_3^- / NH_4^+)

Growth and 'bacoside A' production was studied with ammonium nitrate and potassium nitrate (taken separately) as nitrogen source at 2 g/l. Experiments were performed by inoculating the roots into 250 ml Erlenmeyer flasks containing 30 ml of basal MS liquid medium. The cultures were incubated under complete darkness at 25 °C and an initial pH of 6.0. Roots were harvested after 25 d for the estimation of biomass and 'bacoside A' production.

3.11.2.2. Plackett – Burman design

Plackett - Burman design is a powerful and efficient mathematical approach to determine the effect of medium constituents on secondary metabolite production. At first stage screening the factors by Plackett – Burman design involves both determination of parameters that have positive influence on production and elimination of those that have negative or no influence. Once the components critical to production are screened, second step involves determination of optimum concentration of each component for maximum product formation. The variables selected in this study were glucose, potassium nitrate, ammonium sulphate, potassium dihydrogen ortho-phosphate, magnesium sulphate and calcium chloride. The variables were investigated and experiments were carried out at 25 °C for 25 d. Each variable was set at two levels, high level and low level (table 3.8). The experimental design is given in table (3.9).

Table 3.8 Two levels (high or low) of medium components studied using Plackett - Burman design

Components	Symbol	Low level (g/l)	High level (g/l)
Glucose	A	15.0	60.0
Potassium nitrate	B	2.0	8.0
Ammonium sulphate	C	0.60	2.70
Pottasium dihydrogen ortho-phosphate	D	0.08	0.34
Magnesium sulphate	E	0.18	0.74
Calcium chloride	F	0.22	0.88

Experimental runs were performed according to the design and response was recorded. Biomass accumulation and 'bacoside A' production was estimated after 25 d of culture period. Significant parameters were selected from the data and studied further by Response surface method.

Table 3.9 Plackett – Burman design matrix for screening the variables influencing biomass and 'bacoside A' production

std	Run	A	B	C	D	E	F
1	10	1	1	1	1	-1	-1
2	5	1	-1	1	-1	-1	-1
3	6	-1	1	1	-1	1	-1
4	7	-1	-1	-1	1	-1	1
5	12	1	1	-1	-1	1	1
6	2	1	1	-1	-1	-1	1
7	4	-1	1	1	1	-1	1
8	11	-1	-1	1	-1	1	1
9	3	-1	1	-1	1	1	-1
10	8	-1	-1	-1	-1	-1	-1
11	1	1	-1	-1	1	1	-1
12	9	1	-1	1	1	1	1

3.11.2.3. Response surface methodology

The interactive effects of four significant factors: A (Glucose), B (Potassium nitrate), C (Potassium dihydrogen ortho-phosphate), and D (Magnesium sulphate) on the response, namely, biomass accumulation and 'bacoside A' production using RSM was studied. Central composite design (CCD) developed by the Design Expert software, version 7.0.0.0 (Stat Ease Inc. Minneapolis, USA, trial version) was adopted for this study.

Each one of the above independent variables A, B, C and D was taken at a central coded value considered as zero and studied at five different levels. A matrix consisting of 30 experiments with 6 replicates at the centre point generated by the software was performed for optimizing the biomass and 'bacoside A' production in hairy roots (Table 3.10).

Experiments were carried out in 250 ml Erlenmeyer flasks containing 30 ml of the culture medium (pH 6.0). The flasks were sterilized by autoclaving at 120 °C for 20 min, inoculated with the culture under aseptic conditions, and incubated at 25 °C for 25 d, on an orbital shaker set at 60 rpm under complete dark. At the end of the incubation period, root biomass (FW/DW) was recorded and 'bacoside A' production was quantified. All experiments were carried out in triplicate and the data represented the mean. Following second-order polynomial equation describes the relationship between the dependent and independent variables were described. The model was statistically analyzed. Analysis of variance (ANOVA) involved Fischer's *FF* test to judge the model's overall significance, associated probability values, and coefficient of determination to measure the regression model's goodness of fit. The fitted polynomial equation was further expressed in the form of 3D and contour plots which depicted the interactions graphically.

Table 3.10 Combinations of independent variables of medium ingredients as indicated by central composite design

Std	Runs	Glucose	KNO_3	KH_2PO_4	$MgSO_4$
1	7	15.00	2.00	0.08	0.18
2	26	60.00	2.00	0.08	0.18
3	12	15.00	8.00	0.08	0.18
4	18	60.00	8.00	0.08	0.18
5	20	15.00	2.00	0.34	0.18
6	13	60.00	2.00	0.34	0.18
7	4	15.00	8.00	0.34	0.18
8	30	60.00	8.00	0.34	0.18
9	2	15.00	2.00	0.08	0.74
10	27	60.00	2.00	0.08	0.74
11	16	15.00	8.00	0.08	0.74
12	10	60.00	8.00	0.08	0.74
13	6	15.00	2.00	0.34	0.74
14	3	60.00	2.00	0.34	0.74
15	15	15.00	8.00	0.34	0.74
16	28	60.00	8.00	0.34	0.74
17	9	0.00	5.00	0.21	0.46
18	19	82.50	5.00	0.21	0.46
19	1	37.50	0.00	0.21	0.46
20	29	37.50	11.00	0.21	0.46
21	11	37.50	5.00	0.00	0.46
22	24	37.50	5.00	0.47	0.46
23	14	37.50	5.00	0.21	0.00
24	8	37.50	5.00	0.21	1.02
25	25	37.50	5.00	0.21	0.46
26	23	37.50	5.00	0.21	0.46
27	17	37.50	5.00	0.21	0.46
28	21	37.50	5.00	0.21	0.46
29	5	37.50	5.00	0.21	0.46
30	22	37.50	5.00	0.21	0.46

In order to further validate and confirm the suitability of the model, verification experiments with numerically optimized levels of tested variables were performed.

As higher growth rate of roots has positive effect on secondary metabolites production and hence leads to higher accumulation of ‘bacoside A’. Thus the root growth and amount of ‘bacoside A’ produced was estimated. The root growth and ‘bacoside A’ production was also compared with the root growth and ‘bacoside A’ production before optimization.

3.12. Statistical analysis

All experiments were performed with three replications. Data were analysed by analysis of variance (ANOVA) using GraphPad Prism Version 5.0 (GraphPad Software Inc., San Diego, CA) and means were compared with Duncan’s multiple range test at $P \leq 0.05$.

Chapter 4

Collection and characterization of *Bacopa monnieri* accessions

Results

4.1. Collection of accessions

Fourteen accessions of *B. monnieri* collected from the different locations across India were maintained in nursery at Thapar University. The herbaria vouchers of these accessions were deposited at the herbarium of Department of Botany, Punjabi University, Patiala and were provided with the accession numbers mentioned in Table 3.1.

4.2. Morphological characterization

The growth related parameters varied in the samples collected from different location during different seasons of year (spring, summer, autumn and winter) (Table 4.1). Among all the four sampling seasons, maximum plant height, stem diameter and number of branches per plant were recorded in samples taken during summer (June) (Table 4.1). Minimum values for all these parameters were recorded in the samples taken during the winter (December).

Variations in the morphological features among the different accessions of *B. monnieri* were examined. Significant variation in the morphological characteristics was recorded amongst these accessions (Table 4.1). Maximum plant height, stem diameter and number of branches per plant were observed in case of accession BM1 during all the sampling dates and minimum values for all these parameters were recorded in accession BM14 (Table 4.1).

Table 4.1 Growth and morphological characteristics of the various accessions of *B. monnieri* during different seasons

Accessions	Spring			Summer			Autumn			Winter		
	Plant height (cm)	Stem diameter (mm)	No. of branches	Plant height (cm)	Stem diameter (mm)	No. of branches	Plant height (cm)	Stem diameter (mm)	No. of branches	Plant height (cm)	Stem diameter (mm)	No. of branches
BM 1	53.17	3.41	17.23	60.47	4.98	24.33	41.27	1.11	14.75	29.03	0.87	7.75
BM 2	23.60	2.12	9.63	30.60	3.64	16.36	11.21	1.12	6.73	4.07	0.42	2.73
BM 3	20.52	2.09	3.95	27.72	3.59	10.02	7.78	1.01	1.45	2.13	0.37	0.77
BM 4	38.10	2.85	11.32	41.90	3.95	18.31	26.76	1.55	8.23	15.91	0.68	3.54
BM 5	36.97	1.60	11.32	40.27	3.01	18.31	24.57	0.30	8.23	11.26	0.04	3.53
BM 6	20.21	2.03	2.36	27.23	3.54	9.62	8.11	0.90	0.87	2.01	0.30	0.69
BM 7	51.71	3.04	11.42	58.91	4.14	18.65	39.57	2.14	8.47	26.16	0.81	3.77
BM 8	25.37	1.85	1.73	32.77	3.25	5.32	13.93	0.55	0.63	4.45	0.13	0.73
BM 9	31.77	1.44	6.76	38.56	2.89	13.04	19.57	0.24	3.11	7.61	0.02	2.01
BM 10	41.67	1.94	11.42	48.20	3.41	18.62	29.05	0.74	8.47	16.51	0.24	3.77
BM 11	29.43	1.79	7.76	36.47	3.19	14.61	17.81	0.49	4.42	6.54	0.09	2.22
BM 12	26.67	2.45	6.93	33.90	3.68	13.31	14.37	1.48	3.33	3.89	0.57	2.13
BM 13	20.57	1.92	3.61	27.77	3.32	10.17	8.37	0.62	1.91	1.48	0.19	1.78
BM 14	6.67	1.34	2.12	13.07	2.64	9.01	3.17	0.14	1.01	0.88	0.01	0.41

ANOVA table	SS	DF	MS	F	SS	DF	MS	F	SS	DF	MS	F	SS	DF	MS	F
Seasons	0.67	3	0.37	734.3*	0.89	3	0.42	687.8*	0.77	3	0.33	841.9*	0.79	3	0.19	684.3*
Accessions	7.01	13	0.66	1670*	4.71	13	0.62	12950*	6.71	13	0.51	1092*	6.79	13	0.34	1103*
Seasons X Accession	0.06	39	0.031	3.212*	0.054	39	0.0011	4.792*	0.034	39	0.012	3.272*	0.049	39	0.017	4.312*
Residual	0.02	112	0.0004		0.03	112	0.0004		0.02	112	0.0004		0.006	112	0.0005	

* Significant at $P \leq 0.05$

4.3 Biomass and Harvest Index

Variations in the biomass and harvest index (HI) in the aerial plant parts (runners, stem and leaves) of these accessions were also estimated during the different seasons, i.e. spring (March), summer (July), autumn (September) and winter (December). Significant variation in biomass and harvest index (HI) was recorded during the different seasons (Table 4.2). The maximum average biomass (2.67 g per plant) and HI (0.0032) of all accessions was recorded during summer. Minimum average biomass (1.27 g per plant) and HI (0.0019) was recorded in winter (Fig. 4.1). Nearly a 1.26 fold variation in the HI was observed during winter to summer season (Fig. 4.1). The average HI was higher in summer, thus suggesting the maximum economic yield during this period.

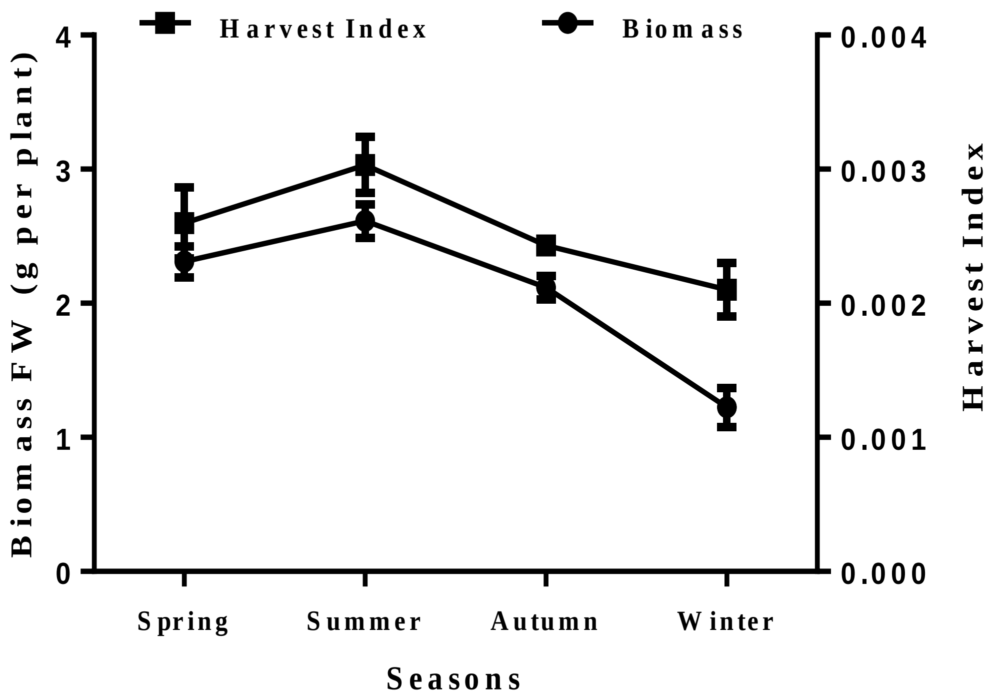


Fig. 4.1 Average biomass (FW) g per plant and harvest index of all accessions of *B. monnieri* in different seasons

Table 4.2 Biomass accumulation and harvest index (HI) of various accessions of *B. monnieri* during different seasons

Accessions	Spring			Summer			Autumn			Winter		
	Biomass (FW)	Biomass (DW)	Harvest Index	Biomass (FW)	Biomass (DW)	Harvest Index	Biomass (FW)	Biomass (DW)	Harvest Index	Biomass (FW)	Biomass (DW)	Harvest Index
BM1	3.89	1.57	0.0040	4.2	1.64	0.0041	3.72	1.43	0.0038	2.67	0.90	0.0033
BM2	2.57	0.99	0.0028	2.73	1.07	0.0030	2.43	0.84	0.0021	1.84	0.59	0.0023
BM3	2.16	0.87	0.0026	2.26	0.76	0.0028	2.01	0.76	0.0014	1.47	0.41	0.0020
BM4	2.68	1.08	0.0029	2.86	1.09	0.0030	2.52	0.97	0.0024	1.98	0.64	0.0024
BM5	2.73	1.17	0.0037	3.2	1.16	0.0038	2.62	1.04	0.0028	2.11	0.71	0.0031
BM6	2.36	0.93	0.0027	2.51	0.91	0.0029	2.21	0.83	0.0018	1.79	0.54	0.0022
BM7	3.89	1.44	0.0040	4.2	1.52	0.0041	3.71	1.32	0.0038	2.59	0.87	0.0033
BM8	1.53	0.41	0.0022	1.78	0.48	0.0023	1.37	0.37	0.0009	0.89	0.21	0.0017
BM9	2.17	0.92	0.0026	2.34	0.82	0.0028	2.01	0.90	0.0014	1.55	0.49	0.0019
BM10	1.93	0.73	0.0025	2.06	0.57	0.0027	1.80	0.69	0.0011	1.31	0.34	0.0018
BM11	2.95	1.31	0.0039	3.7	1.26	0.0041	2.67	1.17	0.0035	2.16	0.81	0.0033
BM12	1.62	0.64	0.0024	1.86	0.41	0.0026	1.59	0.57	0.0010	0.98	0.28	0.0017
BM13	1.23	0.38	0.0020	1.38	0.37	0.0022	1.09	0.26	0.0007	0.72	0.18	0.0014
BM14	1.09	0.24	0.0014	1.26	0.22	0.0016	0.97	0.14	0.0005	0.58	0.09	0.0007

ANOVA table	SS	DF	MS	F	SS	DF	MS	F	SS	DF	MS	F	SS	DF	MS	F
Seasons	2.17	3	0.97	1541.3*	2.01	3	0.84	1324.1*	2.27	3	0.91	1498.1*	1.79	3	0.62	1031.3*
Accessions	21.12	13	2.06	4754*	18.31	13	1.82	2587*	20.11	13	1.51	1152*	1.79	13	21.34	1703*
Seasons X Accession	0.19	39	0.0031	12.532*	0.24	39	0.0027	11.622*	0.21	39	0.0019	13.762*	0.14	39	0.0017	11.871*
Residual	0.0043	112	0.00005		0.0033	112	0.00002		0.0027	112	0.00002		0.0024	112	0.00003	

*Significant at P≤0.05

Amongst accessions, biomass and harvest index (HI) also varied significantly (Table 4.2). Maximum biomass accumulation and HI were observed in case of accession BM1 followed by BM7 during all the sampling seasons. Minimum biomass accumulation and HI were recorded in accession BM14 in all seasons. Therefore, this study clearly established the potential of accessions BM1 and BM7 for high growth and best season for harvest was summer. Two-way anova showed significant differences between the accessions collected from different locations and during different seasons (Table 4.2).

4.4 Relative growth rate

Plant growth plays a key role in the functioning of the terrestrial biosphere and it is typically characterized by relative growth rate (RGR). Thus the relative growth rate in all the accessions was studied during the different seasons (Table 4.3). Considerable variation was recorded in RGR of different accessions of *B. monnieri* during all the seasons (Table 4.3). Among all the accessions, RGR was higher during winter (January) to spring (March), whereas during autumn (September) to winter (December) it was minimum. The RGR values were positive from winter to summer, whereas from summer to winter, these values were negative indicating a period involving senescence (Table 4.3). Amongst the accessions, RGR during all sampling seasons was maximum in BM14 and was found minimum in accession BM1 and BM7 indicating the higher growth potential of these accessions.

Table 4.3 Relative growth rate of various accessions of *B. monnieri* during different seasons

Accession	Relative growth rate			
	Spring	Summer	Autumn	Winter
BM1	0.0041^{dA}	0.0008^{dB}	-0.0013^{aC}	-0.0036^{cD}
BM2	0.0037^{eA}	0.0006^{dB}	-0.0013^{aC}	-0.0031^{bD}
BM3	0.0043^{dA}	0.0004^{dB}	-0.0013^{aC}	-0.0034^{cD}
BM4	0.0033^{eA}	0.0007^{dB}	-0.0014^{aC}	-0.0026^{aD}
BM5	0.0028^{fA}	0.0017^{bB}	-0.0022^{cC}	-0.0024^{aD}
BM6	0.003^{fA}	0.0007^{dB}	-0.0014^{aC}	-0.0023^{aD}
BM7	0.0044^{dA}	0.0008^{dB}	-0.0013^{aC}	-0.004^{dD}
BM8	0.003^{cA}	0.0016^{bB}	-0.0028^{dC}	-0.004^{dD}
BM9	0.0037^{eA}	0.0008^{dB}	-0.0017^{bC}	-0.0028^{bD}
BM10	0.0042^{dA}	0.0007^{dB}	-0.0015^{aC}	-0.0034^{cD}
BM11	0.0034^{eA}	0.0024^{aB}	-0.0035^{eC}	-0.0023^{aD}
BM12	0.0051^{cA}	0.0015^{bB}	-0.0017^{bC}	-0.0053^{eD}
BM13	0.0057^{bA}	0.0013^{cB}	-0.0026^{dC}	-0.0035^{cD}
BM14	0.0181^{aA}	0.0016^{bB}	-0.0028^{dC}	-0.0063^{fD}

Values sharing common lower case letters within the columns and upper case letter within the rows are not significant at $P \leq 0.05$

4.5 Biochemical characterization

4.5.1 Bacoside A content of various accessions of B. monnieri

Wild populations of medicinal plants have been reported to show variation in the levels of active principle. Thus the major components of 'bacoside A' namely 'bacoside A3', 'bacopaside II' and 'bacopasaponin C' were estimated in all the accessions (Table 4.4). Significant variations among accessions were found in terms of 'bacoside A3', 'bacopaside II' and 'bacopasaponin C'. Maximum levels of 'bacoside A3' were recorded in samples of accessions BM1, BM7 and BM11 (0.83 mg/g DW) and minimum level was detected in samples of accession BM14 (0.13 mg/g DW). However, 'bacopaside II' contents were higher in accession BM4 (0.7 mg/g DW) and lowest level of 'bacopaside II' was recorded in samples of accession BM13 (0.09 mg/g DW). Level of 'bacopasaponin C' was much higher as compared to 'bacoside A3' and 'bacopaside II' (Table 4.4). Maximum level of 'bacopasaponin C' was recorded in accession BM1 and BM7 (2.48 mg/g DW) and lowest level of 'bacopasaponin C' was recorded in samples of accession BM14 (0.54 mg/g DW).

The levels of total 'bacoside A' varied in samples collected from different location (Table 4.4). Maximum levels of total 'bacoside A' were recorded in samples of accession BM1 and BM7 (Table 4.4). The minimum levels of total 'bacoside A' were recorded in samples of accession BM14. About 2.5 to 3.0 fold variations were recorded in the levels of total 'bacoside A' across the accessions.

Table 4.4 'Bacoside A' contents (mg/g DW) in various accessions of *B. monnieri*

Accessions	Bacoside A3	Bacopaside II	Bacopasaponin C	Total 'bacoside A'
BM1	0.83[a]	0.63[c]	2.48[a]	3.95[a]
BM2	0.67[e]	0.68[b]	1.47[e]	2.83[f]
BM3	0.53[f]	0.58[d]	1.47[e]	2.60[h]
BM4	0.75[d]	0.70[a]	1.47[e]	2.93[d]
BM5	0.76[b]	0.53[e]	2.37[c]	3.66[c]
BM6	0.67[e]	0.63[c]	1.38[f]	2.69[f]
BM7	0.83[a]	0.63[c]	2.48[a]	3.95[a]
BM8	0.39[g]	0.58[d]	1.24[g]	2.23[k]
BM9	0.67[e]	0.58[d]	1.38[f]	2.64[g]
BM10	0.78[b]	0.14[f]	1.61[d]	2.54[i]
BM11	0.83[a]	0.58[d]	2.47[b]	3.90[b]
BM12	0.39[g]	0.58[d]	1.47[e]	2.46[j]
BM13	0.65[f]	0.09[g]	1.20[h]	1.94[l]
BM14	0.13[h]	0.58[d]	0.54[i]	1.26[m]

Values are average of three replicates. Values sharing common letter are not significantly different at $P \leq 0.05$ by Duncan's multiple range test

Two-dimensional scatter plot of various accessions generated by PCA based on 'bacoside A' revealed that overall variation represented on two components were 82.7 % and 17.3 %, respectively. All accessions fell into two bigger clusters and accession BM14 was separated out (Fig. 4.2). All the populations were positively correlated to component 1, whereas on component 2 one group was positively correlated (accession BM4, BM13, BM6, BM8, BM2, BM9, BM12), whereas other group showed negative correlation (accessions BM5, BM7, BM10, BM1, BM11). Accession BM3 was found to be neutral on component 2.

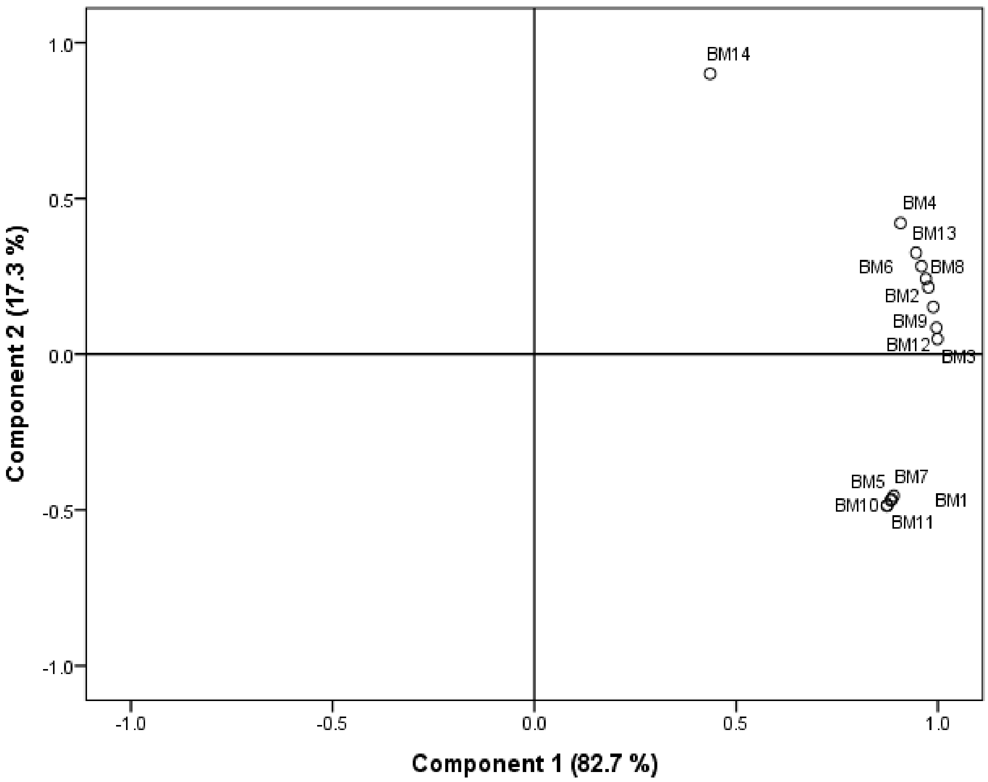


Fig. 4.2 *Scatter plot* of various accessions of *B. monnieri* by principal component analysis (PCA) based on components of 'bacoside A'

All accessions were also grouped using hierarchical cluster analysis (CA) based on 'bacoside A' content. The general structure of dendrogram confirms the existence of five clusters based on the components of ‘bacoside A’ (Fig. 4.3). The first cluster includes accession BM5, BM10 and BM11. The distinction of these samples was determined by the presence of maximum level of ‘bacoside A3’ and low level of ‘bacopasaponin C’. The second cluster includes accession BM1 and BM7 which contains highest level of ‘bacopaside II’ and thus belong to a distinct group. The three accessions BM6, BM13 and BM14 were similar to each other by the presence of low level of ‘bacoside A3’, ‘bacopaside II’ and ‘bacopasaponin C’ and were clustered together. The analysis put the accession BM2, BM9 and BM12 together with the presence of highest conc. of ‘bacopasaponin C’. Accession BM3, BM4 and BM8 were grouped together in one cluster because of the presence of ‘bacoside A3’, ‘bacopaside II’ and 'bacopasaponin C’ at moderate level.

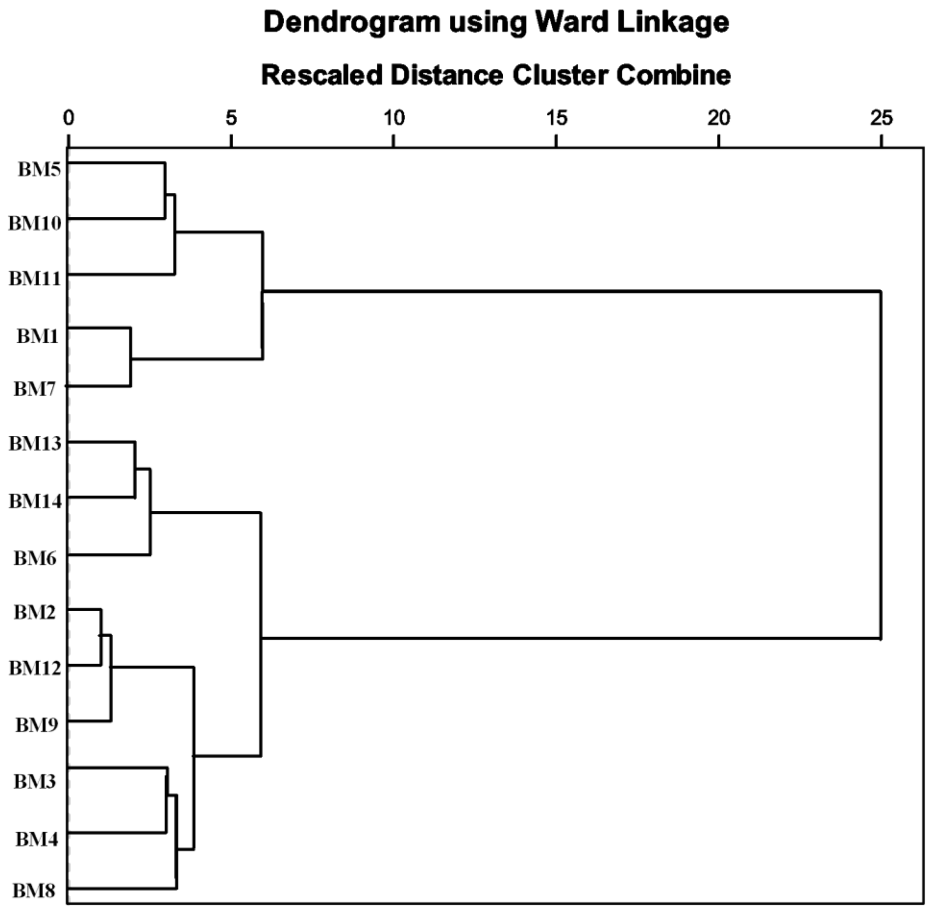


Fig. 4.3 The hierarchical cluster analysis calculated from ‘bacoside A’ content in various accessions of *B. monnieri*

4.5.2 Bacoside A composition in various accessions of B. monnieri during different seasons

The levels of 'bacoside A' varied in samples collected from different location during the different seasons (Fig. 4.4 and Table 4.5). Among all the seasons i.e. spring (March), summer (June), autumn (September) and winter (December), maximum levels of 'bacoside A' were recorded during summer (June) when average monthly temperatures were higher (Figs. 4.4 and 4.5). The minimum levels of 'bacoside A' were recorded during winter when average temperatures were lower (Fig. 4.4). About 2.5 to 3.0 fold variations were recorded in the levels of 'bacoside A' across the accessions.

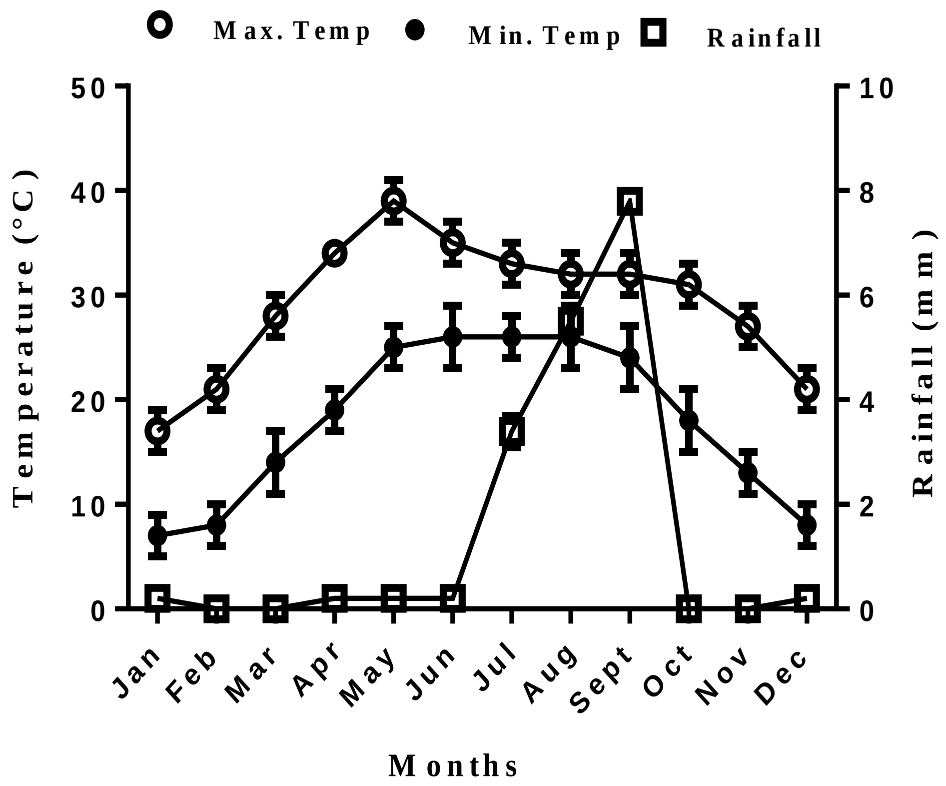
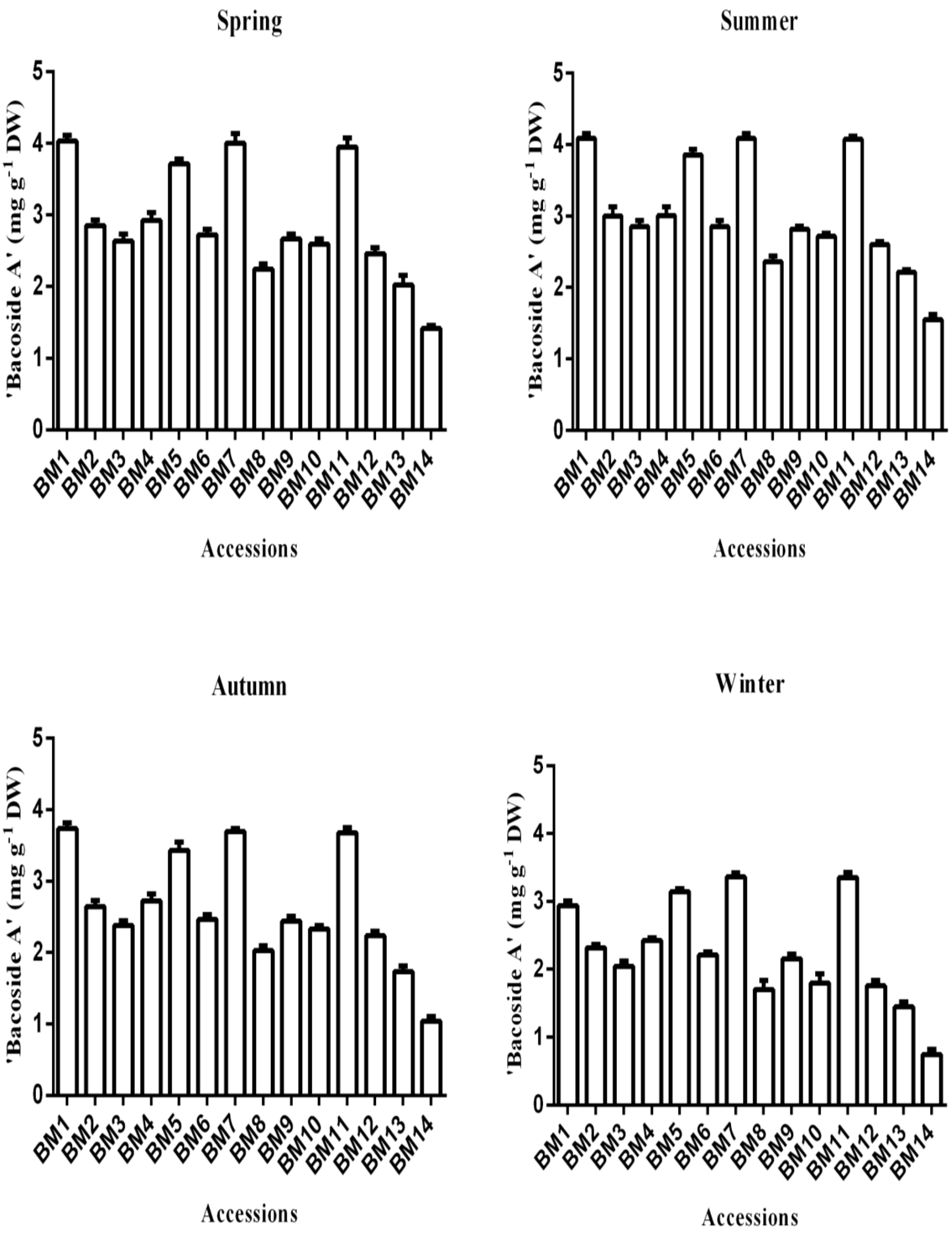


Fig. 4.4 Average monthly maximum temperature, minimum temperature and rainfall during period of study



ANOVA table	SS	DF	MS	F
Seasons	14.29	3	4.76	719.7*
Accessions	93.55	13	7.20	1088*
Seasons X Accession	0.72	39	0.02	2.774*
Residual	0.74	112	0.01	

* Significant at P≤0.05

Fig. 4.5 Content of 'Bacoside A' (mg/g DW) in *B. monnieri* accessions during different seasons

Significant variations in the various components of ‘bacoside A’ were also recorded during the different seasons, i.e. spring, summer, autumn and winter (Table 4.5). Levels of all the three components of ‘bacoside A’ were maximum during summer, whereas minimum levels of these were recorded during winter.

Amongst the accessions, levels of ‘bacoside A3’ during all sampling dates were maximum in accessions BM1 and BM7 and minimum levels were detected in the accession BM14 (Table 4.5). However, maximum ‘bacopaside II’ contents in every season were recorded for accession BM4 and lowest level of ‘bacopaside II’ was recorded in samples of accession BM13. ‘Bacopasaponin C’ content of either season were higher in accession BM1 and BM7 (Table 4.5; Fig. 4.5) and lowest content of ‘bacopasaponin C’ was recorded in the samples of accession BM14. Two-way anova showed significant different amongst the accessions and during different seasons (Table 4.5).

Table 4.5 Comparison of 'bacoside A3', 'bacopaside II' and 'bacopasaponin C' (mg/g DW) components in *B. monnieri* accessions during different seasons

Accessions	Bacoside A3				Bacopaside II				Bacopasaponin C			
	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter
BM1	0.85	0.88	0.78	0.68	0.67	0.72	0.56	0.49	2.51	2.56	2.41	2.26
BM2	0.65	0.69	0.62	0.52	0.72	0.77	0.61	0.55	1.50	1.55	1.41	1.25
BM3	0.52	0.65	0.48	0.38	0.62	0.67	0.51	0.45	1.50	1.55	1.41	1.25
BM4	0.69	0.72	0.70	0.60	0.74	0.79	0.63	0.58	1.50	1.55	1.41	1.25
BM5	0.75	0.78	0.71	0.61	0.57	0.62	0.46	0.41	2.40	2.45	2.30	2.14
BM6	0.66	0.69	0.62	0.52	0.67	0.72	0.56	0.50	1.41	1.46	1.31	1.20
BM7	0.85	0.88	0.78	0.65	0.67	0.70	0.54	0.50	2.51	2.53	2.38	2.23
BM8	0.36	0.39	0.34	0.23	0.62	0.67	0.51	0.46	1.27	1.32	1.17	1.01
BM9	0.66	0.69	0.62	0.52	0.62	0.67	0.51	0.46	1.41	1.46	1.31	1.20
BM10	0.80	0.83	0.73	0.63	0.15	0.20	0.07	0.03	1.64	1.69	1.54	1.15
BM11	0.85	0.88	0.78	0.68	0.62	0.67	0.51	0.46	2.50	2.55	2.40	2.24
BM12	0.36	0.39	0.34	0.08	0.62	0.67	0.51	0.46	1.50	1.55	1.40	1.24
BM13	0.62	0.65	0.61	0.48	0.09	0.14	0.02	0.00	1.37	1.42	1.13	0.97
BM14	0.10	0.13	0.08	0.00	0.62	0.67	0.51	0.46	0.71	0.76	0.43	0.27

ANOVA table	SS	DF	MS	F	SS	DF	MS	F	SS	DF	MS	F
Seasons	0.81	3	0.27	704.3*	1.19	3	0.39	1051*	2.92	3	0.97	1690*
Accessions	7.30	13	0.56	1450*	5.43	13	0.42	1103*	50.99	13	3.92	6807*
Seasons X Accession	0.083	39	0.0021	5.522*	0.033	39	0.0008	2.217*	0.28	39	0.0072	12.65*
Residual	0.04	112	0.0003		0.042	112	0.0003		0.064	112	0.00005	

*Significant at P≤0.05

Two dimensional scatter plot by PCA analysis of average levels of 'bacoside A' components ('bacoside A3', 'bacopaside II' and 'bacopasaponin C') (Table 4.5) during different seasons grouped the samples according to seasons. The two principal components (PC1 and PC2) reported 99.99 % of variation in the entire data set (Fig. 4.6). The extracts from sample of different seasons i.e. spring, summer, autumn and winter were clearly clustered separately (Fig. 4.6) and exhibit remarkable difference in the levels of 'bacoside A' (Fig. 4.4) throughout the year. The samples of summer (June) were only positively correlated on both axes again suggesting an appropriate time for the harvest.

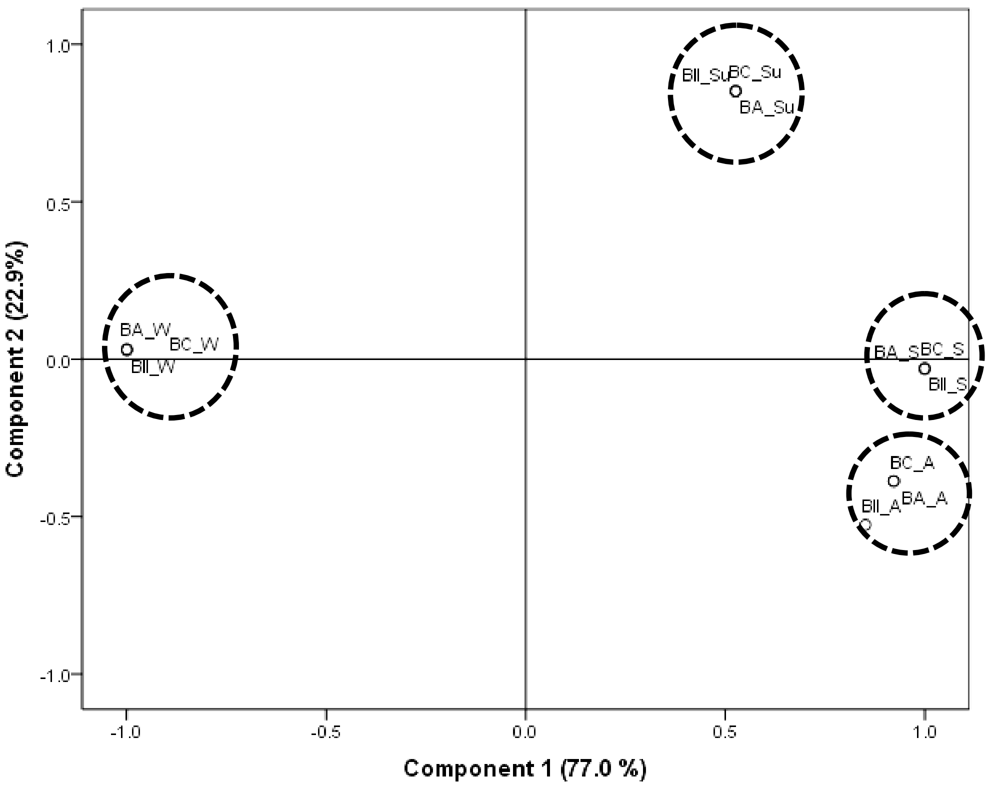


Fig. 4.6 *Scatter plot* of 'bacoside A' components of all the accessions of *B. monnieri* by principal component analysis during different seasons. S: Spring; Su: Summer; A: Autumn; W: Winter; BA: Bacoside A3; BII: Bacopaside II; BC: Bacopasaponin C

4.6 Molecular Characterization

After the biochemical and morphological characterization of *B. monnieri* accessions, genetic diversity of these accessions was studied by PCR based DNA markers such as RAPD and ISSR.

4.6.1 RAPD based fingerprinting

Genetic variations amongst these accessions were studied using fourty primers of RAPD. Out of these, 35 RAPD primers produced reproducible and scorable bands and hence used in this study. All the fourteen accessions produced distinct reproducible bands of amplicons with 35 selected primers. A total of 213 markers were scored using RAPD primers. Amongst 213 markers scored, polymorphic bands recorded for RAPD markers were 49 (23 %). The size of amplified fragments ranged from 250-2500 bp (Table 4.6). The DNA profiles as observed in RAPD are presented in Fig. 4.7. The number of scored bands per primer ranged from 2 (OPA 13) to 13 (OPD 14), with a mean number of 6.08 per primer. The maximum polymorphic markers (6) were scored for primers OPD14 and OPA2 (Table 4.6).

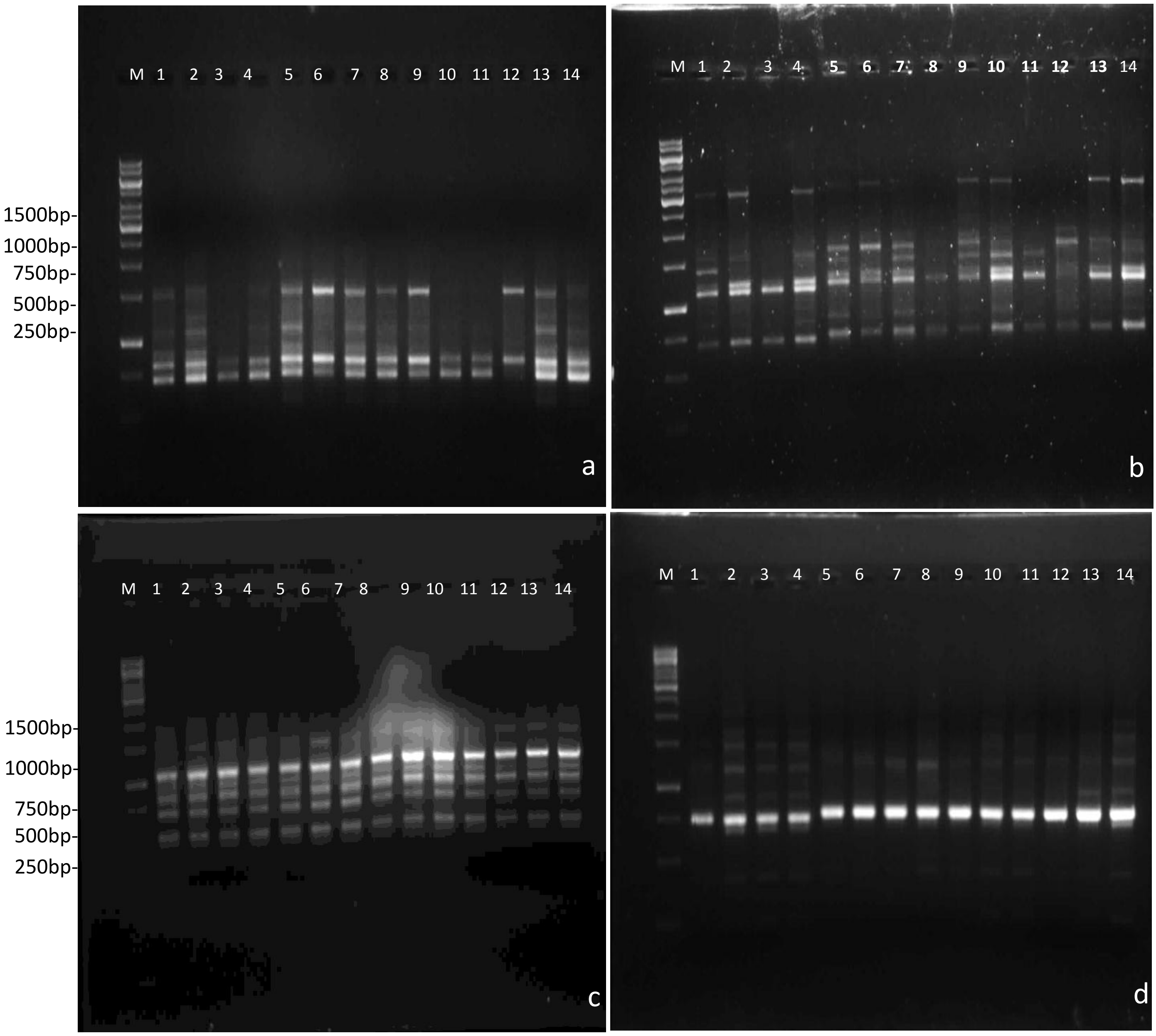


Fig. 4.7 RAPD profile of 14 accessions of *B. monnieri* showing the banding pattern. **a)** RAPD profile using primer OPD 13; **b)** RAPD profile using primer OPD 16; **c)** RAPD profile using primer OPA18 and **d)** RAPD profile using primer OPA5. Lane M: 1 kbp ladder; lanes 1–14: accession BM1–BM14, respectively.

Table 4.6 Sequence of RAPD primers used for amplifications and subsequent amplification of polymorphic bands

S.no	Primer Code	Sequence of Primer	Amplicon size range (bp)	No amplified bands	No of polymorphic bands	Percent Polymorphism
1.	OPD 1	ACC GCG AAG G	250-1000	7	2	28.57
2.	OPD 2	GGA CCC AAC C	500-1500	6	0	0
3.	OPD 3	GTC GCC GTC A	250-1500	4	3	75
4.	OPD 4	TCT GGT GAG G	250-1500	5	0	0
5.	OPD 5	TGA GCG GAC A	600-2000	9	2	22.22
6.	OPD 6	ACC TGA ACG G	500-1500	6	1	16.66
7.	OPD 7	TTG GCA CGG G	600-2000	5	0	0
8.	OPD 8	GTG TGC CCC A	350-2000	7	0	0
9.	OPD 10	GGT CTA CAC C	250-2000	6	0	0
10.	OPD 11	AGC GCC ATT G	300-1500	4	2	50
11.	OPD 12	CAC CGT ATC C	750-2000	5	1	20
12.	OPD 13	GGG GTG ACG A	500-1500	7	1	14.28
13.	OPD 14	CTT CCC CAA G	350-2000	13	6	46.15
14.	OPD 15	CAT CCG TGC T	450-2000	9	1	11.11
15.	OPD 16	AGG GCG TAA G	250-2000	4	1	25
16.	OPD 17	TTT CCC ACG G	250-1000	4	1	25
17.	OPD 18	GAG AGC CAA C	600-3000	9	2	22.22
18.	OPD 19	CTG GGG ACT T	500-2000	9	3	33.33
19.	OPA 20	ACC CGG TCA C	250-2000	12	6	50
20.	OPA 2	TGC CGA GCT G	350-1500	6	1	16.66
21.	OPA 3	AGT CAG CCA C	250-2500	3	0	0
22.	OPA 4	AAT CGG GCT G	250-1000	6	0	0
23.	OPA 8	GTG ACG TAG G	250-1000	4	0	0
24.	OPA 9	GGG TAA CGC C	250-2000	3	0	0
25.	OPA 10	GTG ATC GCA G	500-2000	8	3	37.5
26.	OPA 11	CAA TCG CCG T	250-1500	6	3	50
27.	OPA 12	TCG GCG ATA G	500-1000	6	2	33.33
28.	OPA 13	CAG CAC CCA C	250-2500	2	1	50
29.	OPA 14	TCT GTG CTG G	250-2500	5	0	0
30.	OPA 15	TTC CGA ACC C	250-2000	7	3	42.85
31.	OPA 16	AGC CAG CGA A	750-2500	4	2	50
32.	OPA 17	GAC CGC TTG T	250-2000	6	0	0
33.	OPA 18	AGG TGA CCG T	250-2000	4	0	0
34.	OPA 19	CAA ACG TCG G	500-2000	6	3	50
35.	OPA 20	GTT GCG ATC C	250-2000	6	0	0
				213	**49**	**23.0**

The Jaccard's similarity coefficient of 14 accessions of *B. monnieri* based on RAPD revealed that similarity value amongst accessions ranged from 0.751 to 0.890 indicating moderate levels of genetic similarity (Table 4.7). Maximum similarity value of 0.890 was recorded between BM11 and BM13. Dendrogram constructed using Jaccard's similarity coefficient is shown in Fig. 4.8.

Table 4.7 Jaccard's Coefficient of various accessions of *B. monnieri* using RAPD data

	BM1	BM2	BM3	BM4	BM5	BM6	BM7	BM8	BM9	BM10	BM11	BM12	BM13	BM14
BM1	1.000													
BM2	0.886	1.000												
BM3	0.827	0.825	1.000											
BM4	0.806	0.852	0.862	1.000										
BM5	0.838	0.865	0.856	0.844	1.000									
BM6	0.805	0.803	0.814	0.812	0.834	1.000								
BM7	0.831	0.838	0.765	0.837	0.820	0.817	1.000							
BM8	0.814	0.803	0.804	0.802	0.804	0.820	0.845	1.000						
BM9	0.813	0.820	0.802	0.829	0.822	0.799	0.834	0.837	1.000					
BM10	0.806	0.813	0.824	0.822	0.815	0.774	0.808	0.839	0.829	1.000				
BM11	0.810	0.807	0.754	0.751	0.809	0.796	0.821	0.814	0.832	0.835	1.000			
BM12	0.753	0.770	0.753	0.768	0.751	0.796	0.802	0.805	0.803	0.877	0.840	1.000		
BM13	0.782	0.790	0.773	0.761	0.791	0.816	0.822	0.863	0.874	0.856	0.890	0.882	1.000	
BM14	0.667	0.648	0.640	0.655	0.675	0.699	0.651	0.630	0.679	0.655	0.667	0.704	0.659	1.000

Thirteen accessions were grouped in one major cluster and one accession BM14 was placed separately as an out group in cluster showing less similarity coefficient (0.66) with other accessions. Accessions within main cluster one are further grouped into four clades. The first clade comprised of BM13, BM11, BM12 and BM10. Within the first clade BM13 and BM11 appeared to be closer to each other, with 0.89 similarity coefficient. The second clade comprised of BM9, BM8 and BM7. The third clade consisted of accession BM4 and BM3.

The clade fourth comprised of BM1 and BM2. Two accessions (BM6 and BM5) formed a separate out group in the cluster 1 indicating less genetic similarity (Fig. 4.8).

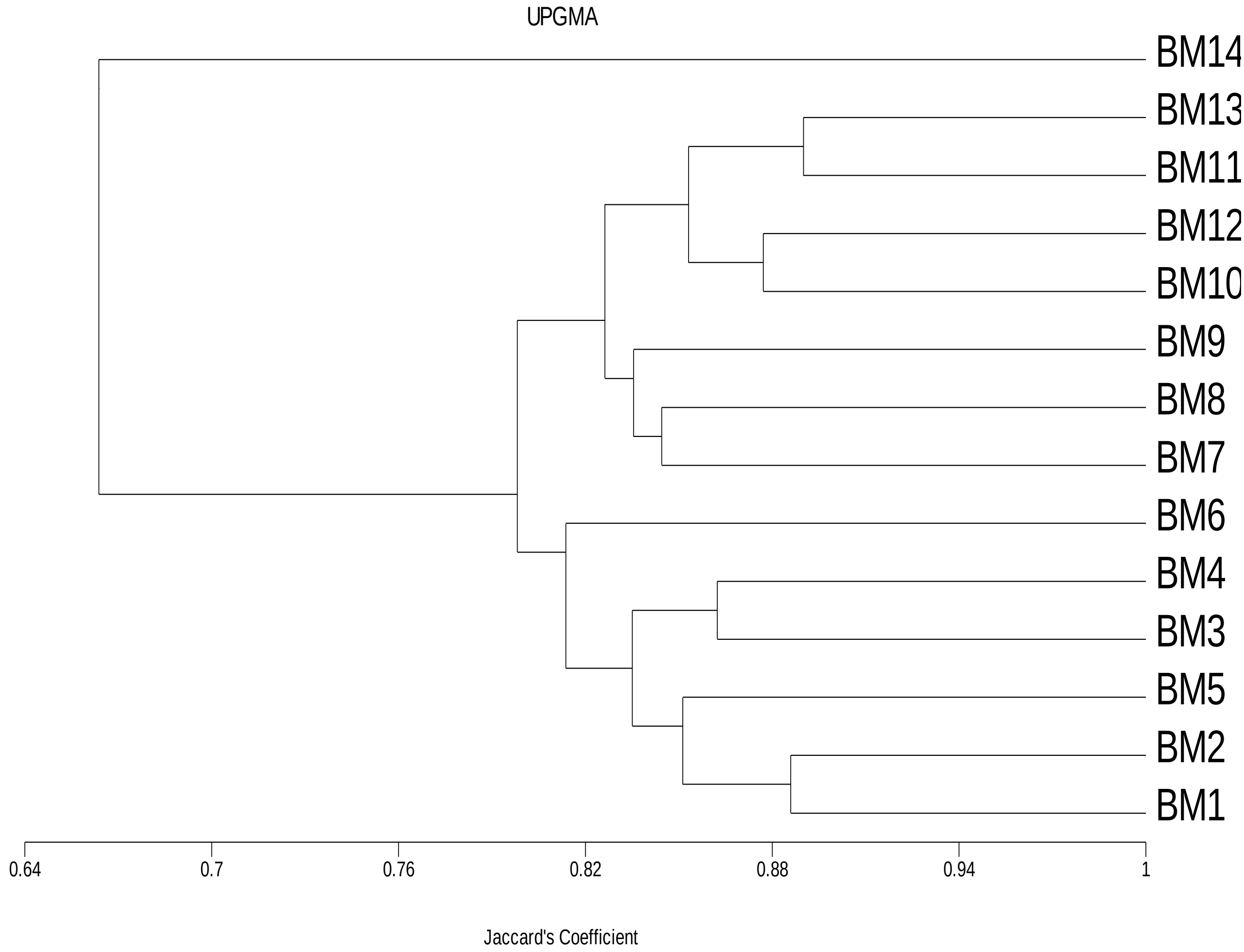


Fig. 4.8 Unweighted pair group method with average (UPGMA) cluster based on Jaccard's coefficient calculated from RAPD data of various accessions of *B. monnieri*

4.6.2 ISSR based fingerprinting

Total 27 ISSR primers were screened for studying genetic variations amongst the fourteen accessions. Out of these, 20 ISSR primers produced reproducible and scorable bands and hence used in this study. The ISSR profile of all the samples is represented in Fig. 4.9. A total of 209 bands were amplified with twenty primers. Polymorphic bands recorded for ISSR markers were 96 (44.9 %). The size of amplified fragment ranged from 250-2500 bp (Table 4.8). In case of ISSR primers, average number of bands produced per primer was 10.45.

Maximum number of ISSR markers (14) was amplified for primers ISSR2, ISSR5 and ISSR10 and maximum polymorphic markers (9) were scored for primers ISSR12, ISSR17 and ISSR20 (Table 4.8).

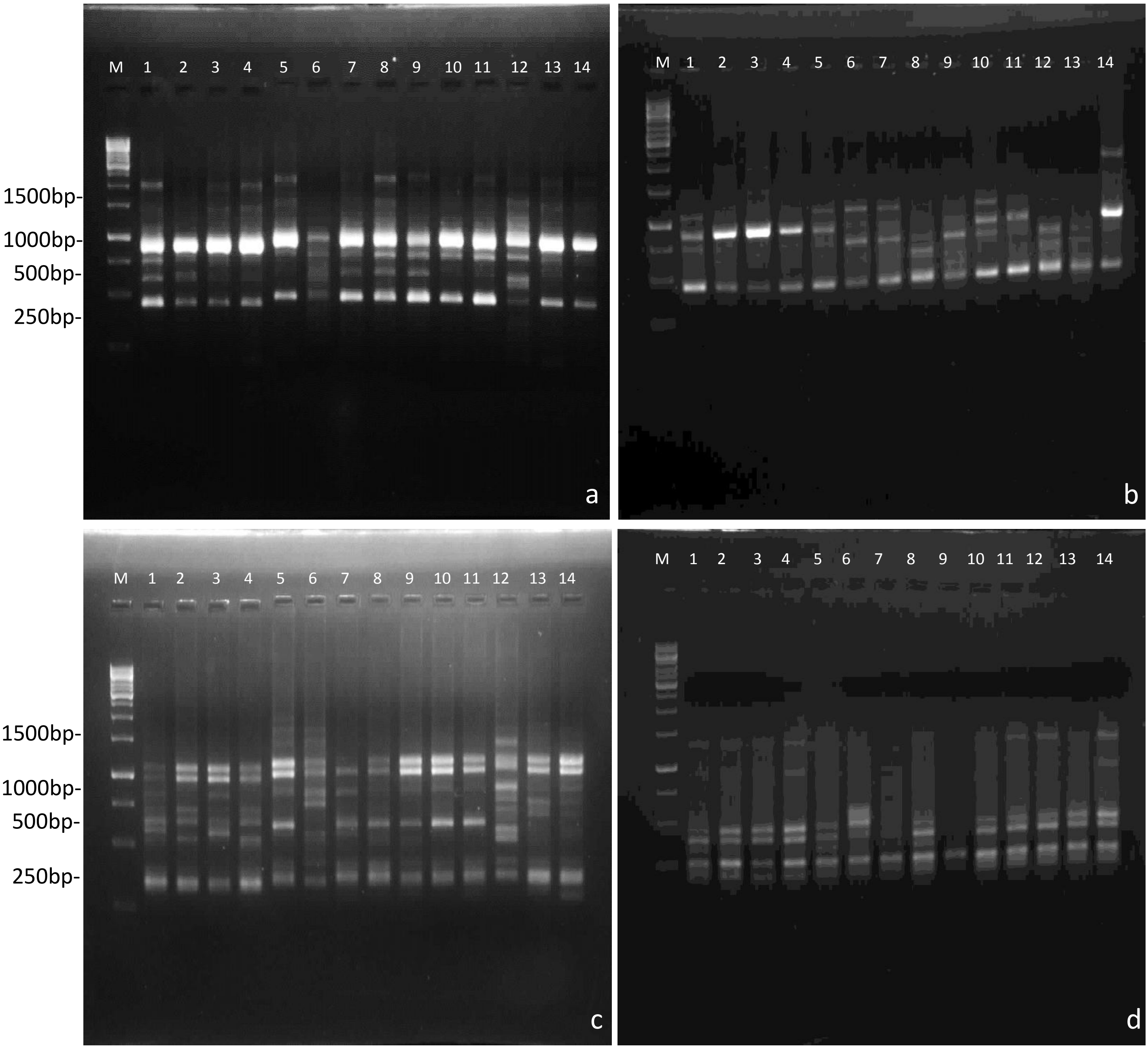


Fig. **4.9** ISSR profile of 14 accessions of *B. monnieri* showing the banding pattern. **a)** ISSR profile using primer ISSR 9; **b)** ISSR profile using primer ISSR 16; ISSR profile using primer ISSR 23 and **d)** ISSR profile using primer ISSR 23. Lane M: 1 kbp ladder; lanes 1–14: accession BM1–BM14, respectively

Table 4.8 Sequence of various ISSR primers used for amplifications and subsequent amplification parameters of polymorphic bands

S.No	Primer Code	Sequence of Primer	Amplicon size range (bp)	No amplified bands	No of polymorphic bands	Percent polymorphism
1	ISSR2	$(GA)_8CG$	450-2000	14	8	57.14
2	ISSR3	$(GA)_8TC$	250-2500	16	8	50
3	ISSR4	$(AC)_8GC$ GC	250-2000	13	5	38.46
4	ISSR5	$(AC)_{10}$	250-2000	14	6	42.85
5	ISSR7	$(CA)_8GC$	250-2000	8	4	50
6	ISSR9	$(GC)_8T$	450-2000	12	7	58.33
7	ISSR10	$(GC)_8A$	250-2000	14	8	57.14
8	ISSR12	$(CT)_8G$	300-2500	13	9	69.2
9	ISSR16	$(GT)_8TC$	250-2000	9	5	55.55
10	ISSR17	$(AT)_8C$	250-2000	12	9	75
11	ISSR18	$(AT)_8G$	250-2000	4	1	25
12	ISSR19	$(AT)_8GC$	250-2000	8	4	50
13	ISSR20	$(AT)_8$	250-2000	11	9	81.81
14	ISSR21	$(GA)_8TG$	250-2000	12	5	41.66
15	ISSR22	$(GA)_8C$	250-2000	11	8	72.72
16	ISSR23	$(GA)_8CT$	250-2500	13	4	30.76
17	ISSR24	$(GA)_8CA$	250-2000	7	2	28.57
18	ISSR25	$(GA)_8CC$	250-2000	9	2	22.22
19	ISSR26	$(GA)_8$ T	250-2000	7	3	42.85
20	ISSR27	$(CT)_8T$	250-2000	9	5	55.55
				209	**94**	**44.9**

The Jaccard's similarity coefficient of the accessions based on ISSR revealed that similarity value amongst accessions ranged from 0.634 to 0.893 indicating moderate levels of genetic similarity (Table 4.9). Maximum similarity value of 0.893 was recorded amongst BM2 and BM3. Dendrogram constructed using Jaccard's similarity coefficient is shown in Fig. 4.10. Thirteen accessions were grouped in one major cluster and one accession BM14 was placed separately as an out group in cluster showing less similarity coefficient (0.7) with other accessions.

Table 4.9 Jaccard's Coefficient of various accessions of *B. monnieri* using ISSR data

	BM1	BM2	BM3	BM4	BM5	BM6	BM7	BM8	BM9	BM10	BM11	BM12	BM13	BM14
BM1	1.000													
BM2	0.859	1.000												
BM3	0.852	0.893	1.000											
BM4	0.841	0.849	0.832	1.000										
BM5	0.841	0.860	0.843	0.851	1.000									
BM6	0.853	0.802	0.835	0.784	0.813	1.000								
BM7	0.829	0.807	0.831	0.829	0.809	0.822	1.000							
BM8	0.835	0.833	0.827	0.806	0.797	0.791	0.853	1.000						
BM9	0.807	0.775	0.798	0.827	0.816	0.830	0.804	0.783	1.000					
BM10	0.781	0.819	0.823	0.821	0.810	0.793	0.851	0.759	0.827	1.000				
BM11	0.832	0.830	0.823	0.791	0.831	0.834	0.820	0.817	0.828	0.843	1.000			
BM12	0.803	0.801	0.814	0.812	0.812	0.815	0.851	0.837	0.829	0.833	0.844	1.000		
BM13	0.779	0.767	0.809	0.837	0.797	0.820	0.825	0.803	0.865	0.838	0.828	0.849	1.000	
BM14	0.671	0.686	0.699	0.716	0.716	0.690	0.686	0.634	0.753	0.735	0.707	0.699	0.703	1.000

Accessions within main cluster are further grouped into four clades. The first clade comprised of BM11 and BM10. The second clade comprised of BM9 and BM13. The clade third comprised of BM12, BM8 and BM7. The clade fourth comprised of BM5, BM4, BM3 and BM2. Within the fourth clade BM3 and BM2 appeared to be closer to each other, with 0.89 similarity coefficient. Two accessions (BM6 and BM1) formed a separate out group in the cluster 1 indicating less genetic similarity (Fig. 4.10).

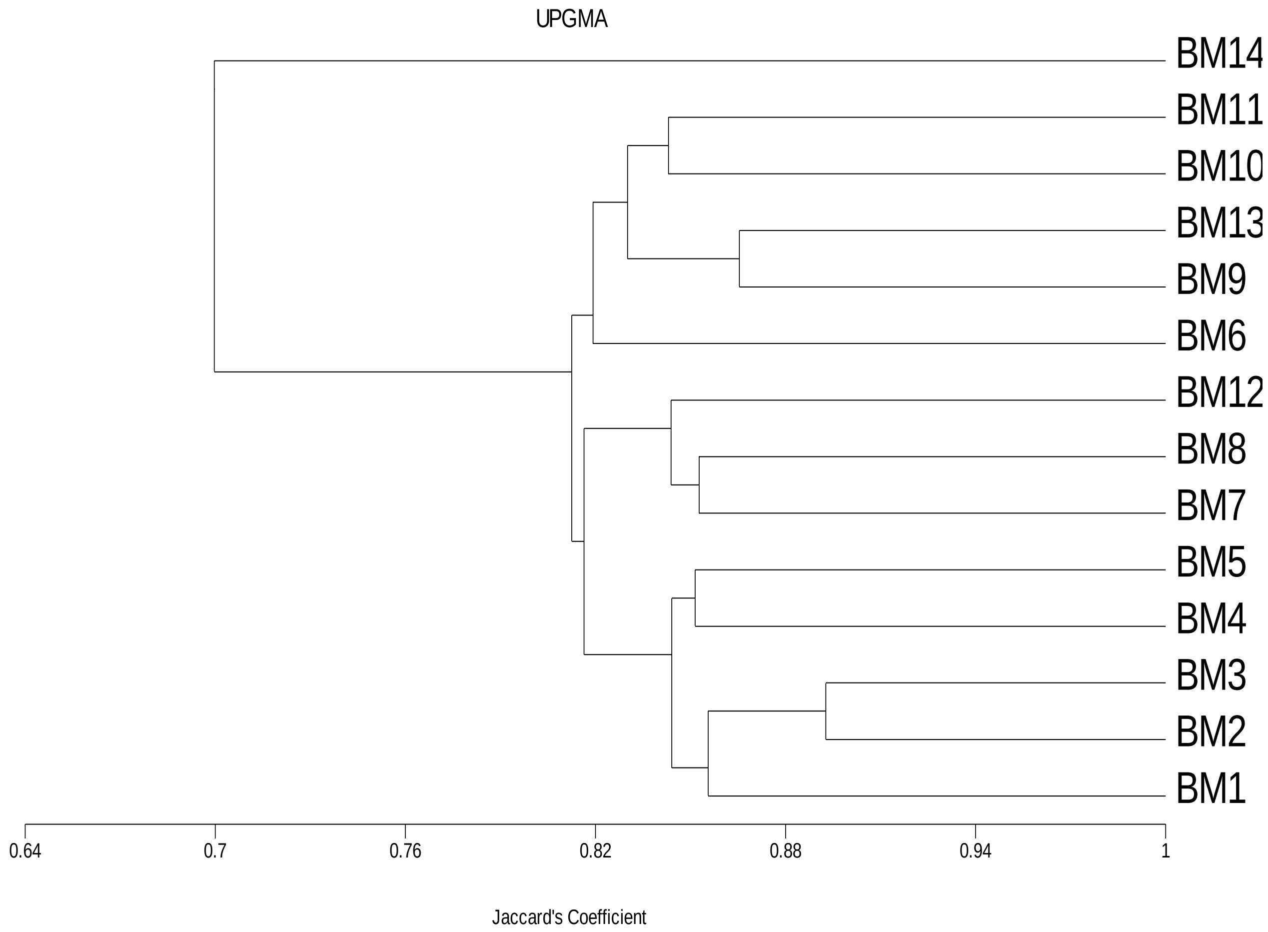


Fig. 4.10 Unweighted pair group method with average (UPGMA) cluster based on Jaccard's coefficient calculated from ISSR data of various accessions *B. monnieri*

4.6.3 Combined RAPD and ISSR fingerprinting

Genetic variations amongst these accessions were also studied using combined data of RAPD and ISSR primers. A total of 422 markers were scored (213 for RAPD primers and 209 for ISSR primers). Amongst 422 markers scored 143 (33.9 %) were polymorphic. Polymorphic bands recorded for RAPD markers were 49 (23 %) and ISSR markers were 96 (44.9 %). The size of amplified fragment ranged from 250-2500 bp (Table 4.6; 4.8).

The Jaccard's similarity coefficient of 14 accessions of *B. monnieri* based on RAPD and ISSR revealed that similarity value amongst accessions ranged from 0.758 to 0.871 indicating moderate levels of genetic similarity (Table 4.10). Maximum similarity value of 0.871 was recorded amongst BM9 and BM13.

Table 4.10 Jaccard's Coefficient of various accessions of *B. monnieri* using combined ISSR and RAPD data

	BM1	BM2	BM3	BM4	BM5	BM6	BM7	BM8	BM9	BM10	BM11	BM12	BM13	BM14
BM1	1.000													
BM2	0.871	1.000												
BM3	0.839	0.859	1.000											
BM4	0.822	0.852	0.849	1.000										
BM5	0.841	0.861	0.848	0.847	1.000									
BM6	0.828	0.805	0.826	0.800	0.823	1.000								
BM7	0.829	0.825	0.798	0.835	0.814	0.821	1.000							
BM8	0.824	0.820	0.817	0.806	0.800	0.807	0.850	1.000						
BM9	0.814	0.800	0.802	0.830	0.818	0.816	0.822	0.812	1.000					
BM10	0.798	0.818	0.825	0.828	0.817	0.786	0.830	0.802	0.835	1.000				
BM11	0.820	0.820	0.789	0.773	0.819	0.817	0.822	0.817	0.832	0.841	1.000			
BM12	0.777	0.787	0.785	0.792	0.781	0.808	0.828	0.823	0.818	0.857	0.843	1.000		
BM13	0.780	0.781	0.792	0.799	0.793	0.820	0.825	0.835	0.871	0.854	0.861	0.867	1.000	
BM14	0.804	0.794	0.802	0.815	0.830	0.833	0.802	0.758	0.856	0.837	0.823	0.840	0.816	1.000

Dendrogram constructed using Jaccard's similarity coefficient is shown in Fig. 4.11. All the thirteen accessions were grouped in two major clades that were got subdivided into smaller groups and one accession BM14 was placed separately as an out group in clades showing less similarity coefficient (Fig. 4.11). The first clade was further divided into three clades, among them first clade comprised of BM1 and BM2. Second clade comprised of BM3, BM4 and BM5. The third clade comprised of BM7 and BM8. BM1 and BM2 appeared to be closer to each other with similarity coefficient of 0.87. The second clade comprised of BM10, BM11, BM12 and BM13. Two accessions (BM6 and BM9) formed a separate out group in a clade 1 and 2 indicating less genetic similarity (Table 4.10).

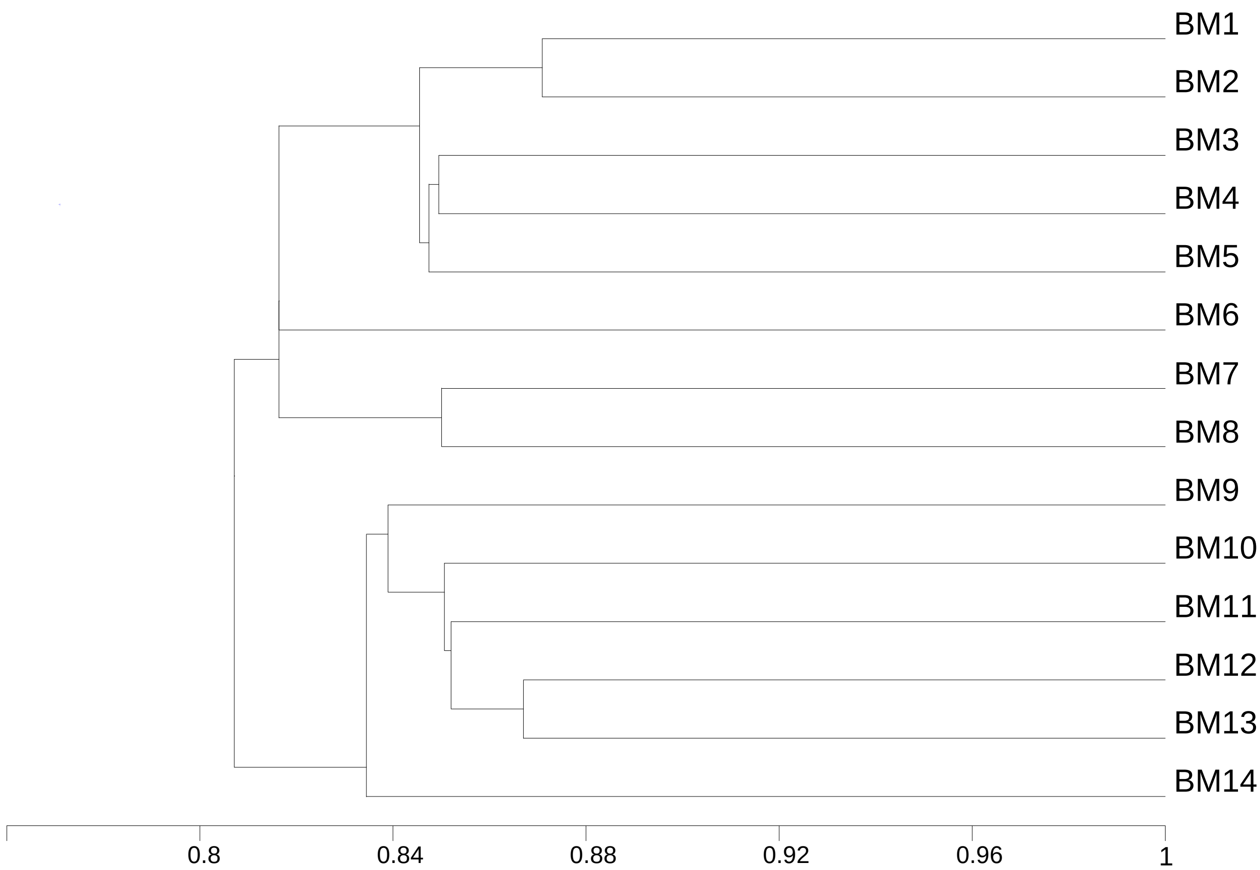


Fig. 4.11 Unweighted pair group method with average (UPGMA) cluster based on Jaccard's coefficient calculated from combined ISSR and RAPD data various accessions of *B. monnieri*

Two component PCA analysis showed 55.0 % and 8.7 % variation on component 1 and component 2 respectively (Fig. 4.12). All the accessions were places similarly as in case of UPGMA dendrogram. In this two dimensional PCA scatter plot also accession BM14 was separated out.

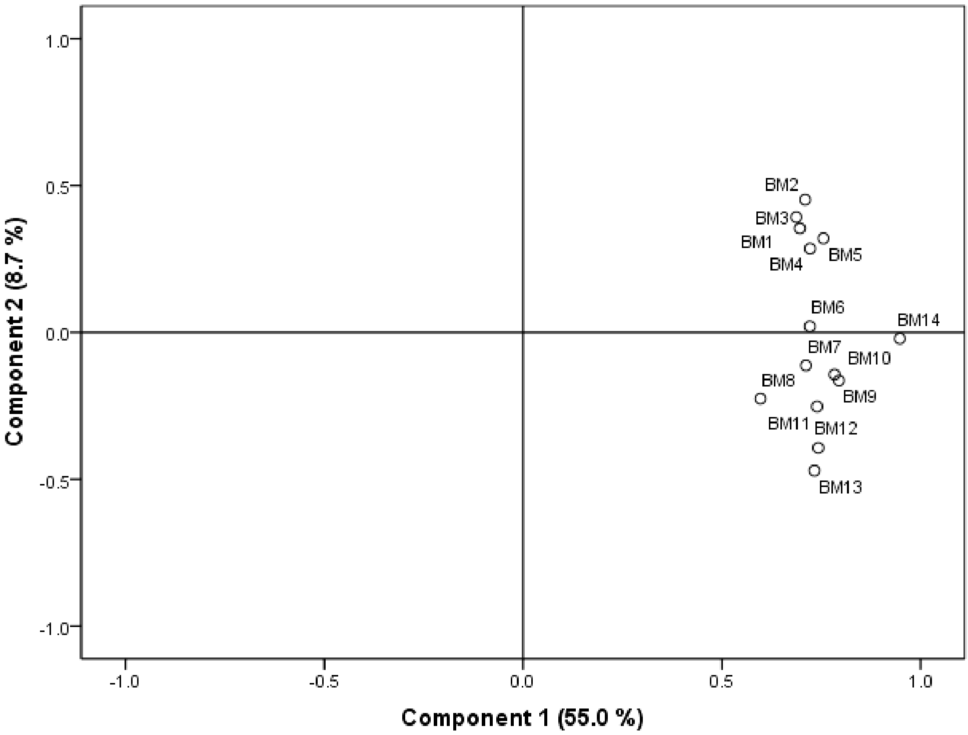


Fig. 4.12 Scatter plot of various accessions of *B. monnieri* by principle component analysis (PCA) of combined RAPD and ISSR data

Discussion

In this study, relationship among 14 accessions of *B. monnieri* collected from various locations of India was studied on the basis of growth, active principle content ('bacoside A') and PCR-based molecular markers (RAPD and ISSR). These studies will be helpful in developing strategy for long-term sustainable utilization of this important medicinal herb and a step towards effective conservation.

Various morphological characters such as plant height, stem diameter and no. of branches per plant were studied in all the accessions during the different seasons i.e. spring, summer, autumn and winter. Morphological features varied significantly among accessions and during different sampling seasons. Earlier, there is only one preliminary report investigating variability in morphological characters among the accessions of *B. monnieri* (Mathur et al., 2003). However, Mathur et al. (2003) studied the quantitative and qualitative characters of accessions of *B. monnieri*. Earlier, variation in morphological characteristics in wild populations of some of the medicinal plants has also been studied (Radusiene et al., 2004; Nadeem et al., 2002, 2007; Kumar et al., 2007; Cirak et al., 2007). Further, it has been suggested that variations in the morphological characters among accessions is due to their genetic diversity (Nadeem et al., 2007). Earlier, Cushman et al. (2006) have also reported that the timing of harvest affected the growth and overall productivity of the American may apple. Similar findings with respect to morphological features and variability have been earlier reported in many plant species (Walker et al., 2001; Kothari et al., 2003; Cirak et al., 2006; Kumar et al., 2007).

Variations in the biomass and harvest index (HI) of these accessions were estimated during all the sampling seasons. Significant variation in biomass and harvest index (HI) was recorded during the different seasons (Table 4.2). Variations in the biomass and harvest index amongst the populations collected from different locations has been reported earlier in many plant species (Bransby et al., 1989; Mosjidis, 1996; Moser et al., 2006; Kruse et al., 2008; Hodgson et al., 2010; Moghaddam et al., 2013). Maximum biomass and HI of all accessions was recorded during summer; however, minimum biomass and HI was recorded winter (Fig. 4.1). Nearly a 1.26 fold variation in the HI was observed during winter to summer season (Fig. 4.1). Earlier, there are few reports pertaining to the effect of different season on biomass and harvest index (Li et al., 2011; Moghaddam et al., 2013). Zhang et al. (2012) studied harvest index (HI) in the six wheat genotypes for a period of two years. They found a correlation between grain yields with harvest index. Hodgson et al. (2010) studied influence of environmental factors on the biomass in the fifteen *Miscanthus* genotypes grown in five locations across Europe. These authors reported a significant variation in the biomass during different seasons and found higher biomass during winter.

Biomass and harvest index (HI) of these accessions also varied significantly among the accession (Table 4.1). Biomass and harvest index variability amongst the populations of different locations has been reported earlier by many researchers (Sladden et al., 1991; Pandey et al., 2007; Nadeem et al., 2007). In the present study, maximum biomass accumulation and HI were observed in case of accession BM1 and BM7 and minimum biomass accumulation and HI were recorded in accession BM14. Therefore, this study clearly established its potential of accessions BM1 and BM7 for domestication. Identification of such high yielding accession is important for cultivation and production of 'bacoside A'. The variation in the biomass and HI has been reported in many crops (Scully and Wallace, 1990; Pilbeam, 1996; Gilbert et al., 2006; White and Wilson, 2006). These authors reported that increase in harvest index is due to the increase in biomass of the plant. Pandey et al. (2007) reported variation in the harvest index in plants of different ages of *P. hexandrum* grown at lower altitude.

Considerable variations were also recorded in RGR of *B. monnieri* of different accessions during all the sampling seasons. Earlier, there are few reports pertaining to the effect of different season on RGR (Karimi and Siddique, 1991; Barradas and Lopez-Bellido, 2009). These authors also reported that relative growth rate was higher during early growth periods, decreasing after summers (June) until the end of the winter season. The negative RGR values observed from summers to winters in this study can be explained by an increase in the number of senesced leaves (Davidson and Campbell, 1984). Amongst the accessions, RGR during all sampling dates was maximum in BM14 and was found minimum in accession

BM1 and BM7. Pandey et al. (2007) reported the RGR as an index of biomass production in *P. hexandrum*. These authors also observed that relative growth rate was maximum in the year when the biomass of the plant was minimum.

Various components of ‘bacoside A’ namely, ‘bacoside A3’, ‘bacopaside II’ and ‘bacosaponin C’ were also detected in all the accessions. ‘Bacoside A3’ content during all sampling seasons were maximum in accessions BM1 and BM7 and minimum levels were detected in the accession BM14 (Table 4.4 and 4.5). However, maximum ‘bacopaside II’ content was recorded for accession BM4 in all seasons and lowest level of ‘bacopaside II’ for BM13. ‘Bacopasaponin C’ content of either season were higher in accession BM1 and BM7 (Table 4.5) and lowest content of ‘bacopasaponin C’ was recorded in the samples of accession BM14.

The levels of ‘bacoside A’ in samples collected from different locations varied significantly (Table 4.4). Earlier, Darokar et al. (2001) reported variation in ‘bacoside A’ content investigating the accessions of *B. monnieri* and reported a total a 14-fold variation in ‘bacoside A’ content of different accessions. In the present study, bacoside content varied from 1.26 to 3.95 mg/g DW, thus showing about three fold variations. Variation in active principle contents in wild populations of medicinal plants has also been reported earlier in other plants including *B. monnieri* (Nadeem et al., 2002, 2007; Naik et al., 2012). However, the study by Naik et al. (2012) was restricted to a smaller geographical area of State Karnataka of India only. In the present study, growth and ‘bacoside A’ content were evaluated in accession after growing at a common location for one year, therefore, the recorded variations in ‘bacoside A’ contents could only be due difference in genetic makeup of these accessions. Further, it has also been suggested that variations in secondary metabolites contents is the function of stress and changing environment (Fernie, 2007).

Similar findings with respect to secondary metabolite variability have been reported earlier in many plant species (Lim et al., 2005; Cirak et al., 2007; Sanaa et al., 2012).

Variations in the components of 'bacoside A' were estimated during different seasons of year, i.e. spring, summer, autumn and winter (Table 4.5). The levels of 'bacoside A' component varied significantly during different sampling seasons (Table 4.5). Levels of all the three components of 'bacoside A' were maximum during summer, whereas minimum levels of these were recorded during winter.

The levels of 'bacoside A' varied in samples collected from different location during the different periods of the year (Fig. 4.4 and Table 4.5). Among all the four seasons i.e. spring (March), summer (June), autumn (September) and winter (December), maximum levels of 'bacoside A' were recorded during summer when average monthly temperature was higher (Fig. 4.4 and 4.5). The minimum levels of 'bacoside A' were recorded during winter when average temperature was minimum (Fig. 4.4 and 4.5). About 2.5 to 3.0 fold variations were recorded in the levels of 'bacoside A' across the accessions. Earlier, there are few reports pertaining to the effect of different season on 'bacoside A' content (Mathur et al., 2002; Phrompittayart et al., 2011). These authors also reported summer as an appropriate season for the harvest of *B. monnieri*. The effect of season on active principle has also been reported in *Hypericum perforatum* (Southwell and Bourke, 2001; Bagdonaite et al., 2012). It was also important to note that the levels of 'bacoside A' and its components in *B. monnieri* were maximum during summer. Therefore, summer could be an appropriate season for the harvest of *B. monnieri*.

Two dimensional scatter plot by PCA analysis of average levels of 'bacoside A' components ('bacoside A3', 'bacopaside II' and 'bacopasaponin C') exhibited remarkable difference in the levels of 'bacoside A' (Fig. 4.6) throughout the year. The samples of summer were only positively correlated on both axes again suggesting an appropriate time for the harvest. Similar findings with respect to secondary metabolite variability have been earlier reported in many plants (Filippini et al., 2010; Bagdonaite et al., 2010; Bagdonaite et al., 2012; Scognamiglio et al., 2014). These authors also observed clustering of secondary metabolite depending upon the season in which plants samples were extracted. This study suggests summer as an appropriate time for the harvest of these plants, both for the extraction of bacosides and preparation of various 'Ayurvedic' formulations.

Documentation and preservation of genetic diversity of natural populations is important for conservation of medicinal plants (Karthikeyan et al., 2011). Genetic diversity was assessed among various accessions of *B. monnieri* using RAPD and ISSR markers. The utility of these markers in studying genetic diversity among wild populations has been highlighted earlier (Pharmawati et al., 2004; Mohapatra and Rout, 2005; Kashyap et al., 2005; Barik et al., 2006; Karthikeyan et al., 2011; Saxena et al., 2011). In the present investigation, 35 RAPD and 20 ISSR primers produced 143 polymorphic bands that unambiguously grouped 14 *B. monnieri* accessions collected from different locations into two major clusters. The level of diversity detected by ISSR (44.9 %) was much higher as compared to the diversity detected by RAPD markers (23 %). This discrimination could be due to the fact that these markers target different parts of the genome (Gajera et al., 2010). The present study reflected higher genetic variations than the earlier reports in *B. monnieri* using RAPD markers (Ramesh et al., 2011; Karthikeyan et al., 2011). In the present study, significant polymorphism (more than 35 %) was observed among the accessions, indicating diverse genetic base of these accessions of *B. monnieri* collected from different wild populations. The higher diversity recorded could be due to intraspecific variations as reported earlier (Nayak et al., 2006). These results are also in agreement with earlier report (Tripathi et al., 2012). Several studies on assessment of genetic diversity of plants using molecular markers have established the correlation between geographical distance and genetic similarity between individuals (Islam, 2004; Kaur et al., 2010; Paul et al., 2013). However, no such correlation could be established in this study. The possible reasons for these differences could be a limited number of markers scored in the earlier studies (Ceasar et al., 2010). Earlier, Cirak et al. (2012) have also reported similar results in *Hypericum orientale*, where no such relations based on geographical area of collection could be established. This seems to be the first study to investigate genetic variation on the larger group of populations spreading over larger geographical area across India. Two-dimensional PCA scatter plot of accessions using RAPD and ISSR data also showed nearly same pattern of grouping as UPGMA clustering. It was interesting to note that accession BM14 was separated in UPGMA dendrogram and two-dimensional PCA analysis performed using biochemical and also molecular data. However, this was not true in case of all accessions. The grouping based on 'bacoside A' content is also in agreement with the molecular marker data.

Conclusion

The aim of the present work was to identify elite genotypes, which can be used for commercial propagation to provide quality raw material and maintain assured and sustainable supply for various Ayurvedic formulations. Fourteen accessions of *B. monnieri* collected from various locations of India were studied on the basis of active principle content ('bacoside A'), biomass, harvest index and PCR-based molecular markers (RAPD and ISSR). These accessions were also studied for the morphological characteristics and relative growth rate. Significant variation in 'bacoside A' contents of the various accessions collected from different locations was observed. These accessions were also significantly different in their morphological characteristics. The RAPD and ISSR markers also documented considerable variations among these accessions and thus morphological, biochemical and molecular data were in line with each other. Further, level of variations recorded with ISSR data was higher as compared to RAPD data. Out of fourteen accessions, accession BM 14 was separated in UPGMA dendrogram and two-dimensional PCA analysis performed using biochemical and also molecular data.

Salient findings

- BM1 and BM7 showed maximum biomass, harvest index, RGR and all morphological features compared to other accession i.e. plant height, stem diameter and number of branches per plant.
- Summers (June) was found to be appropriate time for maximum Relative growth rate, HI and biomass.
- Maximum level of 'bacoside A' was recorded in accession BM1 and BM7 and lowest with BM14.
- 'Bacoside A' was higher in the samples taken during summers when average temperature was higher and it was lower in winters.
- Molecular characterization using RAPD and ISSR markers indicated a moderate level of genetic diversity amongst accessions.
- Dendrogram constructed using RAPD and ISSR data out grouped BM14 accession showing less similarity while UPGMA dendrogram and two-dimensional PCA analysis performed using biochemical and molecular data also out grouped accession BM 14.

Chapter 5

Establishment of aseptic cultures from selected elite clones and selection of cell lines for the production of bacosides

The previous chapter reported the identification of elite accessions with the ability of high growth rate and 'bacoside A' content. It is interestingly clear that for conservation of medicinal plants biotechnological approaches must be followed to enhance the yield and reduced time gap. *In vitro* cell/organ culture could provide an alternative to field-grown plants for the production of pharmaceutically important compounds. Cultures (cell/organ) can thus be used to produce secondary metabolites as well as for commercial propagation (Sivanandhan et al., 2011, 2012). All the accessions in the present study were capable of producing 'bacoside A', whereas higher levels of 'bacoside A' coupled with higher HI was observed in accession BM6. Therefore accessions BM6 was used in this study for the production of bacosides using cell and organ culture.

5.1. Establishment of aseptic cultures

After surface disinfection with mercuric chloride, nodal explants were inoculated on MS medium variously supplemented with BA (0.5-5.0 μM) (Table 5.1). Explants were sub-cultured at every 3 - 4 days for 3 – 4 subculture cycles. After 2 - 3 weeks, about 40 % of the explants showed sprouting from nodes which later on resulted in the formation of shoot clumps after 2 - 3 cycles of sub-culture on MS medium supplemented with PGR's (Fig 5.1). Experiments were carried out to study the effect of different concentrations of BA on sprouting of explants. The concentration of cytokinin influenced sprouting from nodal explant, higher concentrations of BA (more than 2.5 µM) did not favour sprouting from nodal explants (Table 5.1). Maximum sprouting of explants was observed on medium supplemented with 2.5 µM BA (Table 5.1). Following successful initiation of culture, newly formed shoots were excised from the explant and further cultured on MS medium supplemented with 2.5 µM BA to increase the number of shoots.

Table 5.1 Effect of different concentrations of BA on shoot induction from nodal explants of *B. monnieri* on MS medium

Plant growth regulator BA (µM)	Percent explants showing shoot induction
0.5	8.8 ± 0.11[f]
1.0	11.1± 0.18[e]
1.5	13.3 ± 0.17[d]
2.0	17.7 ± 0.19[c]
2.5	30.5 ± 0.14[a]
5.0	23.2 ± 0.16[b]

Data was recorded after 3-4 weeks of inoculation of nodal explants. Means with the same letter within columns are not significantly different according to Duncan's multiple range test ($P < 0.05$), values are mean ± standard deviation

5.1.1. Shoot organogenesis

Shoot organogenic potential of various accessions was studied using leaf explants on MS medium supplemented with 12.5 µM BA and 1.0 µM 2, 4-D (Table 5.2). Significant variation with respect to shoot organogenesis was recorded among various accessions. Maximum shoot organogenic potential was recorded in case of accession BM6, where 96 % explants showed shoot organogenesis with an average of 39.8 shoots per explant. In case of accession BM3, only 36.3 % explants showed shoot organogenesis with 11.9 shoots per explant. In case of accession BM14 minimum number of shoots (2.8) regenerated per explant (Table 5.2; Fig. 5.2).

Fig. 5.1 (a) Elite plants of *B. monnieri* used in the present study for establishment of aseptic culture and subsequent experiments (**b)** Newly formed shoots of *B. monnieri* on MS medium supplemented with 2.5 μM BA **(c-d)** Shoot multiplication on MS medium supplemented with 2.5 μM BA

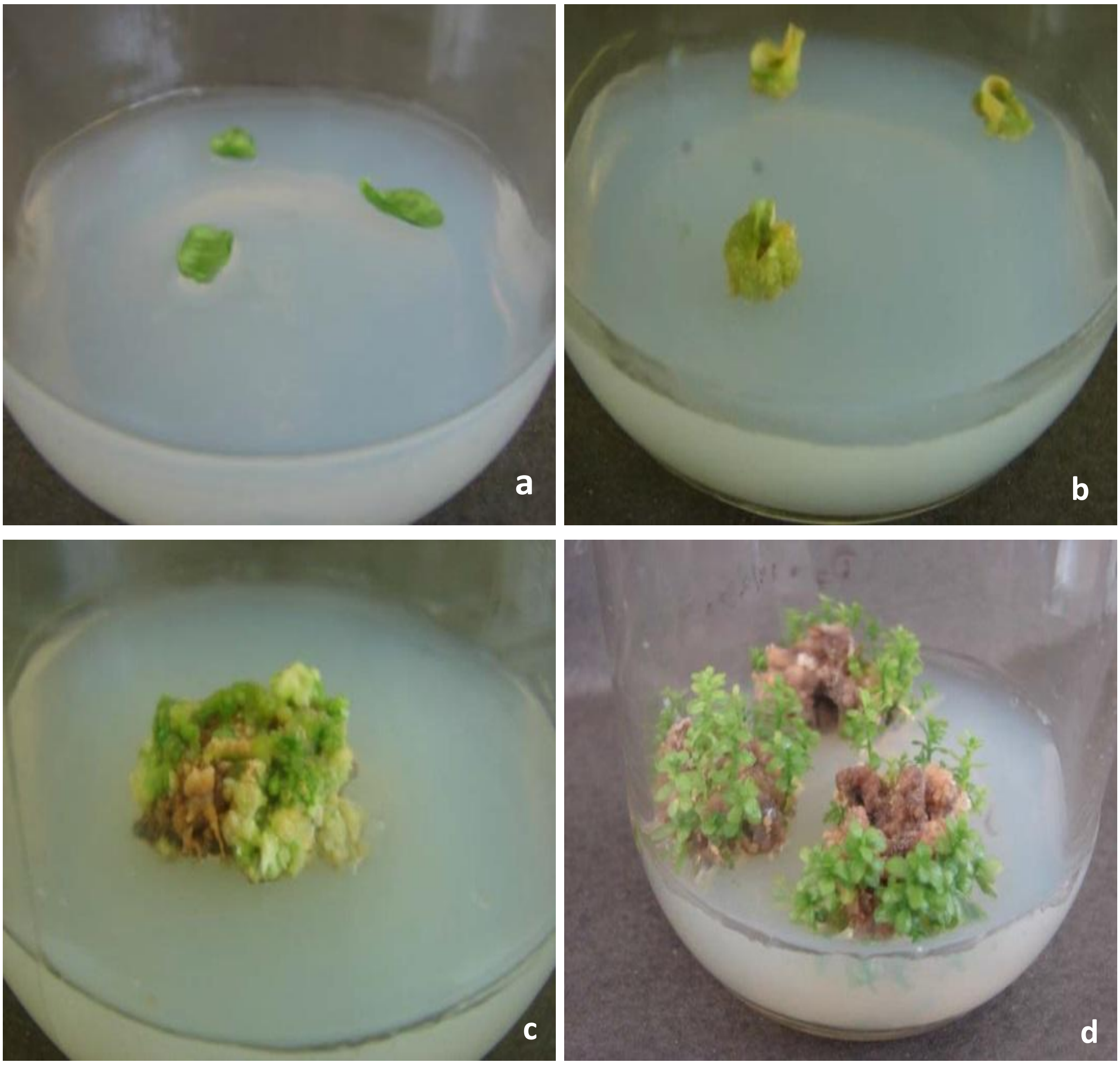


Fig. 5.2 Shoot organogenesis of *B. monnieri* using MS medium containing 12.5 µM BA and 1.0 µM 2, 4-D **(a)** Leaf explants of *B.monnieri* inoculated (adaxial side down) in media after injury **(b)** Explant swelling and the suberization of cut ends with slight callusing as observed after a week **(c)** 4-5 week old callus initiated from leaf explants on MS medium supplemented with 12.5 µM BA and 1.0 µM 2, 4-D **(d)** Multiple shoots regenerated from callus on MS medium supplemented with 12.5 µM BA and 1.0 µM 2, 4-D

Table 5.2 Comparison of shoot organogenic potential of leaf explants of various accessions of *B. monnieri* on MS medium supplemented with 12.5 µM BAP and 1.0 µM 2, 4-D

Accessions	Percent explants showing shoot regeneration	No. of shoots per Explant
BM1	77.5^{d}	20.4 ± 2.1^{c}
BM 2	75.2^{f}	16.3 ± 1.6^{d}
BM 3	36.3^{m}	14.1 ± 1.3^{g}
BM 4	86.3^{c}	15.3 ± 1.4^{f}
BM 5	95.4^{b}	24.6 ± 2.3^{b}
BM 6	96.5^{a}	39.8 ± 4.8^{a}
BM 7	63.4^{j}	13.8 ± 1.2^{h}
BM 8	73.7^{g}	12.6 ± 0.7^{i}
BM 9	52.1^{l}	11.9 ± 1.6^{j}
BM 10	72.8^{h}	15.8 ± 0.7^{e}
BM 11	53.6^{k}	5.7 ± 0.2^{k}
BM 12	63.9^{i}	3.7 ± 0.02^{m}
BM 13	76.4^{e}	3.9 ± 0.01^{l}
BM 14	72.6^{h}	2.8 ± 0.01^{n}

Data were recorded after 8 weeks of culture. Means followed by the same letter are not significantly different at $P \leq 0.05$ by Duncan's multiple range test

5.1.2. Rooting of microshoots

A high rooting efficiency of microshoots was observed on basal MS medium (Table 5.3). A minimum rooting efficiency of microshoots (93 %) was recorded for accession BM5 and 100 % rooting of shoots was recorded in accessions BM1, BM2, BM7, BM10 and BM14. Number of roots per shoot varied from 1.3 in accession BM8 and a maximum of 4.0 was recorded in many accessions (Table 5.3).

Table 5.2 Comparison of rooting efficiency of shoots of various accessions of *B. monnieri* on basal MS medium

Accessions	Percent shoots rooting	Number of roots/explants	Root length (cm)
BM1	100	3.6±0.3[b]	0.3±0.08[e]
BM2	100	4±0.5[a]	0.5±0.05[de]
BM3	97	4±0.0[a]	0.6±0.05[de]
BM4	98	4±1.0[a]	0.56±0.21[de]
BM5	93	3.6±0.6[b]	0.5±0.05[de]
BM6	95	4±0.5[a]	0.85±0.02[d]
BM7	100	2.6±0.8[d]	0.7±0.03[d]
BM8	99	1.3±0.3[e]	1.1±0.11[c]
BM9	95	3.6±0.6[b]	1.9±0.14[a]
BM10	100	3.3±0.3[b]	0.6±0.05[de]
BM11	99	3.6±0.3[b]	0.67±0.4[de]
BM12	98	4±0.0[a]	0.76±0.06[d]
BM13	98	3±0.5[c]	1.5±0.12[b]
BM14	100	4±0.5[a]	1.4±0.05[b]

Data were recorded after two weeks of inoculation. Means followed by the same letter are not significantly different at $P \leq 0.05$ level by Duncan's multiple range test

5.2. Establishment of callus and cell suspension cultures

5.2.1. Establishment of callus cultures

For the establishment of callus cultures leaf explants were inoculated on MS medium supplemented with different concentrations and combinations of NAA and KIN. Leaf explant developed calli with varying morphology on different combinations of MS medium (Table 5.4). The calli obtained were compact, green, non-morphogenic initially, which became friable after 3 - 4 subcultures on same medium (Table 5.4; Fig. 5.3). Among all the media combinations used, friable green callus was observed on MS medium supplemented with NAA (5.0 µM) and KIN (1.15 µM) (Fig. 5.3 d). MS medium supplemented with NAA (7.5 µM) and KIN (3.5 µM) resulted in yellowish, friable callus (Fig. 5.3 h; Table 5.4).

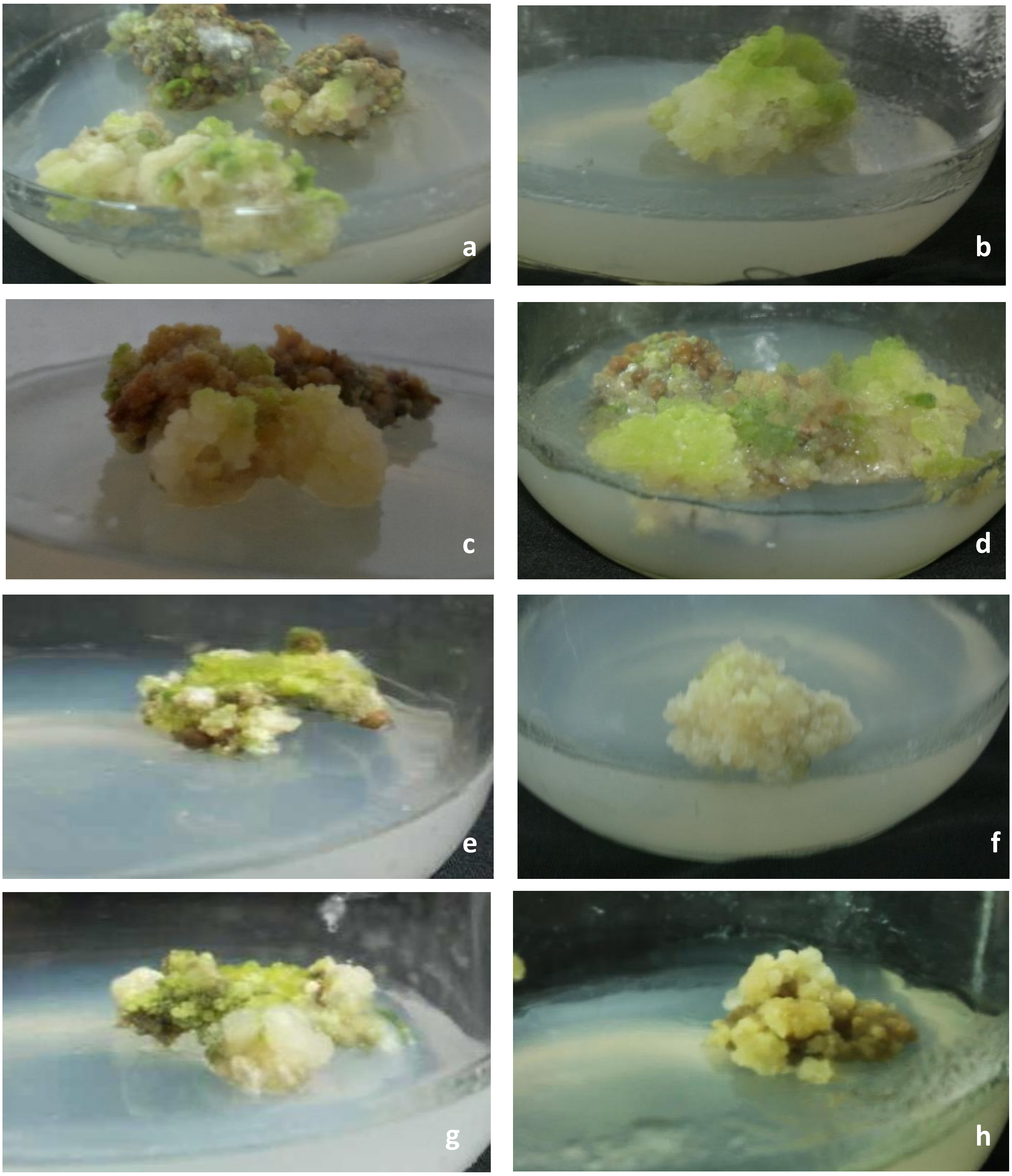


Fig. 5.3 Growth of callus showing different morphology from *B. monnieri* leaves on MS media variously supplemented with PGR's (explained in table 5.4)

Table 5.4 Growth of callus derived from *B. monnieri* leaves under different combinations of growth regulators. Growth was determined 15 d after inoculation

Media Code	NAA (µM) conc.	Kinetin (µM) Conc.	Callus morphology	Figure 5.3
B1	2.5	1.15	Greenish yellow and compact	(a)
B2	2.5	2.3	Slightly yellow and friable	(b)
B3	2.5	3.5	Yellowish and friable	(c)
B4	5.0	1.15	Greenish yellow and friable	(d)
B5	5.0	2.3	Yellowish and friable	Not shown in figure
B6	5.0	3.5	Greenish and compact	(e)
B7	7.5	1.15	Yellowish	(f)
B8	7.5	2.3	Greenish and compact	(g)
B9	7.5	3.5	Yellowish and friable	(h)

5.2.2 Establishment of cell suspension cultures

Calli obtained from leaf explants on different media combinations were used for the establishment of cell suspension cultures (Table 5.5). The calli grown on B4 media (NAA at 5.0 µM and Kinetin at 1.15 µM) showed maximum cell growth in suspension culture (5.5 g/l FCW and 1.78 g/l DCW) and 'bacoside A' production of 5.56 mg/g DCW ('bacoside A3' 0.68 mg/g DCW, 'bacopaside II' 3.02 mg/g DCW, 'bacopasaponin C' 1.63 mg/g DCW and 'bacopaside X' 0.23 mg/g DCW) (Table 5.5; Fig. 5.4). The detailed data on the effect of various PGR's combination on cell proliferation and 'bacoside A' production is provided in Table 5.5. Calli cultured on B3 medium (Table 5.6), showed 4.8 g/l (FCW) and 1.52 g/l (DCW), and 'bacoside A' content of 3.89 mg/g DCW ('bacoside A3', 'bacopaside II', 'bacopasaponin C' and 'bacopaside X' were documented as 0.43, 2.05, 1.09 and 0.22 mg/g DCW, respectively) (Fig. 5.5).

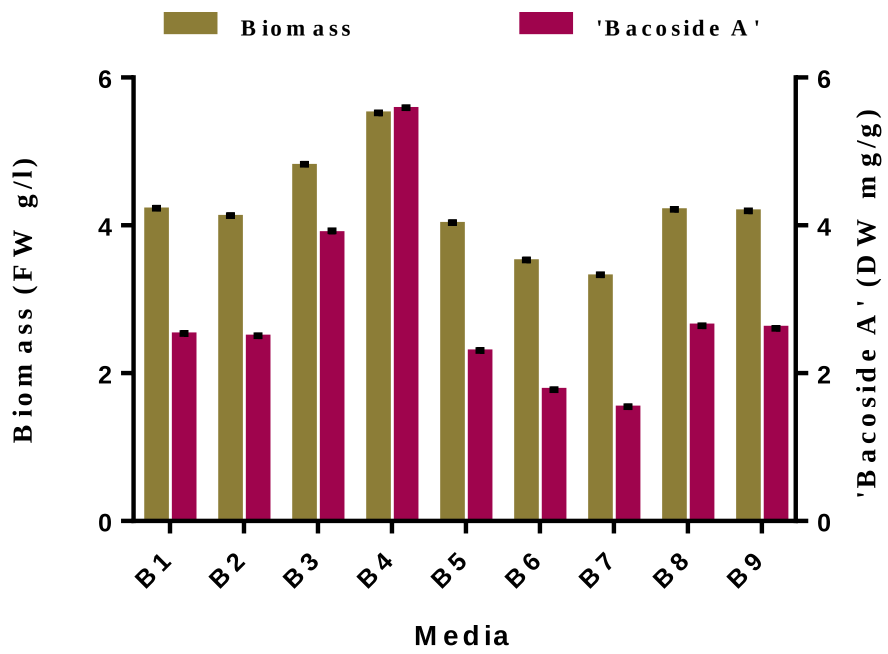


Fig 5.4 Effect of different concentrations of auxins and cytokinins on cell biomass and 'bacoside A' production by cell suspension cultures. Bars represent means ± SD (n =3)

Table 5.5 Influence of different concentrations of auxins and cytokinins on cell biomass accumulation (g/l) and 'bacoside A' production (mg/g DW) in cell suspension cultures of *B. monnieri*

Media	FCW	DCW	Total 'bacoside A'	Bacoside A3	Bacopaside II	Bacopasaponin C	Bacopaside X
B1	5.01±0.37[c]	1.39±0.13[d]	2.52±0.32[d]	0.31±0.08[d]	1.47±0.11[d]	0.55±0.04[d]	0.19±0.01[a]
B2	4.14±0.31[c]	1.37±0.19[d]	2.47±0.26[e]	0.29±0.10[e]	1.45±0.04[d]	0.54±0.01[d]	0.19±0.02[a]
B3	4.80±0.28[b]	1.52±0.21[b]	3.89±0.11[b]	0.43±0.14[b]	2.05±0.23[b]	1.09±0.17[b]	0.22±0.03[a]
B4	5.52±0.37[a]	1.78±0.21[a]	5.56±0.27[a]	0.68±0.13[a]	3.02±0.17[a]	1.63±0.21[a]	0.23±0.10[a]
B5	4.08±0.16[c]	1.24±0.06[f]	2.28±0.27[f]	0.25±0.06[f]	1.44±0.05[e]	0.41±0.02[f]	0.18±0.04[b]
B6	3.52±0.26[d]	1.17±0.12[g]	1.76±0.16[g]	0.26±0.15[e]	0.83±0.03[f]	0.58±0.11[c]	0.09±0.00[c]
B7	3.31±0.17[d]	1.08±0.07[h]	1.52±0.13[h]	0.21±0.07[g]	0.74±0.07[g]	0.49±0.07[e]	0.08±0.00[c]
B8	5.09±0.22[c]	1.43±0.22[c]	2.63±0.22[c]	0.34±0.12[c]	1.53±0.12[c]	0.61±0.12[c]	0.15±0.01[b]
B9	4.12±0.26[c]	1.31±0.16[e]	2.61±0.11[c]	0.37±0.16[c]	1.56±0.06[c]	0.41±0.16[f]	0.19±0.06[a]

Values bearing different letters in the same columns are significant at P<0.05. Values are mean ± SD (n =3).

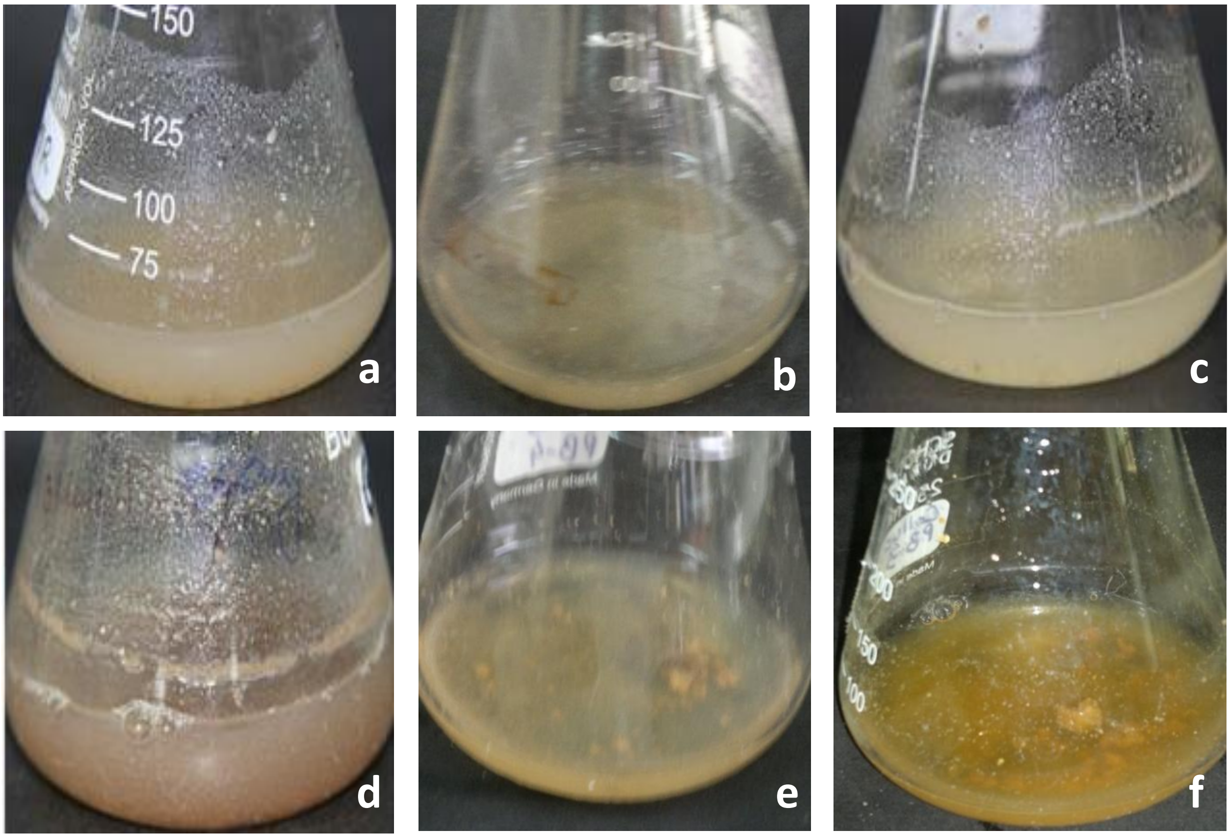


Fig. 5.5 Cell suspension cultures of *B. monnieri* on different media combinations (a: B4, b: B3, c: B8, d: B5, e: B6 and f: B7). The details of media composition are provided in Table 5.4.

5.3. Establishment of hairy root cultures

5.3.1. Establishment of hairy roots

Hairy roots were induced from leaf and internode segments using five different strains of *A. rhizogenes*. Strain SA79 was found to be more effective for induction of hairy roots as compared to other strains from both leaf and intermodal segments. Maximum root induction was recorded from leaf explants (52 %) when infected with strain SA79, whereas root induction was minimum from intermodal segments infected with strain MTCC 532 (Table 5.6).

Table 5.6 Effect of explants and strains of *A. rhizogenes* on induction of hairy roots in *B. monnieri*

***A. rhizogenes* strains**	**% explants showing root induction**	
	Internode	**Leaf**
SA79	38.5±0.23^{aD}	52.1±0.75^{aC}
A4	26.9±.05^{cD}	43.7±0.35^{cB}
R1000	31.5±0.14^{bD}	48.2±0.64^{bC}
MTCC 532	9.7±0.32^{eD}	22.6±0.47^{eB}
MTCC 2364	17.4±0.21^{dD}	31.9±0.15^{dB}

Means sharing common lower case letters within the column and upper case letter within the row are not significant at $P \leq 0.05$.

Among five strains of *A. rhizogenes* used, root induction frequency from leaf explants was higher as compared to internodal segments (Table 5.6; Fig. 5.6). Maximum root induction was observed from leaf explants infected with strain SA79; hence these were used to study the effect of various factors on root induction in subsequent experiments.

5.3.2. Factors affecting hairy root induction

Various factors studied namely bacterial density, incorporation of acetosyringone in infection medium, and co-cultivation period influenced hairy root induction. Incorporation of 100 µM acetosyringone in the medium used for re-suspension of bacterial pellet increased frequency of root induction in both leaf and internodal explants (Table 5.7). The numbers of days required for induction of roots were also significantly reduced from 15-18 days to 3-8 days (Table 5.7).

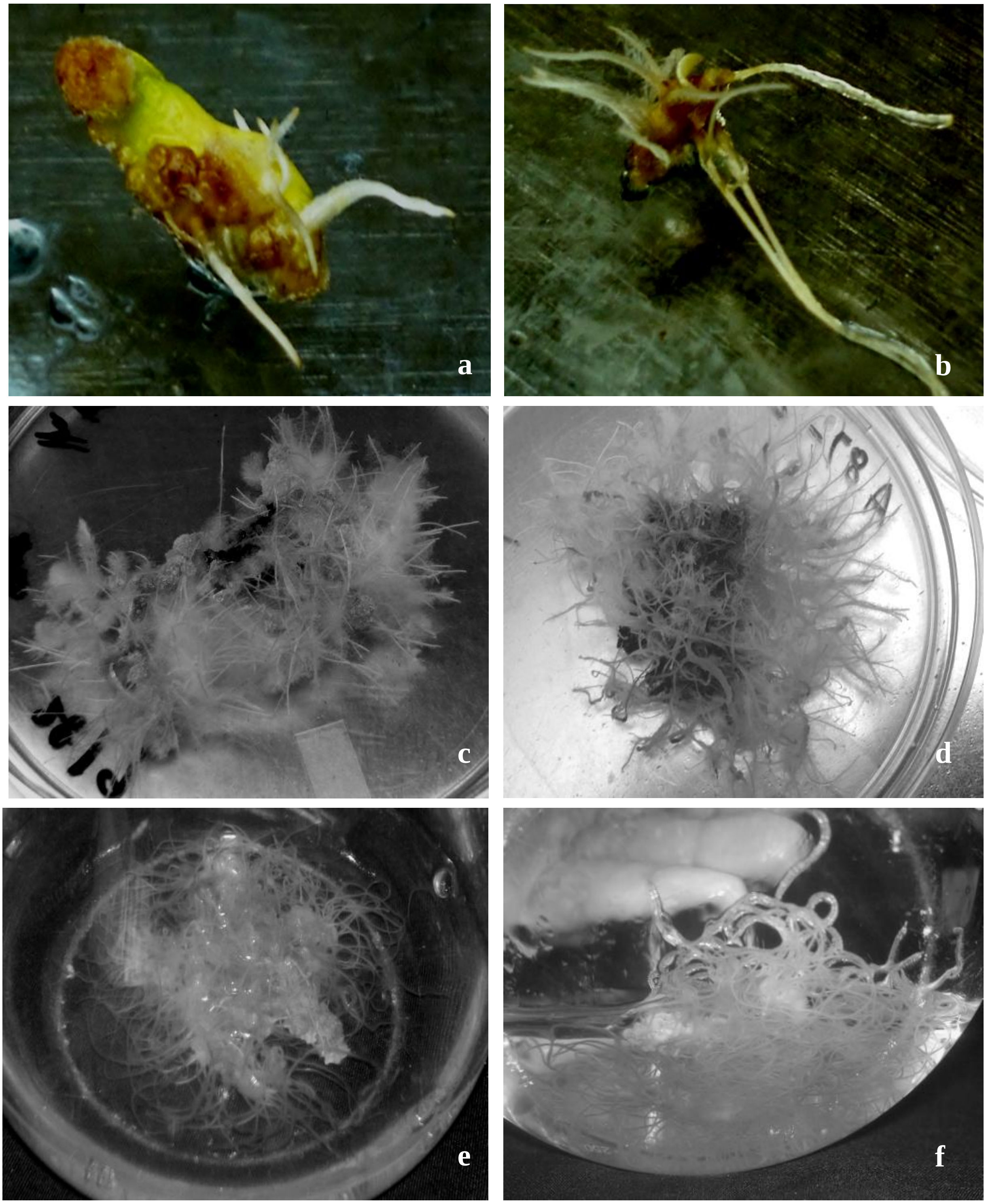


Fig. 5.6 Hairy root induction in *B. monnieri* on basal MS medium: (a) leaf explant showing root induction after 5 days of infection with *A. rhizogenes* strain SA79, (b) explant showing rapid root growth, (c-d) root growth after 21 days of subculture on basal MS medium, (e-f) 3 week old hairy roots in liquid shake culture

Table 5.7 Effect of explants, strains of *A. rhizogenes* and acetosyringone (AS) on induction of hairy roots of *B. monnieri*

A. rhizogenes **Strains**	**Days for induction of roots**		**% explants showing root induction**			
	With AS	**Without AS**	**Internode**		**Leaf**	
			With AS	**Without AS**	**With AS**	**Without AS**
SA79	3-8	15-18	55.09±3.23^{aB}	38.5±0.23^{aD}	75.1±0.30^{aA}	52.1±0.75^{aC}
A4	4-6	13-15	30.38±0.38^{cC}	26.9±.05^{cD}	65.25±0.70^{cA}	43.7±0.35^{cB}
R1000	5-9	14-18	49.13±0.44^{bB}	31.5±0.14^{bD}	72.13±0.88^{bA}	48.2±0.64^{bC}
MTCC 532	6-9	15-20	14.13±0.44^{eC}	9.7±0.32^{eD}	48.13±0.88^{eA}	22.6±0.47^{eB}
MTCC 2364	7-9	15-20	29.25±0.49^{dC}	17.4±0.21^{dD}	53.75±0.37^{dA}	31.9±0.15^{dB}

Means sharing common lower case letters within the columns and upper case letter within the rows are not significant at $P \leq 0.05$.

The bacterial density of suspension used for infection of leaf explants also influenced root induction (Fig. 5.7). Among five different densities of bacterial suspension tested (OD_{600} = 0.2, 0.4, 0.6, 0.8 and 1.0), maximum frequency of hairy root induction (62 % leaf explants) was observed when a bacterial density was 0.6 (Fig. 5.7).

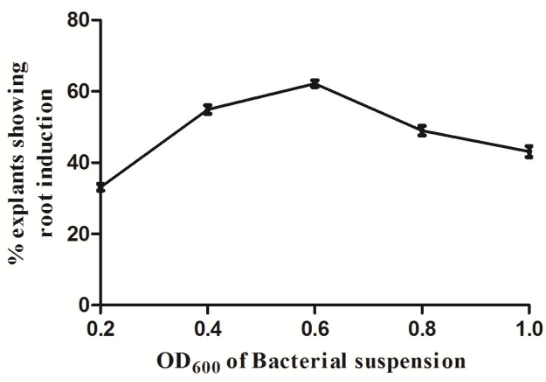


Fig. 5.7 Effect of bacterial density on root induction from leaf explants using *A. rhizogenes* strain SA79

The time period of infection with *A. rhizogenes* also influenced the frequency of root induction. Among the different infection periods tested (0, 5, 10, 15 and 20 minutes); maximum frequency of leaf explants (78 %) was induced to root when an infection time of 10 minutes was given (Fig. 5.8). Similarly co-cultivation period following infection also influenced root induction from leaf explants (Fig. 5.9). A co-cultivation period of two days was found to be suitable for root induction from 61.9 % of leaf explants. The extended co-cultivation period (> 3 days) adversely affected health of the explant.

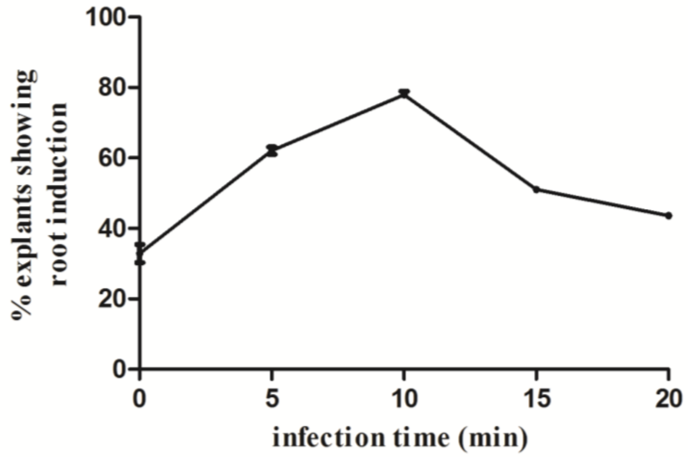


Fig. 5.8 Effect of infection time on root induction from leaf explants using *A. rhizogenes* strain SA79

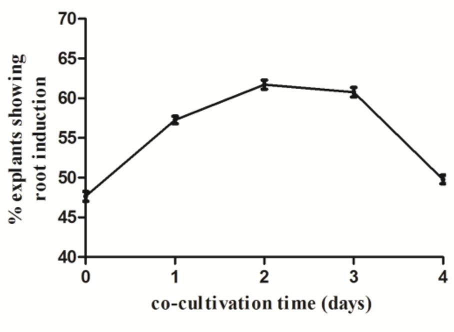


Fig. 5.9 Effect of co-cultivation time on root induction from leaf explants using *A. rhizogenes* strain SA79

5.3.3. Molecular analysis

Integration of T-DNA in the genome of hairy roots of *B. monnieri* was confirmed by PCR amplification of DNA fragment of 380 bp using primers specific to a fragment of *rolB* gene (Fig. 5.10 a). The *rolB* gene is an integral component of T-DNA of Ri plasmid and is responsible for the induction of roots. Genomic DNA from various lines of putative hairy roots showed amplification of this fragment, indicating the presence of *rolB* gene (Fig. 5.10 a). Amplification of this DNA fragment was not observed from DNA isolated from untransformed roots (negative control). Size of DNA fragment amplified from the Ri plasmid (positive control) was similar to the one amplified from hairy roots (Fig. 5.10 a). Further, elimination of bacteria from the tissues was ascertained by testing the presence of DNA fragments specific to *virD1* gene and 16S rDNA in DNA isolated from various lines of hairy roots by PCR amplification. Amplification of DNA fragments specific to *virD1* gene and 16S rDNA was not observed from genomic DNA of various lines of hairy roots (Fig. 5.10 b, c) indicating the absence of bacterial DNA contaminations. However, a fragment of about 440 bp specific to *virD1* gene (Fig. 5.10 b) and 1500 bp specific to 16S rDNA was amplified from bacterial plasmid DNA and chromosomal DNA respectively. These results confirmed the absence of *Agrobacterium* in the established root cultures.

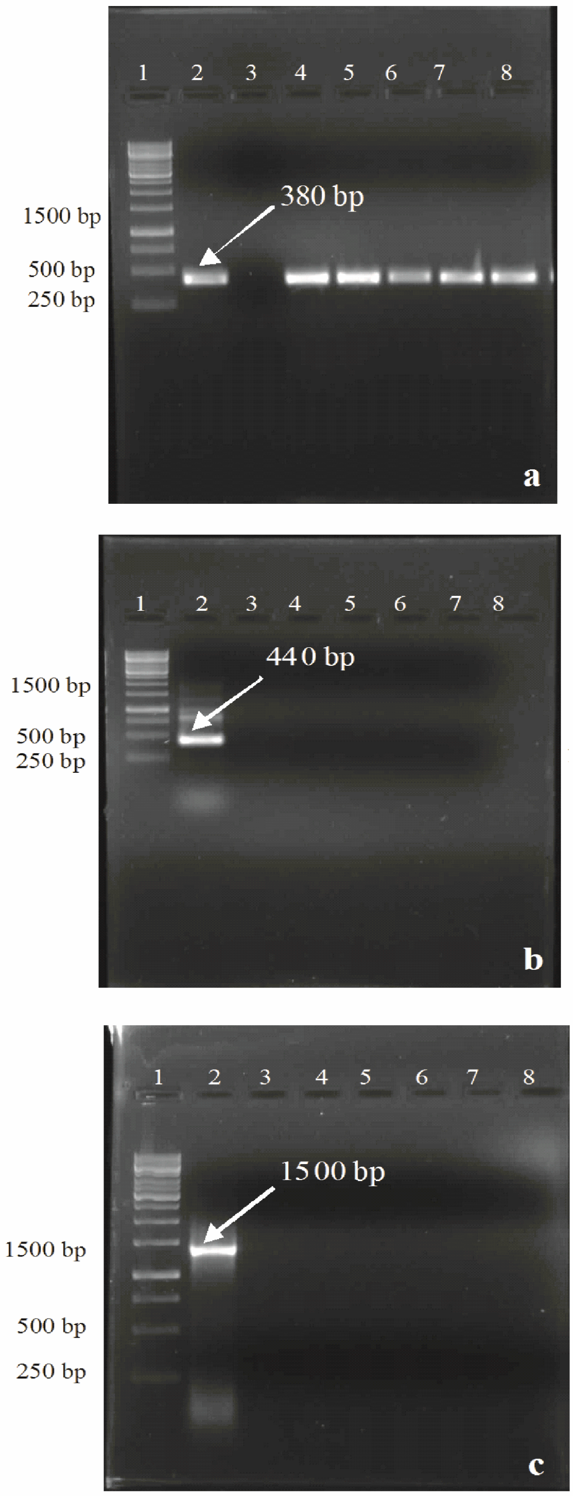


Fig. 5.10 Amplification of **(a)** fragment of 380 bp of *rolB* gene **(b)** fragment of 440 bp of *virD1* gene **(c)** 1500 bp of 16S rRNA in hairy root lines of *B. monnieri*. Lane 1 = 1000 bp DNA ladder, Lane 2 = amplification from plasmid DNA (chromosomal DNA of *A. rhizogenes* in case of 16S rDNA), Lane 3 = negative control (DNA from non-transformed roots), Lane 4 – 8 hairy roots induced by *A. rhizogenes* strains (A4, MTCC 532, R1000, SA 79, MTCC 2364).

5.3.4. Morphology and growth characteristics

At the end of the culture period, hairy roots of different lines established using different strains of *A. rhizogenes* were found to vary in morphology and growth pattern based on root elongation, lateral root density and root thickness (Fig. 5.11). Roots induced by strains MTCC 2364 and MTCC 532 were thin (Fig. 5.11 a, b), whereas hairy roots induced by strains R1000 and SA79 were thick (Fig. 5.11 c, d). Strain A4 induced hairy roots were thin in morphology and also developed intervening callus masses (Fig. 5.11 e).

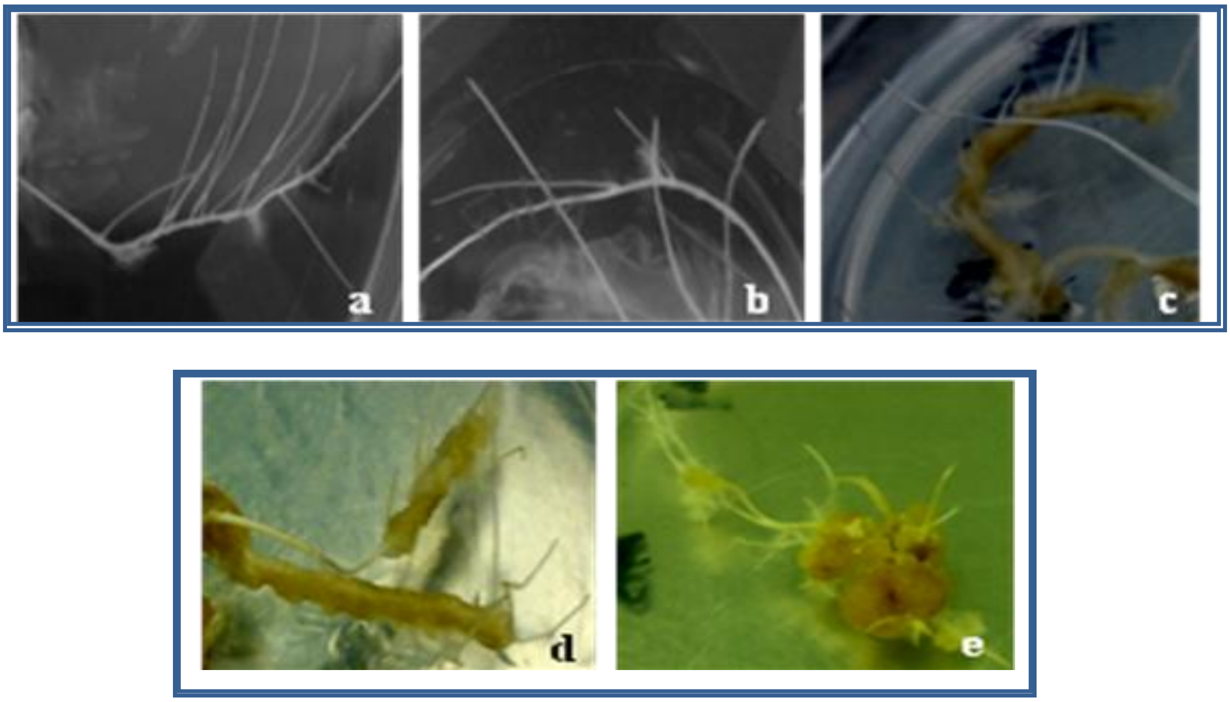
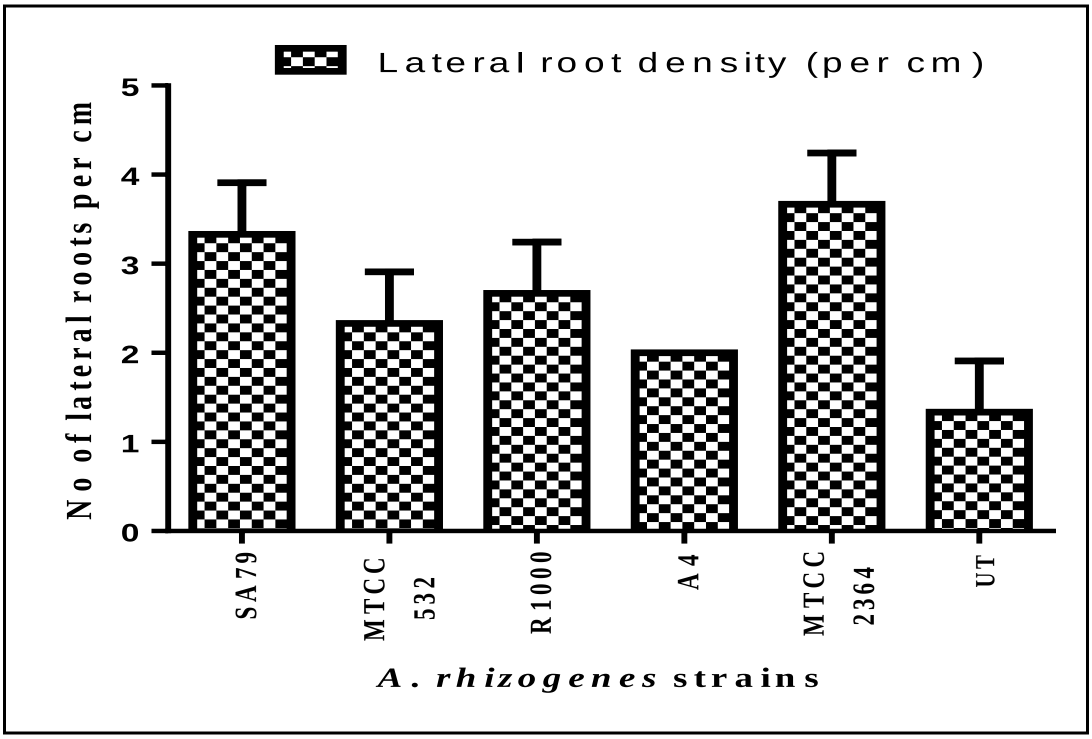


Fig. 5.11 Morphological variations amongst five different lines of hairy roots established using *A. rhizogenes* strains (a) MTCC 2364, (b) MTCC 532, (c) SA79, (d) R1000 and (e) A4. The roots were grown on MS medium and photographed after 21st day of the growth

Lateral root density varied significantly among lines of hairy roots induced by strains of *A. rhizogenes* used and maximum density of lateral roots was observed in roots induced by strain MTCC 2364 (3.6 roots per cm). Minimum lateral root density was recorded in roots induced by strain A4 (2.0 roots per cm). Root elongation per cycle also varied significantly amongst lines of roots induced by different strains of *A. rhizogenes*. Maximum root elongation (4.5 cm per subculture cycle of 30 days) was observed in hairy roots induced by strain MTCC 2364 (Fig. 5.12).



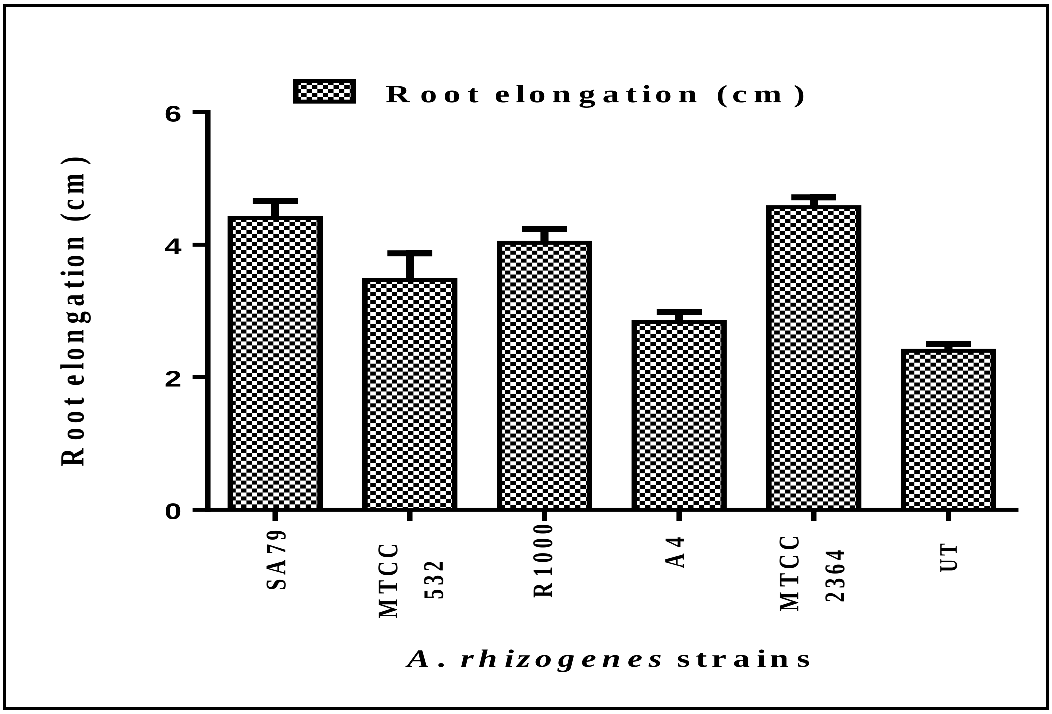


Fig 5.12 Effect of strains of *A. rhizogenes* on the (a) lateral root density and (b) root elongation on basal MS medium

5.3.5. Growth Analysis

All lines of hairy roots accumulated higher biomass (g/l) over the untransformed roots except with the only exception of hairy root line established using strain A4 (Table 5.8). However, there were significant differences with respect to biomass accumulation by various lines of hairy roots. Maximum biomass accumulation (6.8 g/l) was recorded in the cultures of hairy roots induced by strain MTCC 2364, which showed about 10-fold increment in dry weight of roots (Table 5.8) and also showed about 5.5 folds more biomass accumulation than untransformed roots in a single subculture cycle of 30 days.

Table 5.8 Effect of *A. rhizogenes* strains on accumulation of biomass from hairy root cultures of *B. monnieri*.

A. rhizogenes **strains**	**FW after 4 weeks (g/l)**	**DW after 4 weeks (g/l)**	**Growth ratio (Folds growth)**
SA79	3.7 ± 0.1^{b}	0.38 ± 0.001^{b}	5.06
A4	1.2 ± 0.02^{f}	0.098 ± 0.004^{f}	1.31
R1000	1.78 ± 0.08^{d}	0.184 ± 0.06^{d}	2.45
MTCC 532	2.8 ± 0.01^{c}	0.308 ± 0.004^{c}	4.10
MTCC 2364	6.8 ± 0.03^{a}	0.739 ± 0.05^{a}	9.86
Un-transformed	1.32 ± 0.02^{e}	0.107 ± 0.002^{e}	1.42

Cultures were grown in 250 ml Erlenmeyer's flasks containing 30 ml medium for 30 days. Dry weight of the initial inoculum of roots was 0.075(g/l). Means sharing common letters within the columns are not significant at $P \leq 0.05$.

5.3.6. 'Bacoside A' content

The 'bacoside A' content also varied significantly amongst the various lines of hairy roots established using different strains of *A. rhizogenes.* An important observation of the present study is higher levels of 'bacoside A' content accumulated by the most of the lines of hairy root as compared to untransformed roots (Table 5.9). Hairy root line induced by strain MTCC2364 accumulated about five fold higher (10.02 DW mg/g) 'bacoside A' content than untransformed roots. The 'bacoside A' content in roots induced by strain A4 (1.25 DW mg/g) was only lower than untransformed roots.

Table 5.9 'Bacoside A' content (mg/g DW) in hairy roots of *B. monnieri* induced by different strains of *A. rhizogenes*

A. rhizogenes strains	Total 'bacoside A' (mg/g DW)	'Bacoside A' components			
		Bacoside A3	**Bacopaside II**	**Bacopaside X**	**Bacosaponin C**
SA79	4.88^{b}	1.32^{b}	1.27^{c}	0.90^{b}	1.39^{c}
A4	1.25^{f}	0.26^{f}	0.37^{f}	0.36^{e}	0.26^{f}
R1000	3.15^{d}	0.51^{d}	1.18^{d}	0.28^{d}	1.18^{d}
MTCC 532	4.78^{c}	0.83^{c}	1.41^{b}	0.56^{c}	1.98^{b}
MTCC 2364	10.02^{a}	2.12^{a}	2.80^{a}	1.48^{a}	3.62^{a}
Un-transformed	2.24^{e}	0.32^{e}	1.12^{e}	0.08^{f}	0.72^{e}

Means sharing common letters within the columns are not significant at $P \leq 0.05$.

Discussion

Despite the clear potential of biotechnological tools for enhancing the secondary metabolites production and commercial importance of secondary metabolites, there has been a slow progress on *B. monnieri*. In this study, calli and cell suspension cultures of *B. monnieri* were established on different media combinations. Hairy root lines of *B. monnieri* using various strains of *A. rhizogenes* were also established. Subsequently, the potential of 'bacoside A' production by cell lines and hairy root lines were studied. Cell and organ cultures with higher levels of ‘bacoside A’ have the potential of up-scaling in the bioreactors for the production of ‘bacoside A’. This can be helpful to reduce the pressure on the wild populations. Thus may also help in conservation on one hand and assured supply of raw material on other hand.

Micropropagation, at present is one of the most efficient and reliable method for mass multiplication of true to type plants (Altman and Loberant, 1998; Sharma and Ramamurthy, 2000). The number of species cultured *in vitro* has been steadily increasing over the last two decades (Bonga and Durzan, 1987; Vasil and Vasil, 1980; Hartman et al., 1990; Palni et al., 1998; Brar et al., 2013). In the present study, the cultures were established from nodal segments. The effect of different concentration of BA was tested on shoot induction. Earlier, workers have focused to study the effect of cytokinins and their concentrations in different plant species (Hutchinson and Zimmerman, 1987; Kaur et al., 1999; Sood et al., 2002). In the present study also BA alone was tested for shoot multiplication and elongation in *B. monnieri*. All the tested concentrations promoted shoot multiplication. However, BA was found to be more effective at a concentration of 2.5 μM (Table 5.1). Earlier, beneficial effect of BA for shoot induction was reported in *B. monnieri* (Tiwari et al., 2001; Banita et al., 2005). Moreover, higher concentrations of cytokinins are known to suppress apical dominance and thus stimulate shoot multiplication (Letham and Palni, 1983; George, 1996).

The shoot organogenic potential of leaf explants was studied and it varied significantly among different accessions (Table 4). It was important to note that such differences were not specific to climatic conditions of the place of collection ruling out the possibility of sole role of environmental factors in these variations as observed by Tripathi et al. (2012). The 96.5 % of explants taken from accession BM6 showed shoot organogenesis with 39.8 shoots per explant, whereas 36.3 % explants from accession BM3 showed shoot organogenesis with 11.9 shoots per explant. Earlier, Pandey et al. (1994) studied the effect of genotype on shoot organogenic potential and rooting potential and concluded that significant variance is due to genotypes. There are several reports available on the variation of organogenic potential of explants of genetically diverse populations (Guo and Cao, 1982; Khanna and Raina, 1988; Hsia and Korban, 1996).

Rooting is one of the most important parameters for the vegetative and also *in vitro* propagation of important plants with an aim to preserve the genetic diversity in such genetically diverse populations. Similarly, rooting potential of these accessions also varied significantly. Although, many factors are known to influence the rooting of microshoots, genetic makeup of plant has been reported as one of the most important factors (Aggarwal et al., 2011). Vahdati et al. (2004) in their study on three persian walnut cultivars found significant difference in the rooting potential of microshoots. Dick et al. (1996) also emphasized the effect of genotype on the variation in the rooting ability of *Calliandra calothyrsus*. Provenance variation in rooting ability was found, with seedlings collected in San Ramon provence, Nicaragua, averaging 76 %, while seedlings from Huehutetenango, Guatemala, showed only 12 % rooting. This difference in potential of rooting of different accessions could be due to the difference in the levels of endogenous auxins and/or differential sensitivity of the tissue towards auxins.

Plant cell cultures are an attractive alternative source to whole plant which offers the potential for the production of high-value secondary metabolites. In the present study, callus cultures were established on MS media containing different combinations of NAA and KIN. These callus cultures varied in the morphology (Table 5.4). The concentration of auxin/cytokinin ratio has been reported as one of the most important factors for callus establishment (Mantell and Smith, 1983). Nath and Buragihain (2005) in their study on *Centella asiatica* found significant difference in the callus morphology on medium with different concentrations of auxin and cytokinin ratio. Parkash et al. (2005) also emphasized the effect of PGR's on the variation in the callus establishment of *Azadirachta indica.* In the present study, higher concentration of NAA and lower concentration of KIN favoured formation of friable green callus. Auxins are found to favour growth and development of a cell (Normanly et al., 1995) and production of secondary metabolites is determined by cell proliferation and differentiation (George et al., 2008). Zenk et al. (1977) found that high auxin level stimulates cell growth in the cell suspension cultures of *Catharanthus roseus*. High auxin concentrations were also found favourable for the growth of calli and cell suspension culture in case of *Withania somnifera* (Sivanandhan et al., 2012).

Cell suspension cultures of *B. monnieri* were established using the friable callus cultures. In the present study, concentrations of auxin and cytokinin influenced the biomass accumulation and 'bacoside A' content (Table 5.5). It is reported that in cultures, secondary metabolite production is a function of cell growth (George et al., 2008) and cell growth depends on the media PGRs combination and concentrations (Deus and Zenk, 1982). The type and concentration of auxin and cytokinin alters biomass accumulation and secondary metabolite production (Mantell and Smith, 1983). In cell suspension culture of *B. monnieri* NAA (5.3 µM) in combination with KIN (2.3 µM) was found to be favourable for the active proliferation of friable calli (Rahman et al., 2002). However, these authors did not study any 'bacoside A' production in the suspension cultures of *B. monnieri.* In this study, 'bacoside A' was detected and quantified in cell suspension culture. In a similar study, Sivanandhan et al. (2012) studied the effect of different concentrations of auxin and cytokinin on biomass accumulation and withanolides production in cell suspension cultures of *Withania somnifera.* Several reports are available on variability in cell growth and secondary metabolite production by cell suspension cultures. (Yamakawa et al., 1983; Zhao et al., 2001; Parkash et al., 2005; Cheng et al., 2006; Nagella and Murthy, 2010; Singh and Chaturvedi, 2012).

Hairy roots induced by *A. rhizogenes* offer promise for production of valuable secondary metabolites in many plants (Flores et al., 1999; Kim et al., 2002; Tiwari et al., 2007). In this study, hairy roots were established from *B. monnieri,* an important endangered medicinal plant of India with the aim to exploit these roots for the production of 'bacoside A'. Such type of roots has been established from many important medicinal plants and evaluated for the production of active principle (Giri et al., 2001; Chen et al., 2007; Tiwari et al., 2007; Madhulatha et al., 2007; Sujatha et al., 2013) and has played important role in the conservation of such plants. Hairy roots grow faster than normal roots and accumulate higher amounts of secondary metabolites. These roots grow faster and have great potential for large-scale production of biomass in bioreactors and stable production for pharmaceutical and other important molecules. With hairy root cultures, the year-round production of pharmaceuticals is possible; the extraction of active component is less expensive, less laborious and eco-friendly (Flores et al., 1999; Bourgaud et al., 2001; Rao and Ravishankar, 2002; Sevon and Oksman-Caldentey, 2002). The use of hairy root cultures as a raw material will not only reduce the dependence of pharmaceutical industry on wild population of plants but also ensures the availability of high value compounds without affected by seasonal variations.

In the present study, root induction potential of the various strains of *A. rhizogenes* was studied and virulence of these strains varied significantly (Table 5.6). Maximum explants were induced to root following infection with strain SA79 followed by strain R1000. The differential degree of infectivity of various strains of *A. rhizogenes* has also been reported earlier in many plants (Otani et al., 1996; Kumar and Rajam, 2007; Tiwari et al., 2007; Ooi et al., 2013). This could be due to differential virulence of these strains and/or host specificity

(Porter, 1991; Zehra et al., 1999). Tao and Li (2006) studied transformation of *Torenia fournieri* by *Agrobacterium rhizogenes* using four strains namely, R1000, R1601, A4 and R1205. Amongst these strains, R1000 was found to most efficient for hairy root induction with inducing roots in 90 % explants. Tiwari et al. (2007) also reported higher virulence of R1000 and SA79 strain in *Gentiana macrophylla.* However, root induction frequency was higher when strain A4 was used to induce roots in *Solanum mammosum* (Ooi et al., 2013). Giri et al. (2001) also found the higher frequency of strain A4 for the induction of hairy roots in *Artemisia annua* for the production of artemisinin. Thus it is important to test different bacterial strains for such studies.

In this study, higher percent of leaf explants were induced to root as compared to internodal segments (Table 5.6). Explant dependent response of root induction following *A. rhizogenes* have been reported earlier in some plants (Alpizar et al., 2006; Kang et al., 2006). This could be due to reported higher number of competent cells produced from these explants, which are ideal targets for hairy root induction (Potrykus, 1990). Leaf explants have been widely used for hairy root induction in plants (Batra et al., 2004; Tao and Li, 2006), and were often superior to other explant types (Tiwari et al., 2007; Gangopadhyay et al., 2008). Nagella et al. (2013) studied hairy toot induction in *Gymnema sylvestre* using seedling explants namely roots, stems, hypocotyls, cotyledonary nodal segments, cotyledons and young leaves. Amongst these explants, cotyledons and leaves showed better response to root induction following infection with *A. rhizogenes* strain KCTC 2703. Sujhata et al. (2013) also reported higher efficiency of leaf explant for hairy root induction in *Artemisia vulgaris*.

The various factors namely bacterial density, acetosyringone, infection time and co-cultivation period also influenced hairy root induction. Earlier, these factors has been studied and found to influence the process of T-DNA delivery to the plant tissues (Aggarwal et al., 2013).

Addition of acetosyringone in the bacterial cell suspension before infection increased the root induction frequency from both the explants (leaf and internode segment) in the present study (Table 5.6). Acetosyringone, is a phenolic compound, has been known to enhance transformation efficiency by the induction of *vir* genes of *Agrobacterium* (Atkinson and Gardner, 1991). These results are in line with the earlier reports (Barik et al., 2005; Kumar and Rajam, 2005, Krishna et al., 2010). Aggarwal et al. (2013) also reported that acteosyringone acts as an activator which increased the transient GUS expression from 62.2 % to 68.8 %. Earlier, Henzi et al. (2000) studied the effect of acetosyringone on transformation of broccoli. These authors concluded that significant enhancement in the transformation frequency is due to the addition of aetosyringone. Chen et al. (2007) also reported that activation of *A. rhizogenes* by acetosyringone before co-culturing strongly promoted hairy root induction in *Nicotiana tabaccum*. These results are in line with the present observation were addition of acetosyringone enhanced the transformation frequency.

Another factor, the influence of bacterial cell density on the root induction frequency from leaf explant was studied. Amongst different bacterial cell suspension densities tested, maximum root induction was observed at cell density of 0.6 OD_{600} (Fig. 5.7). These results are in line with the earlier reports (Barik et al., 2005; Jian et al., 2009). At higher bacterial densities, the explants were observed to turn necrotic which may be due to reported increased production of toxic compounds due to bacterial overgrowth (Sonia et al., 2007). Aggarwal et al. (2013) reported maximum transient GUS activity in explants that were infected with the bacterial suspension having an OD_{590} of 0.8. At higher bacterial density, the decrease in transient GUS activity was reported.

The effect of different infection times with bacterial culture on the root induction from leaf explant was also studied. It was found that an infection time of 10 min is optimum for the induction of roots from the leaf explant (Fig. 5.8). The varying effect of infection time on root induction in different plants has been reported earlier (Jun et al., 2007; Aggarwal et al., 2013).

Co-cultivation is one of the most important steps for *Agrobacterium*-mediated transformation of plants (James et al., 1993). Co-cultivation period also influenced root induction from leaf explant significantly (Fig. 5.9). A co-cultivation period of 2 days was found to induce the roots in maximum percent of explants. These results are in line with earlier report of Aggarwal et al. (2013), where transient GUS expression in *B. monnieri* following *A. tumefaciens* infection was found to be maximum with co-cultivation period of 2 days. The tissue necrosis was observed when co-cultivation period of more than 3 days was given. The prolonged co-cultivation periods is reported to have deleterious effect on explants (James et al., 1993). Earlier, Karthikeyan et al. (1996) reported the effect of co-cultivation period on T-DNA delivery in *Vigna mungo*.

Morphology and growth pattern of roots established using different strains of *A. rhizogenes* were found to vary significantly (Fig. 5.11). Maximum lateral root density and root elongation was observed in hairy roots induced by strain MTCC 2364 (Fig. 5.12). It has been suggested that, different morphotypes obtained using different *A. rhizogenes* strains may be due to differential expression of T-DNA genes, variable copy numbers and positional integration of T-DNA in the host genome (Cho et al., 1998). Tiwari et al. (2007) reported that roots induced from different strains of *A. rhizogenes* in *Gentiana macrophylla* showed differences in the growth pattern and type of branching (highly-branched to less-branched). Thimmaraju et al. (2008) in their study on *Beta vulgaris* (L.) hairy roots induced by different strains of *A. rhizogenes* on morphometric and biochemical characterization showed significant difference in morphometric, morphological and functional characteristics of hairy roots. Their growth characteristics, pigment content, levels of endogenous auxin and T-DNA copy number showed significant differences probably due to the physiological status of the host cell rather than the T-DNA copy number.

The growth and biomass accumulation of different lines of hairy root induced by different strains of *A. rhizogenes* also varied significantly (Table 5.7). Hairy roots induced by strain MTCC 2364 showed higher growth and accumulated about 5 folds more biomass as compared to untransformed roots. The slowest growth was recorded in hairy roots induced by strain A4 (Table 5.7). Earlier, roots induced by different strains of *A. rhizogenes* differed in total root elongation, lateral root density and biomass accumulation in *Gentiana macrophylla* (Tiwari et al., 2007). Ooi et al. (2013) used five different strains of *A. rhizogenes* for the induction of hairy roots and observed difference in the growth of roots induced by different strains.

The content of 'bacoside A' also varied significantly amongst different lines of hairy root and normal root (Table 5.8). 'Bacoside A' content was highest in hairy roots induced with strain MTCC 2364, which accumulated about 4.5 folds more 'bacoside A' as compared to untransformed roots. Although, the variations in secondary metabolite content in hairy roots induced by different strains of *A. rhizogenes* have also been reported earlier (Giri et al., 2001), yet the higher levels observed in the present study is a significant observation. Such root lines can be utilized for the efficient production of 'bacoside A'. A line of hairy roots induced by strain A4 was found to accumulate lowest levels of 'bacoside A' (lower than that of untransformed roots). Earlier, strain A4 induced roots in *Beta vulgaris* also showed lower accumulation of secondary metabolite and biomass also than the roots induced by the strain LMG-150 (Thimmaraju et al., 2008).

So, it can be concluded that cell and organ culture has the potential to enhance the biomass and 'bacoside A' levels. Such cultures with higher levels of 'bacoside A' have the potential of up-scaling in the bioreactors for the production of 'bacoside A'.

Conclusion

Callus, cell suspension and hairy root cultures of *B. monnieri* have been established using accession BM6. Cell suspension cultures induced on B4 (NAA at 5.0 µM and KIN at 1.15 µM) medium and MTCC 2364 induced roots showed maximum biomass accumulation and 'bacoside A' production and were finally selected for further work on various process parameters that are dealt in detail in subsequent chapters. These cell and organ cultures have the potential of up-scaling the production of 'bacoside A' and can be helpful to reduce the pressure on the wild populations.

Salient findings

- High-frequency shoot induction was achieved on MS medium supplemented with 2.5 µM BA.
- Maximum shoot organogenic potential from leaf explant was recorded in case of accession BM6, where 96 % explants showed shoot organogenesis.

- Maximum rooting efficiency of microshoots (100 %) was recorded for accession BM1, BM2, BM7, BM10 and BM14.
- Friable green calli were observed from leaf explants cultured on MS medium supplemented with NAA at 5.0 µM and KIN at 1.15 µM.
- Among the different combination of auxins and cytokinins tested, B4 combination (NAA at 5.0 µM and KIN at 1.15 µM) showed maximum cell growth (5.5 g/l FCW and 1.78 g/l DCW) and 'bacoside A' production (5.56 mg/g DCW).
- *A. rhizogenes* strain SA79 was found to be more virulent than other strains in inducing hairy roots.
- Root induction frequency from leaf explants was higher as compared to intermodal segments.
- Infection period of 10 mins with cultures grown at $O.D_{600}$- 0.6 and co-cultivation period of two days resulted maximum root induction.
- Incorporation of 100 µM acetosyringone in the bacterial culture enhanced root induction frequency.
- Maximum biomass accumulation and 'bacoside A' were recorded in roots induced by strain MTCC 2364 with about 10-fold and five-fold increment respectively.

Chapter 6

Optimization of factors for enhanced cell growth and ‘bacoside A’ by using cell suspension cultures

This chapter indicated successful establishment of cell suspension on different medium. In order to enhance the production of ‘bacoside A’, medium and other factors need to be optimized. Cell culture lines with higher potential of cell growth and ‘bacoside A’ production will be used for optimization studies. The basic parameters which affect the secondary metabolite production include medium composition, pH, carbon source, nitrogen source, agitation and light were optimized using one-vairable-at-a-time (OVAT) approach. Further, an attempt was made to enhance the cell growth and production of ‘bacoside A’ by further optimizing the media components through Response Surface Methodology (RSM).

6.1. Optimum of conditions for ‘bacoside A’ production

6.1.1. Effect of carbon sources

Effect of sucrose and glucose at a concentration of 20 g/l have been evaluated on cell biomass and ‘bacoside A’ content. Glucose was found to be better carbon source for cell growth than sucrose (Fig. 6.1). Maximum cell biomass (6.04 g/l FCW and 1.92 g/l DCW) was found in medium containing glucose as carbon source (Fig. 6.1). Table 6.1 represents the production of ‘bacoside A’ (‘bacoside A3’, ‘bacopaside II’, ‘bacopasaponin C’ and ‘bacopaside X’) by cell suspension cultures of *B. monnieri* in B4 medium (MS basal medium supplemented with NAA at 5.0 µM and KIN at 1.15 µM) with different carbon sources. Medium supplemented with glucose (2 % w/v) higher level of ‘bacoside A’ (6.58 mg/g DCW) was detected. The levels of various components of ‘bacoside A’ viz ‘bacoside A3’ 1.06 mg/g DCW, ‘bacopaside II’ 3.32 mg/g DCW, ‘bacopasaponin C’ 1.84 mg/g DCW and ‘bacopaside X’ 0.36 mg/g DCW, respectively were detected in B4 medium containing glucose as a carbon source. The B4 medium with sucrose as carbon source accounted for lower cell growth and ‘bacoside A’ production.

Table 6.1 Effect of glucose and sucrose on cell growth (g/l) and ‘bacoside A’ (mg/g DCW) production in cell suspension cultures of *B. monnieri*

Carbon Source (2%)	FCW	DCW	Total bacoside A	Bacoside A3	Bacopaside II	Bacopasaponin C	Bacopaside X
Sucrose	5.29±0.21[b]	1.37±0.07[b]	5.47±0.23[b]	0.70±0.01[b]	3.04±0.19[b]	1.65±0.11[b]	0.23±0.04[b]
Glucose	6.04±0.18[a]	1.92±0.05[a]	6.58±0.28[a]	1.06±0.04[a]	3.32±0.24[a]	1.84±0.18[a]	0.36±0.04[a]

Values bearing different letters in the same columns are significant at $P<0.05$. Values are mean ± SD (n =3).

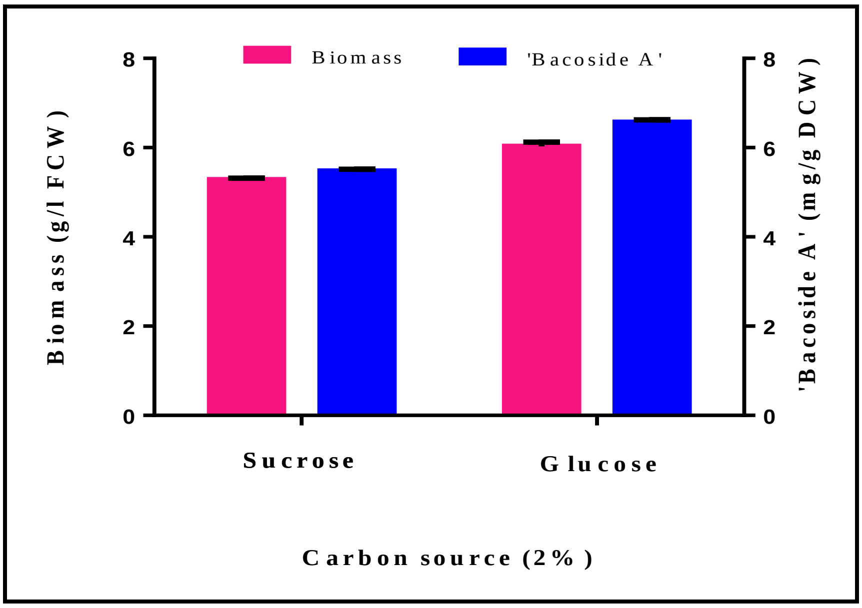


Fig. 6.1 Effect of glucose and sucrose on cell biomass and ‘bacoside A’ production by cell suspension cultures of *B. monnieri*. Bars represent means ± SD (n =3)

6.1.2. Effect of nitrogen sources on cell growth and 'bacoside A' production

The data for the effect of NH_4^+/NO_3^- ratio on cell growth and 'bacoside A' levels in cell suspension cultures is presented in Table 6.2. The increase in NO_3^- concentration significantly increased FCW, DCW and 'bacoside A' levels. Thus, the NO_3^- was found to have a beneficial effect on cell proliferation and 'bacoside A' levels. Furthermore, maximum cell biomass (6.10 g/l FCW and 2.01 g/l DCW) and 'bacoside A' levels of 6.72 mg/g DCW ('bacoside A3' 1.09 mg/g DCW, 'bacopaside II' 3.36 mg/g DCW, 'bacopasaponin C' 1.87 mg/g DCW and 'bacopaside X' 0.40 mg/g DCW) was found in medium containing NO_3^- as sole source of nitrogen (Table 6.2; Fig. 6.2). When the ratio of NH_4^+/NO_3^- was altered in the cultured medium, a significant effect was recorded in both cell growth and 'bacoside A' level in cell suspension cultures (Fig. 6.2).

Table 6.2 Effect of ammonia to nitrate ratio on cell growth (g/l) and 'bacoside A' (mg/g DCW) production in cell suspension cultures of *B. monnieri*

Nitrogen	Nitrogen ratios	FCW	DCW	Total bacoside A	Bacoside A3	Bacopaside II	Bacopasaponin C	Bacopaside X
NO_3^-	60	6.10±0.12[a]	2.01±0.0[a]	6.72±0.21[a]	1.09±0.11[a]	3.36±0.21[a]	1.87±0.15[a]	0.40±0.09[a]
NO_3^-/NH_4^+	50:10	5.22±0.27[b]	1.76±0.0[b]	6.09±0.23[b]	1.05±0.07[b]	3.04±0.18[b]	1.65±0.1[b]	0.35±0.11[b]
NO_3^-/NH_4^+	40:20	4.75±0.27[c]	1.69±0.0[c]	5.43±0.12[c]	0.91±0.01[c]	2.87±0.12[c]	1.38±0.19[c]	0.27±0.01[c]
NO_3^-/NH_4^+	30:30	4.24±0.14[d]	1.62±0.0[d]	4.63±0.15[d]	0.84±0.04[d]	2.35±0.01[d]	1.21±0.02[d]	0.23±0.04[d]
NO_3^-/NH_4^+	20:40	3.87±0.23[e]	1.43±0.1[e]	2.97±0.19[e]	0.49±0.01[e]	1.41±0.21[e]	0.81±0.01[e]	0.19±0.06[e]
NO_3^-/NH_4^+	10:50	3.21±0.06[f]	1.36±0.0[f]	1.82±0.01[f]	0.28±0.08[f]	0.82±0.15[f]	0.64±0.07[f]	0.09±0.03[f]
NH_4^+	60	2.12±0.14[g]	1.17±0.0[g]	0.91±0.01[g]	0.17±0.01[g]	0.43±0.06[g]	0.24±0.01[g]	0.07±0.00[g]

Values bearing different letters in the same columns are significant at $P<0.05$. Values are mean ± SD (n =3).

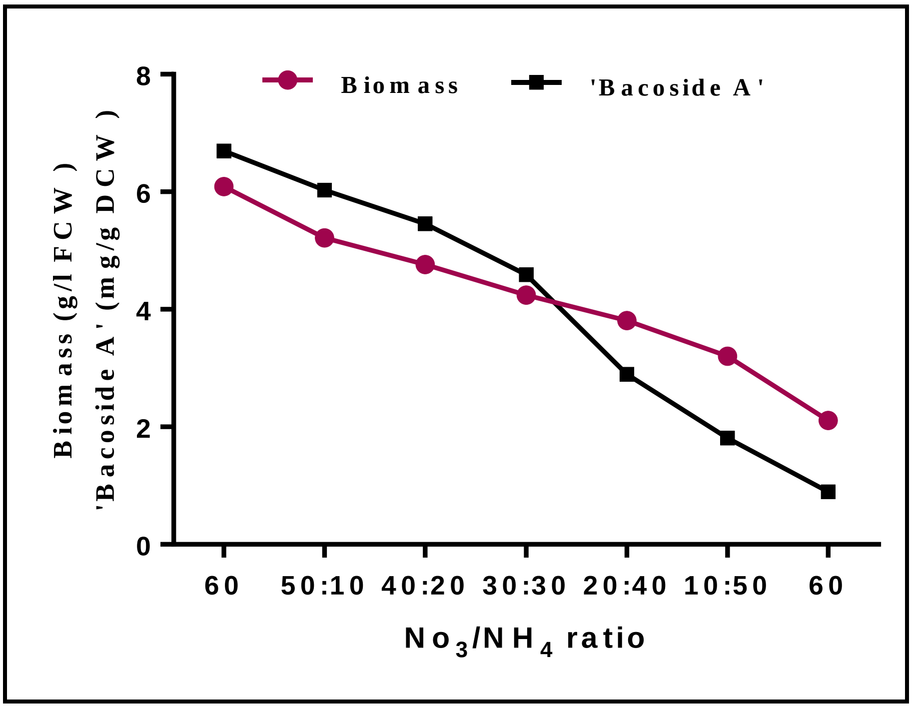


Fig. 6.2 Effect of different ammonia to nitrate ratio on cell growth and ‘bacoside A’ production by cell suspension cultures. Bars represents means ± SD (n =3)

6.1.3. Effect of agitation speed on cell biomass and ‘bacoside A’ production in cell suspension culture

Table 6.3 and Fig. 6.3 depicts the effect of agitation speed on cell biomass and ‘bacoside A’ production in cell suspension culture of *B. monnieri*. Of the different agitation speeds tested, the highest cell biomass (6.40 g/l FCW and 2.06 g/l DCW) and ‘bacoside A’ content was recorded in cell suspension culture at 120 rpm. The higher levels of various components of ‘bacoside A’ namely ‘bacoside A3’ 1.18 mg/g DCW, ‘bacopaside II’ 3.21 mg/g DCW, ‘bacopasaponin C’ 2.07 mg/g DCW and ‘bacopaside X’ 0.40 mg/g DCW, respectively were also detected at 120 rpm under shaking (Table 6.3). The cell suspension culture under static condition displayed the lowest cell biomass and ‘bacoside A’ production (Fig. 6.3), showing the absolute importance of aeration.

Table 6.3 Effect of agitation speed on cell biomass (g/l) and 'bacoside A' (mg/g DCW) production in cell suspension cultures of *B. monnieri*

Agitation speed	FCW	DCW	Total bacoside A	Bacoside A3	Bacopaside II	Bacopasaponin C	Bacopaside X
Control	1.78±0.07[f]	0.21±0.0[f]	2.24±0.32[f]	0.30±0.08[f]	1.08±0.01[f]	0.74±0.04[f]	0.12±0.01[f]
80	6.10±0.31[c]	1.64±0.1[c]	5.38±0.41[c]	0.73±0.09[c]	2.89±0.24[c]	1.49±0.14[c]	0.27±0.02[c]
100	6.23±0.33[b]	1.87±0.1[b]	5.56±0.35[b]	0.83±0.14[b]	2.81±0.30[b]	1.70±0.27[b]	0.22±0.07[b]
120	6.40±0.37[a]	2.06±0.2[a]	6.86±0.37[a]	1.18±0.29[a]	3.21±0.27[a]	2.07±0.27[a]	0.40±0.01[a]
140	4.34±0.22[d]	0.76±0.0[d]	4.84±0.27[d]	0.61±0.06[d]	2.64±0.15[d]	1.38±0.02[d]	0.21±0.04[d]
160	3.67±0.26[e]	0.51±0.0[e]	4.09±0.16[e]	0.54±0.15[e]	2.15±0.33[e]	1.22±0.11[e]	0.18±0.01[e]

Values bearing different letters in the same columns are significant at P<0.05. Values are mean ± SD (n =3).

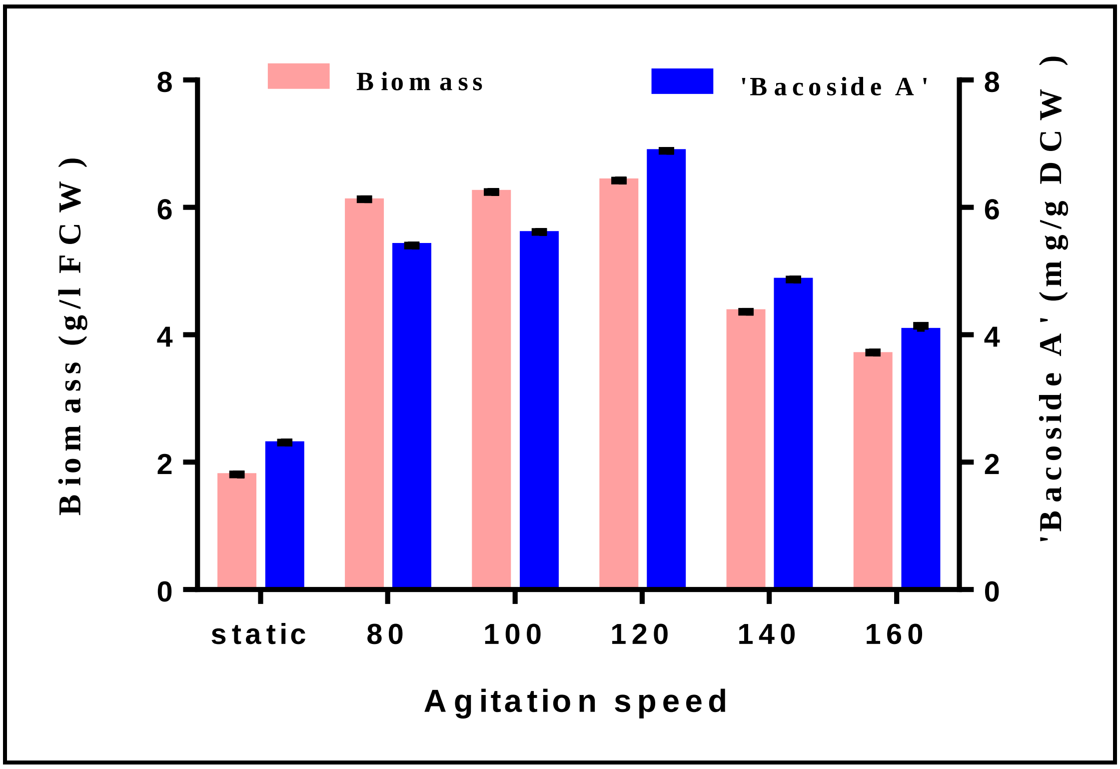


Fig. 6.3 Effect of different agitation speed on cell biomass and 'bacoside A' production by cell suspension cultures. Bars represent means ± SD (n =3)

6.1.4. Effect of light illumination on cell growth and 'bacoside A' production

Light is an important parameter which can affect the growth and accumulation of secondary metabolites. In the present study, cell suspension cultures established from leaf explant responded differently to 8 h dark period and 24 h dark period. Higher cell growth (6.78 g/l FCW and 2.23 g/l DCW) and 'bacoside A' production of 7.02 mg/g DCW was achieved when cultures were incubated in total darkness (Table 6.4; Fig. 6.4). The higher levels of 'bacoside A' components namely 'bacoside A3' 1.12 mg/g DCW, 'bacopaside II' 3.45 mg/g DCW, 'bacopasaponin C' 1.97 mg/g DCW and 'bacopaside X' 0.49 mg/g DCW, were detected in complete darkness (Table 6.4).

Table 6.4 Optimization of light illumination conditions for cell growth (g/l) and 'bacoside A' (mg/g DCW) production in cell suspension cultures of *B. monnieri*

Light/dark regime (h/day)	FCW	DCW	Total bacoside A	Bacoside A3	Bacopaside II	Bacopasaponin C	Bacopaside X
16/8h	6.10±0.24[b]	2.01±0.12[b]	6.77±0.23[b]	1.09±0.01[b]	3.36±0.29[b]	1.87±0.16[b]	0.44±0.04[b]
24h darkness	6.78±0.31[a]	2.23±0.25[a]	7.02±0.28[a]	1.12±0.14[a]	3.45±0.24[a]	1.97±0.18[a]	0.49±0.01[a]

Values bearing different letters in the same columns are significant at $P<0.05$. Values are mean ± SD (n =3)

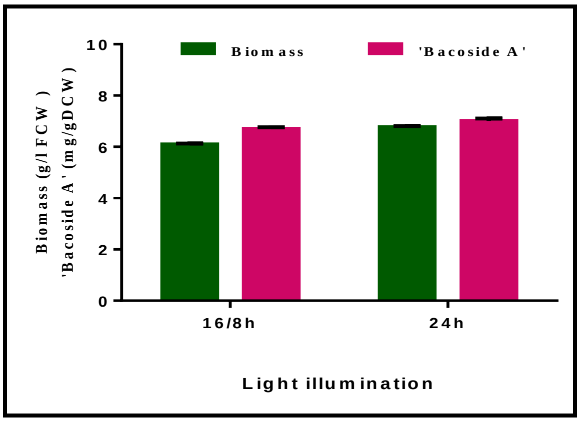


Fig. 6.4 Effect of light illumination on cell growth and 'bacoside A' production in cell suspension cultures of *B. monnieri*. Bars represent means ± SD (n =3)

6.1.5. Growth kinetics of *B. monnieri* suspension cultures

Time course accumulation of cell growth and 'bacoside A' content is shown in Fig. 6.5. The results revealed that the cell biomass increased until 21st day, which is also associated with an increase in 'bacoside A' production, indicating growth associated product formation. The maximum cell growth (7.08 g/l FCW and 2.27 g/l DCW) and 'bacoside A' production of 7.12 mg/g DCW was obtained after 21st day, which declined thereafter (Fig. 6.5).

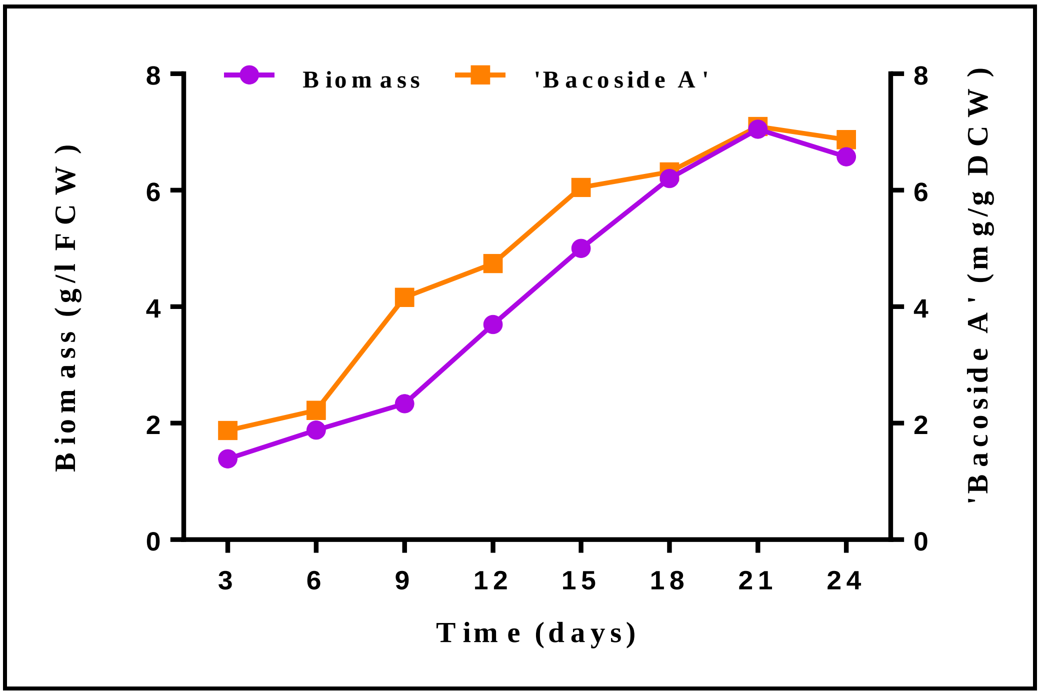


Fig. 6.5 Time course study of 'bacoside A' production and cell growth in cell suspension cultures of *B. monnieri*

6.1.6. Effect of medium pH on cell growth and 'bacoside A' production

pH of the medium was found to effect the cell growth and 'bacoside A' production significantly. Among the different pH range of medium tested, the maximum cell biomass (7.10 g/l FCW and 2.31 g/l DCW) and 'bacoside A' production of 7.23 mg/g DCW were recorded on medium with pH 6.0. The levels of 'bacoside A' components namely 'bacoside A3' 1.16 mg/g DCW, 'bacopaside II' 3.44 mg/g DCW, 'bacopasaponin C' 2.28 mg/g DCW and 'bacopaside X' 0.35 mg/g DCW was recorded on medium with pH 6.0 (Table 6.5; Fig. 6.6).

Table 6.5 Effect of medium pH on cell growth (g/l) and 'bacoside A' (mg/g DCW) production in cell suspension cultures of *B. monnieri*

Medium pH	FCW	DCW	Total bacoside A	Bacoside A3	Bacopaside II	Bacopasaponin C	Bacopaside X
4.0	4.62±0.37[g]	1.21±0.22[g]	5.21±0.32[g]	0.66±0.02[g]	2.87±0.21[f]	1.47±0.16[f]	0.21±0.02[d]
4.5	4.87±0.26[f]	1.37±0.17[f]	5.34±0.11[f]	0.72±0.09[f]	2.87±0.32[f]	1.49±0.02[f]	0.26±0.04[c]
5.0	5.43±0.18[d]	1.82±0.09[d]	6.68±0.31[d]	0.97±0.10[d]	3.24±0.29[d]	2.17±0.22[d]	0.30±0.07[b]
5.5	6.78±0.33[b]	2.16±0.21[b]	7.12±0.27[b]	1.11±0.13[b]	3.40±0.14[b]	2.27±0.17[a]	0.34±0.01[a]
6.0	7.10±0.24[a]	2.31±0.16 [a]	7.23±0.19[a]	1.16±0.03[a]	3.44±0.35[a]	2.28±0.12[a]	0.35±0.01 [a]
6.5	5.89±0.14[c]	1.87±0.12[c]	6.88±0.35[c]	1.06±0.15[c]	3.32±0.23[c]	2.20±0.01[b]	0.30±0.09[b]
7.0	5.17±0.26[e]	1.51±0.04[e]	6.27±0.21[e]	1.01±0.22[e]	3.00±0.27[e]	1.93±0.17[e]	0.28±0.04[b]

Values bearing different letters in the same columns are significant at P<0.05. Values are mean ± SD (n =3).

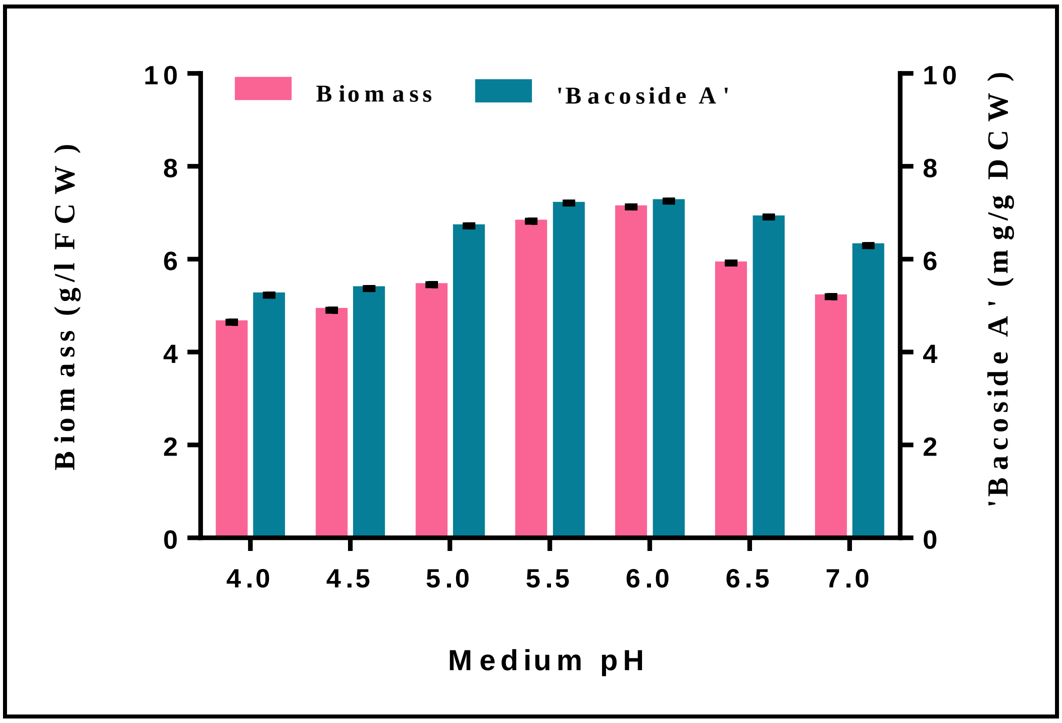


Fig. 6.6 Effect of medium pH on cell growth and 'bacoside A' production in cell suspension cultures of *B. monnieri*. Bars represent means ± SD (n =3)

6.1.7. Effect of medium strength on cell growth and 'bacoside A' production

Effect of medium strength on cell biomass and 'bacoside A' levels in cell suspension cultures were studied. Of the different medium strength tested, the highest cell biomass (6.98 g/l FCW and 2.23 g/l DCW) was recorded in full strength MS medium (Table 6.6). In addition, the quantities of 'bacoside A' were also considerably enhanced and the following were noted: 'bacoside A3' 1.09 mg/g DCW, 'bacopaside II' 3.43 mg/g DCW, 'bacopasaponin C' 2.14 mg/g DCW and 'bacopaside X' 0.37 mg/g DCW in full strength MS medium (Table 6.6; Fig. 6.7). The cell suspension cultures with one-fourth and double medium strength showed the lowest cell biomass and 'bacoside A' production.

Table 6.6 Effect of medium strength on cell growth (g/l) and 'bacoside A' (mg/g DCW) production in cell suspension cultures of *B. monnieri*

Medium strength	FCW	DCW	Total bacoside A	Bacoside A3	Bacopaside II	Bacopasaponin C	Bacopaside X
0.25	5.21±0.37[e]	1.39±0.22[e]	5.62±0.32[d]	0.88±0.02[c]	2.91±0.21[d]	1.47±0.16[d]	0.21±0.02[d]
0.5	6.34±0.26[c]	1.87±0.17[c]	5.93±0.11[c]	0.91±0.09[b]	3.10±0.32[c]	1.49±0.02[d]	0.30±0.04[b]
0.75	6.71±0.18[b]	2.13±0.09[b]	6.17±0.31[b]	0.92±0.10[b]	3.25±0.29[b]	2.21±0.22[b]	0.31±0.07[b]
1	6.98±0.33[a]	2.23±0.21[a]	7.03±0.27[a]	1.09±0.13[a]	3.43±0.14[a]	2.27±0.17[a]	0.34±0.01[a]
1.5	5.32±0.24[d]	1.53±0.16[d]	5.51±0.19[e]	0.86±0.03[d]	2.95±0.35[e]	2.28±0.12[a]	0.25±0.01[c]
2	4.81±0.14[f]	1.16±0.12[f]	5.47±0.35[f]	0.86±0.15[d]	2.83±0.23[e]	2.17±0.01[c]	0.18±0.09[a]

Values bearing different letters in the same columns are significant at P<0.05. Values are mean ± SD (n =3)

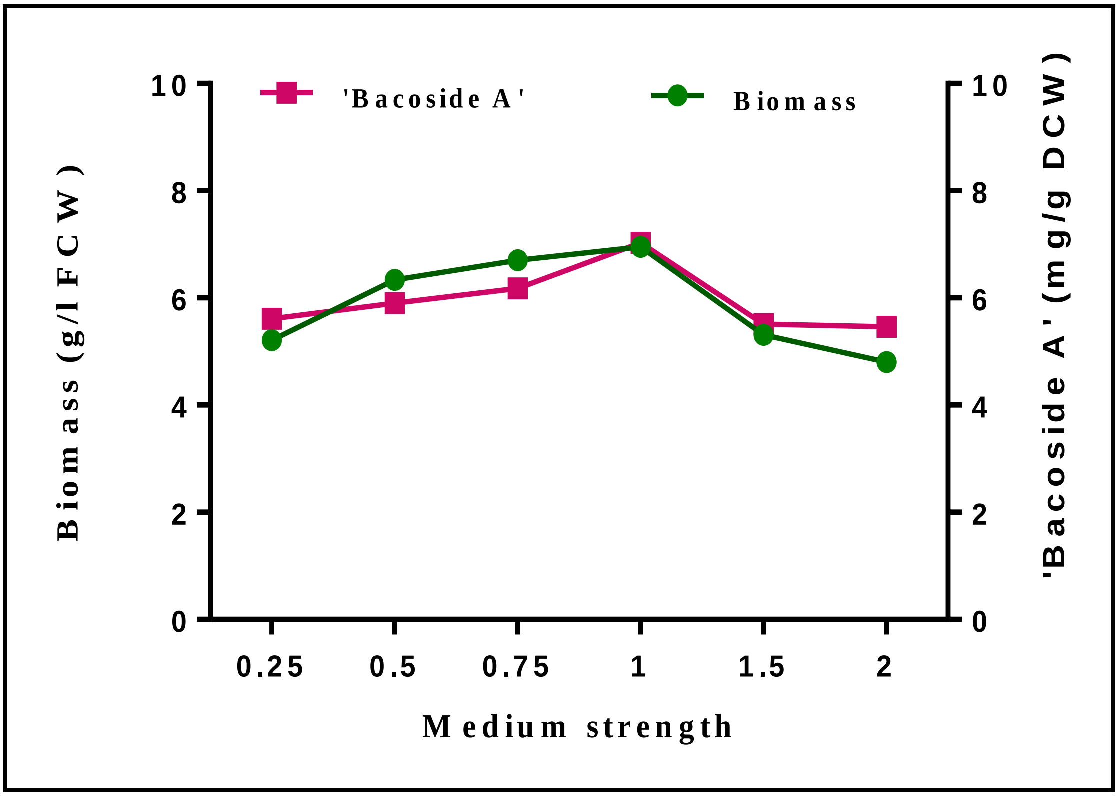


Fig. **6.7** Effect of medium strength on cell growth and 'bacoside A' production in cell suspension cultures of *B. monnieri*. Bars represent means ± SD (n =3)

6.2. Optimization of medium components for enhancing cell growth and 'bacoside A' production using RSM

6.2.1. Plackett – Burman design

Plackett - Burman experiment was used to investigate the nutrient variables in the media constituents affecting the fresh cell wt. (FCW) and biomass accumulation at two levels in total of 8 experiments. The variables and their selected levels are given in table 6.7. The responses (FCW and 'bacoside A' production) obtained after 21 d in each of the experiments (Table 6.8) were subjected to analysis by the Design-Expert software.

Table 6.7 The two levels (higher and lower) of medium components used in the Plackett-Burman design

Components	Low (-1) level	High(+1) level
Glucose (g/l)	15.00	60.00
Potassium nitrate (mM)	15.00	90.00
Potassium dihydrogen ortho-phosphate (g/l)	0.50	2.50
Magnesium sulphate (g/l)	0.18	0.74
Calcium chloride (g/l)	0.22	0.88
Inoculum (g/l)	2.00	8.00

The data were analysed to find the significant factors by F test. The results of Plackett - Burman experiment indicated that there was a wide variation in FCW (4.82 g/l to 12.78 g/l) and 'bacoside A' content (6.32 g/l to 7.75 g/l) in the different trials (Table 6.8). Significant rise in the FCW and 'bacoside A' content was seen with different combinations of the factors studied.

These variations reflected the importance of media optimization for higher FCW and 'bacoside A' production. ANOVA table of different factors involved in FCW and 'bacoside A' content production demonstrated that the model was significant (Table 6.9).

Table 6.8 Plackett - Burman design matrix for screening the variables influencing FCW and 'bacoside A' production in cell suspension cultures

Experiment	Glucose (g/l)	KNO_3 (mM)	K_2HPO_4 (g/l)	$MgSO_4.7H_2O$ (g/l)	$CaCl_2.2H_2O$ (g/l)	Inoculum (g/l)	Responses	
							FCW (g/l)	Bacoside A (mg/g)
1	15.00	90.00	0.50	0.18	0.88	2.00	7.02	7.32
2.	15.00	15.00	0.50	0.74	0.88	8.00	4.82	6.32
3.	60.00	15.00	0.50	0.18	0.22	8.00	7.86	7.01
4.	60.00	90.00	2.50	0.74	0.88	8.00	12.78	7.75
5.	15.00	15.00	2.50	0.18	0.22	8.00	7.17	7.23
6.	60.00	15.00	2.50	0.18	0.88	2.00	12.32	7.67
7.	60.00	90.00	0.50	0.74	0.22	2.00	12.74	7.53
8.	15.00	15.00	2.50	0.74	0.22	2.00	9.67	7.08

The medium components with a p value < 0.05 at 5 % level were considered to have greater impact on the FCW and 'bacoside A' content. The data of regression analysis for Plackett - Burman design demonstrated that out of the six variables studied, 4 variables had significant influence on FCW and 'bacoside A' content as evidenced by their p value and were found to be concentration of glucose, potassium nitrate, potassium di-hydrogen ortho-phosphate and inoculum.

Table 6.9 Analysis of variance (ANOVA) table for FCW and 'bacoside A' content by different factors using Plackett - Burman design

Symbol	Factors	t Coefficient		Studied Effect		Contribution (%)		Prob > F p-Value	
		FCW	Bacoside A	FCW	Bacoside A	FCW	Bacoside A	FCW	Bacoside A
A	Glucose	+29.72	+20.78	4.32	0.55	45.24	25.62	0.0220	0.0424
B	KNO_3	+17.87	+12.62	2.27	0.48	12.51	19.94	0.0418	0.0480
C	KH_2PO_4	+25.87	+15.78	3.81	0.72	35.19	44.76	0.0249	0.0321
D	$MgSO_4.7H_20$	-0.78	-0.94	-0.85	-0.27	1.73	2.39	0.1113	0.1369
E	$CaCl_2.2H_20$	-0.25	+0.71	-0.060	0.097	8.76	0.81	0.7567	0.2290
F	Inoculum	+9.23	-9.46	1.47	-0.17	5.27	6.38	0.0442	0.0346
	Model	-	-	-	-	-	-	0.0389	0.0595

$MgSO_4.7H_20$ and $CaCl_2.2H_20$ had a negative effect on FCW and 'bacoside A' content (as indicated by the negative *t* value and studied effect) and with "Prob>F" (*p* value) more than 0.1. These two variables were included at a fixed concentration level in the optimized medium. Although the PB design could be successfully used for the reasonable prediction of the significant level of the different variables affecting the response (FCW and 'bacoside A' production), some of the significant interactive effects of the chosen variables (two-factor interaction) were not clear in its complex structure. Because of this reason, the actual main effects of these variables may have been influenced. So, the significant components (glucose,

KNO_3, KH_2PO_4 and inoculum) whose interactive effects were identified by PB design were further studied precisely by RSM.

6.2.2. Response surface methodology for optimizing concentrations of medium components

Statistical designs serve as effective tools for determining main as well as interactive influences of different parameters on production of secondary metabolite. Response surface methodology (RSM) is a collection of certain statistical tools for making effective experimental design, building models, studying the effect of factors and searching for optimal conditions for desirable responses (Myers and Montgomery, 2002). Many important secondary metabolite production using cell cultures have been studied by RSM (Zhong and Wang, 1998; Pavlov et al., 2000; Das et al., 2001; Prakash and Srivastava, 2005).

In the present study, Central composite design (CCD) for four independent variables was used to obtain the combination for optimizing the response within the region of three dimensional observation spaces. CCD provides the information regarding optimum level of each variable along with their interactions with other variables and their effect on secondary metabolite production. To examine the effect of these independent variables on FCW and 'bacoside A' content, central composite factorial design of $2^4 = 16$ plus 6 centre points and eight axial points predicted 30 experiments, which were performed (Table 6.10).

Table 6.10 Central composite experimental design matrix with experimental and predicted value for medium optimization

Trials	Actual values				Response			
	Glucose (g/l)	KNO_3 (mM)	KH_2PO_4 (g/l)	Inoculum (g/l)	Experimental		Predicted	
					FCW	Bacoside A	FCW	Bacoside A
1	60.00	15.00	3.00	8.00	14.08	10.57	15.03	10.99
2	15.00	15.00	3.00	2.00	3.95	2.08	4.76	2.81
3	37.50	52.50	2.00	5.00	10.42	6.53	10.06	8.39
4	60.00	90.00	3.00	2.00	4.08	6.02	5.45	6.76
5	15.00	15.00	1.00	2.00	1.95	1.08	2.24	2.09
6	37.50	52.50	2.00	5.00	10.26	8.73	10.06	8.39
7	15.00	15.00	3.00	8.00	13.08	8.37	12.73	8.25
8	60.00	90.00	1.00	2.00	3.78	7.83	2.93	7.72
9	37.50	52.50	2.00	5.00	11.08	6.01	10.06	8.39
10	15.00	90.00	1.00	2.00	3.08	7.28	2.66	7.75
11	60.00	15.00	3.00	2.00	4.85	6.51	2.62	6.17
12	15.00	90.00	1.00	8.00	7.04	7.73	8.07	7.84
13	15.00	90.00	3.00	8.00	9.67	7.03	9.74	8.53
14	37.50	52.50	2.00	5.00	10.46	7.98	10.06	8.39
15	15.00	15.00	1.00	8.00	13.98	4.73	13.13	4.88
16	60.00	90.00	1.00	8.00	13.07	7.03	12.79	7.19
17	15.00	90.00	3.00	2.00	8.09	6.81	7.26	5.79
18	60.00	15.00	1.00	8.00	17.88	5.82	17.51	6.61
19	60.00	15.00	1.00	2.00	1.73	5.05	2.18	4.44
20	60.00	90.00	3.00	8.00	13.88	10.11	12.39	8.88
21	37.50	52.50	2.00	11.00	20.38	11.03	20.70	10.45
22	37.50	52.50	2.00	0.00	3.38	6.23	3.84	6.07
23	37.50	52.50	2.00	5.00	8.99	9.59	10.06	8.39
24	37.50	0.00	2.00	5.00	8.18	7.73	8.67	6.56
25	82.50	52.50	2.00	5.00	4.88	7.02	5.73	7.32
26	00.00	52.50	2.00	5.00	5.38	7.02	5.08	5.58
27	37.50	52.50	2.00	5.00	9.78	9.1	10.06	8.39
28	37.50	52.50	0.00	5.00	9.38	8.87	9.95	4.33
29	37.50	127.5	2.00	5.00	4.12	9.09	4.52	8.97
30	37.50	52.50	4.00	5.00	11.51	6.98	12.06	6.73

The response (FCW and ‘bacoside A’ content) for each experiment were analysed by linear multiple regression and graphical analysis using Design-Expert software. The mathematical models incorporating the interactive effect of these nutrients were proposed for FCW (Model 1) and ‘bacoside A’ production (Model 2) and the following equation was obtained:

Model 1:

$$\textbf{FCW} = +10.06 + 0.64\,A - 0.56\,B + 0.53\,C + 4.46\,D + 0.084\,AB - 0.52\,AC + 1.11\,AD + 0.52\,BC - 1.37\,BD - 0.73\,CD - 1.40\,A^2 - 1.11\,B^2 + 0.24\,C^2 + 0.43\,D^2$$

Model 2:

$$\textbf{`Bacoside A'} = +8.39 + 0.68\,A + 0.89\,B + 0.60\,C + 1.23\,D - 0.60\,AB + 0.25\,AC - 0.16\,AD - 0.67\,BC - 0.68\,BD + 0.66\,CD - 0.61\,A^2 - 0.30\,B^2 - 0.71\,C^2 - 0.099\,D^2$$

where A is glucose, B is potassium nitrate, C is potassium dihydrogen ortho-phosphate, and D is inoculum, respectively.

The statistical analysis of the model equations was performed by ANOVA. The ANOVA of linear regression model equation demonstrated that model equation was highly significant as evident from value of “Model Prob > F” less than 0.0001 (Table 6.11). In this case A, B and D are significant model terms.

The goodness of fit of the models was checked by the determination coefficient (R^2). Normally, a regression model with R^2 value higher than 0.8 is considered as having a very high correlation. R^2 value for FCW and ‘bacoside A’ content is 0.97 and 0.80 respectively, so it is reasonable to use the regression model to analyse the trends in the response. The values of the determination coefficients indicated that only 3 % and 20 % of the total variation for each response was not explained by the respective models.

Table 6.11 ANOVA table for FCW and 'bacoside A' content using CCD of response surface methodology

Source	Sum of squares		df		Mean square		F- value		Prob> F	
	FCW	Bacoside A	FCW	Bacoside A	FCW	Bacoside A	FCW	Bacoside A	FCW	Bacoside A
Model	635.00	109.69	14	14	45.36	7.83	35.38	4.34	<0.0001	0.0039
A	9.26	10.22	1	1	9.28	10.22	7.24	5.67	0.0168	0.0310
B	6.46	16.40	1	1	6.46	16.40	5.04	9.09	0.0403	0.0087
C	5.22	6.78	1	1	5.22	6.78	4.07	3.67	0.0620	0.0717
D	443.2	33.61	1	1	443.22	33.61	345.8	18.63	<0.0001	0.0006
AB	0.11	5.70	1	1	0.11	5.70	0.089	3.16	0.7697	0.0958
AC	4.32	1.01	1	1	4.32	1.01	3.37	0.56	0.0864	0.4670
AD	19.74	0.39	1	1	19.74	0.39	15.40	0.21	0.0014	0.6497
BC	4.34	7.22	1	1	4.34	7.22	3.38	4.00	0.857	0.0639
BD	30.00	7.30	1	1	30.00	7.30	23.41	4.05	0.0002	0.0626
CD	8.54	7.01	1	1	8.54	7.01	6.66	3.88	0.0209	0.0675
A^2	43.13	8.00	1	1	43.13	8.00	33.65	4.43	<0.0001	0.0525
B^2	22.72	1.67	1	1	22.72	1.67	17.72	0.93	0.0008	0.3512
C^2	0.91	8.35	1	1	0.91	8.35	0.71	4.63	0.4134	0.0482
D^2	4.11	0.22	1	1	4.11	0.22	3.21	0.12	0.0936	0.7341
Lack of fit	16.70	16.67	10	10	1.67	1.67	3.30	0.80	0.0996	0.6426

The value of the adjusted determination coefficient (adjusted R^2 =0.94 and 0.61) was also reasonably high, supporting a high significance of the model. Predicted R^2 of 0.82 and 0.33 demonstrated reasonable predictability of the model for FCW and 'bacoside A' content.

At the same time, the relatively low values of the coefficient of variation (CV=12.94 and 18.66 for FCW and 'bacoside A' content) with adequate precision values 23.13 and 9.37, indicated an acceptable precision and reliability of the experiments.

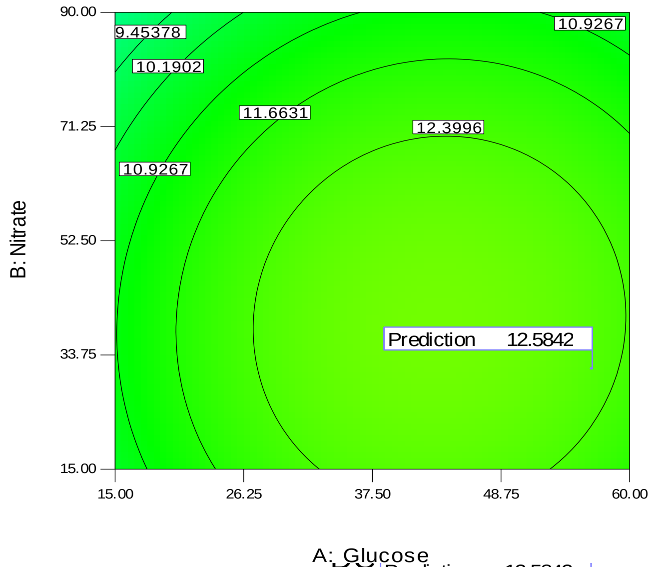
The three dimensional response surface plots and contour plots were employed to demonstrate the interaction among various factors (Fig. 6.8; 6.9). Each graph represents an infinite number of combinations of all the test variables with each other.

Maximum FCW and 'bacoside A' content was seen in with concentration of glucose = 56.74 g/l, potassium nitrate= 31.37mM, potassium dihydrogen ortho-phosphate= 2.91 g/l and inoculum = 6.60 g/l. The developed model predicted a FCW 13.02 g/l and 'bacoside A' content of 9.84 mg/g which was experimentally verified and yielded a FCW of 12.58 g/l and 'bacoside A' content of 9.79 mg/g. Thus, indicated a strong agreement between predicted and experimental value. In comparison with the pre-optimized media where the 5.3 g/l FCW and 'bacoside A' content of 5.56 mg/g was produced, optimized media had enhanced FCW and 'bacoside A' content.

It was verified that optimized media led to 2.5-fold increase in the FCW and 1.7-fold increase in levels of 'bacoside A'. So, it has been undoubtedly proved that with the application of RSM, the FCW and 'bacoside A' production has been enhanced significantly.

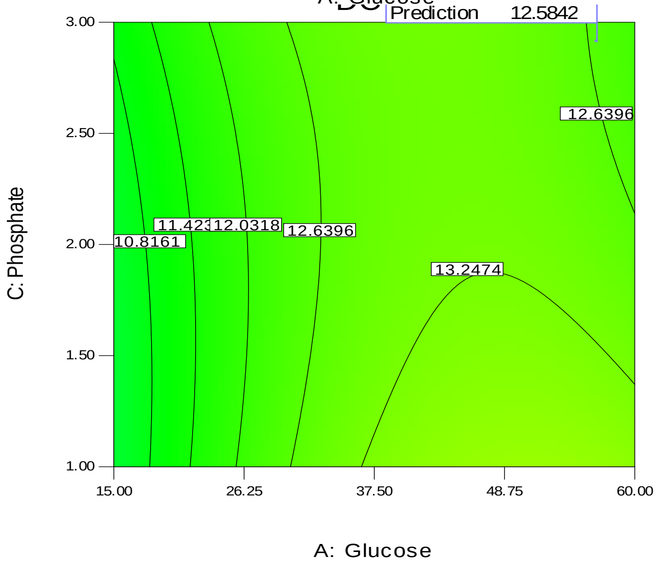
(a)

Actual Factors
C: Phosphate= 2.91
D: Inoculum= 6.60



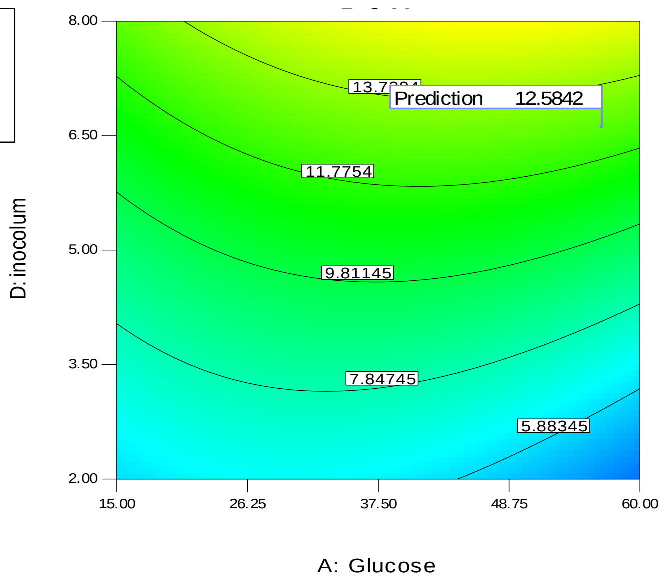
(b)

Actual Factors
B: Nitrate= 31.37
D: Inoculum= 6.60



(c)

Actual Factors
B: Nitrate= 31.37
C: Phosphate= 2.91



(d) **Actual Factors**
A: Glucose= 56.74
D: Inoculum= 6.60

(e) **Actual Factors**
A: Glucose= 56.74
C: Phosphate= 2.91

(f) **Actual Factors**
A: Glucose= 56.74
B: Nitrate= 31.37

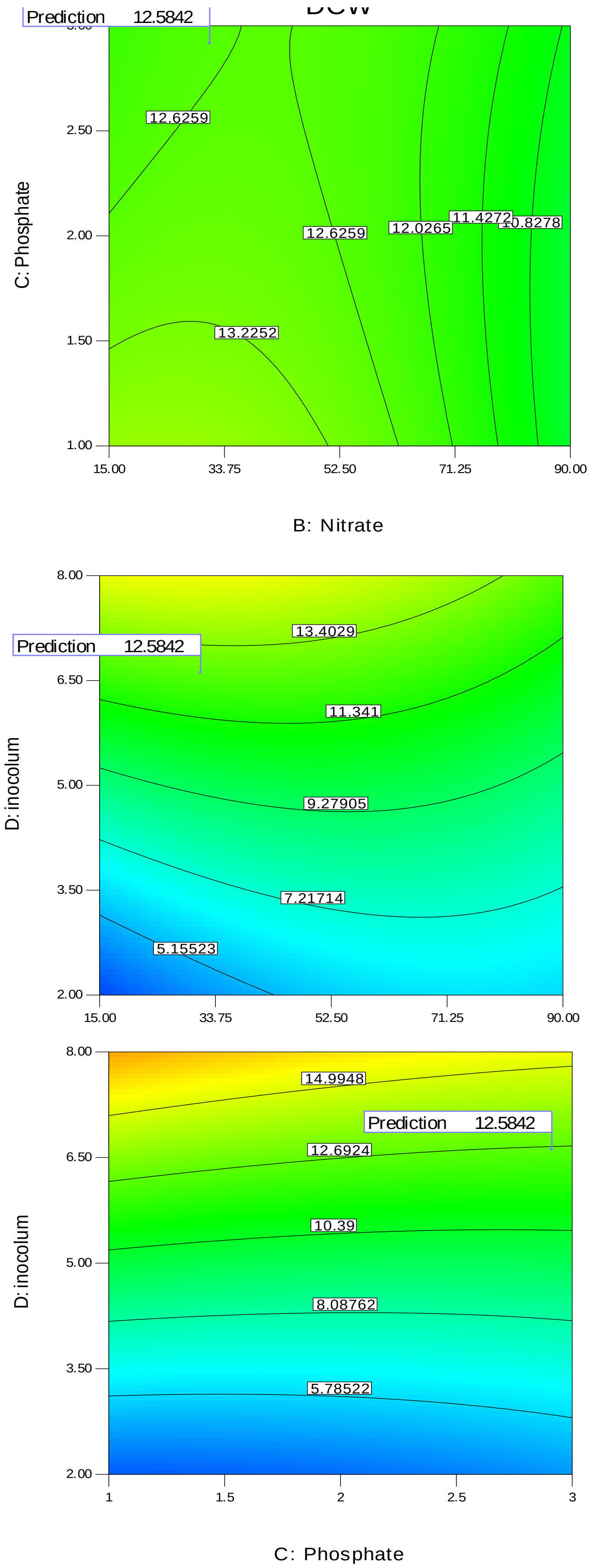
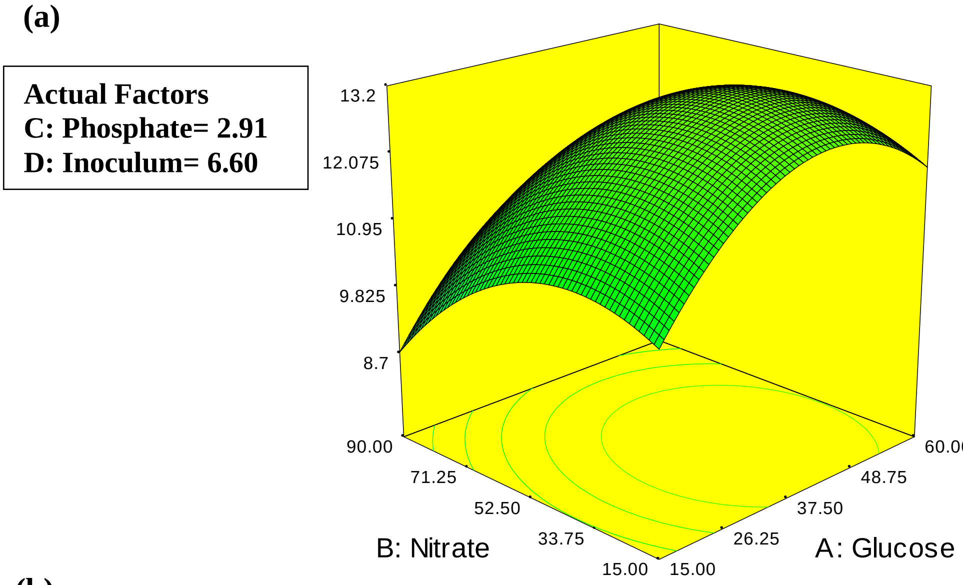
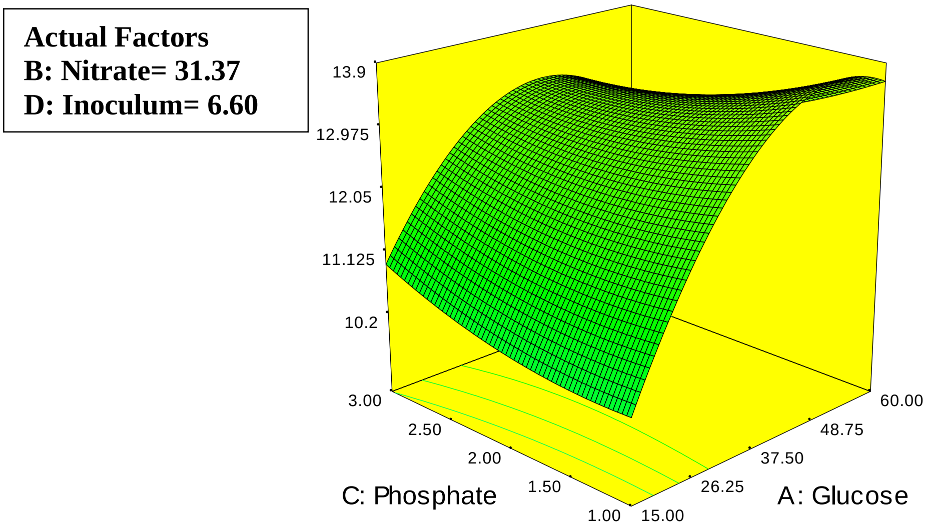


Fig. 6.8 Contour plot between effect of (a) glucose and potassium nitrate (b) glucose and potassium dihydrogen ortho-phosphate (c) glucose and inoculum (d) potassium nitrate and potassium dihydrogen ortho-phosphate (e) potassium nitrate and inoculum (f) potassium dihydrogen ortho-phosphate and inoculum on fresh cell weight (FCW)

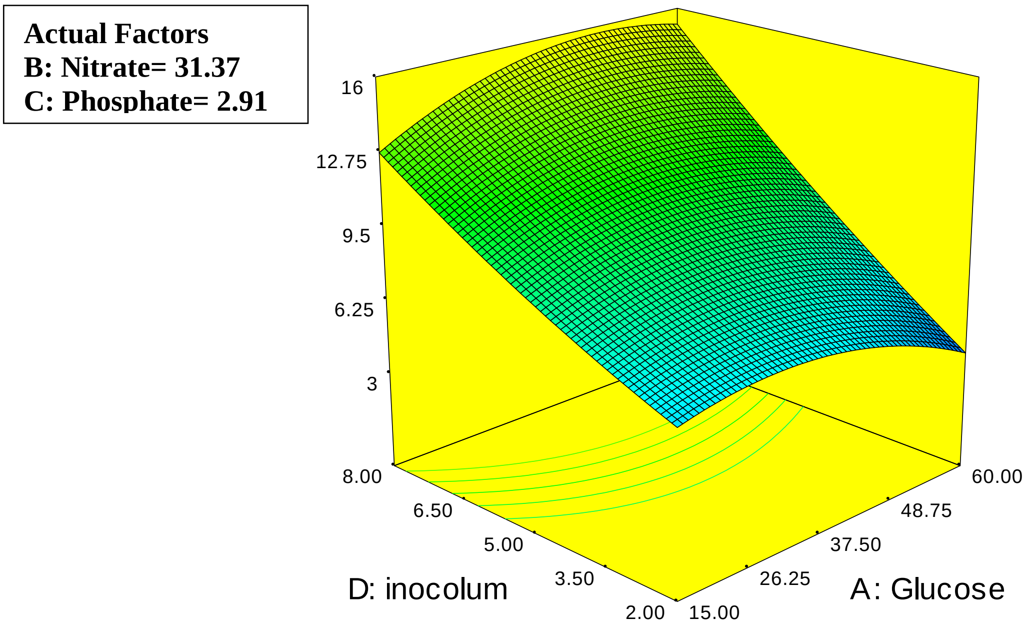
(a)


Actual Factors
C: Phosphate= 2.91
D: Inoculum= 6.60
13.2
12.075
10.95
9.825
8.7
90.00
71.25
52.50
33.75
15.00
B: Nitrate
15.00
26.25
37.50
48.75
60.00
A: Glucose

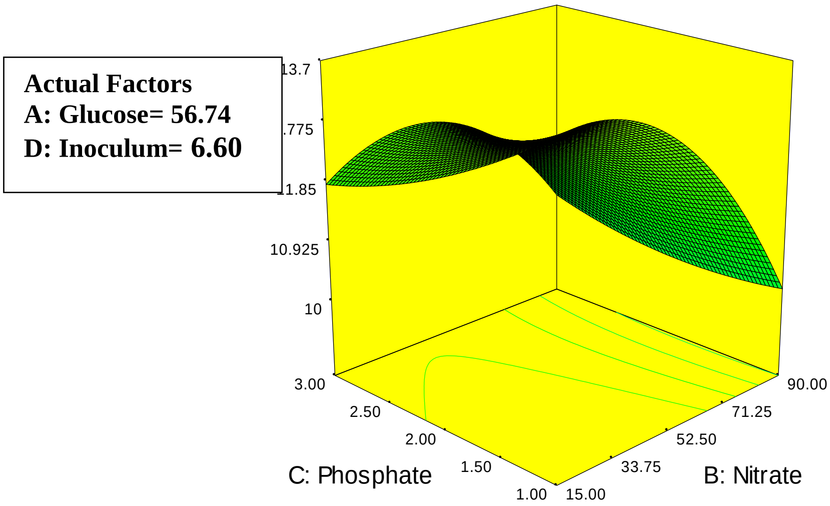
(b)


Actual Factors
B: Nitrate= 31.37
D: Inoculum= 6.60
13.9
12.975
12.05
11.125
10.2
3.00
2.50
2.00
1.50
1.00
C: Phosphate
15.00
26.25
37.50
48.75
60.00
A: Glucose

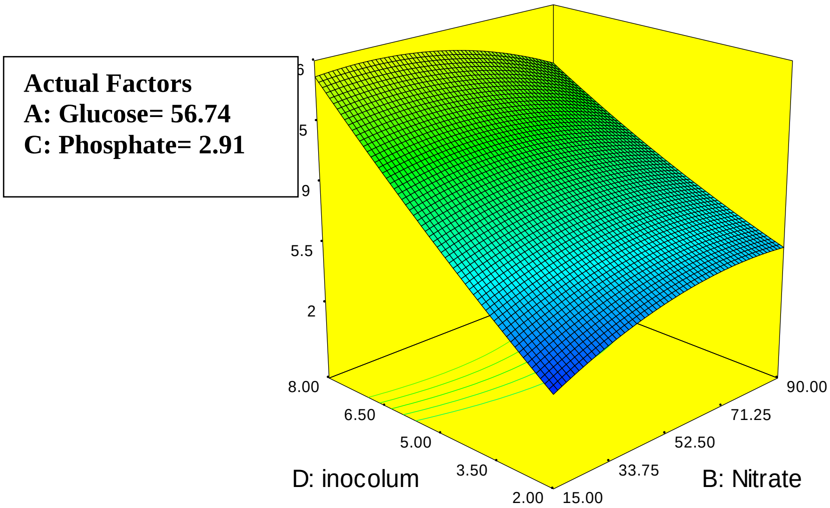
(c)


Actual Factors
B: Nitrate= 31.37
C: Phosphate= 2.91
16
12.75
9.5
6.25
3
8.00
6.50
5.00
3.50
2.00
D: inocolum
15.00
26.25
37.50
48.75
60.00
A: Glucose

(d)



(e)



(f)

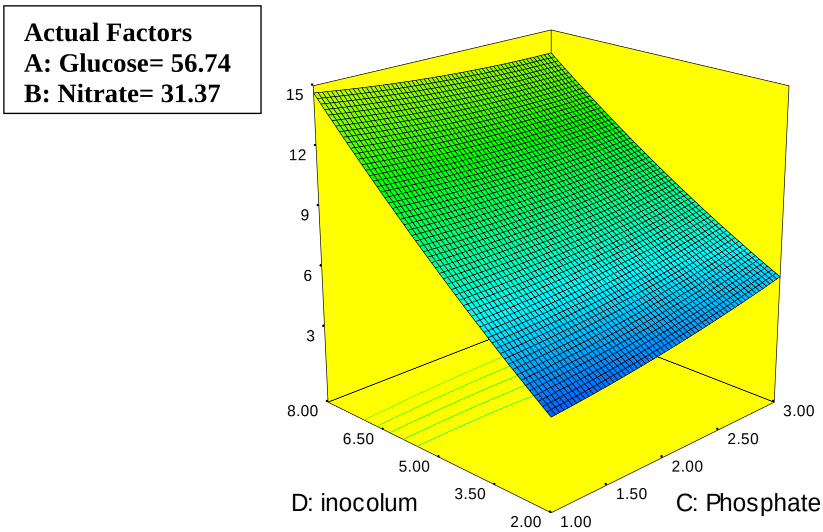
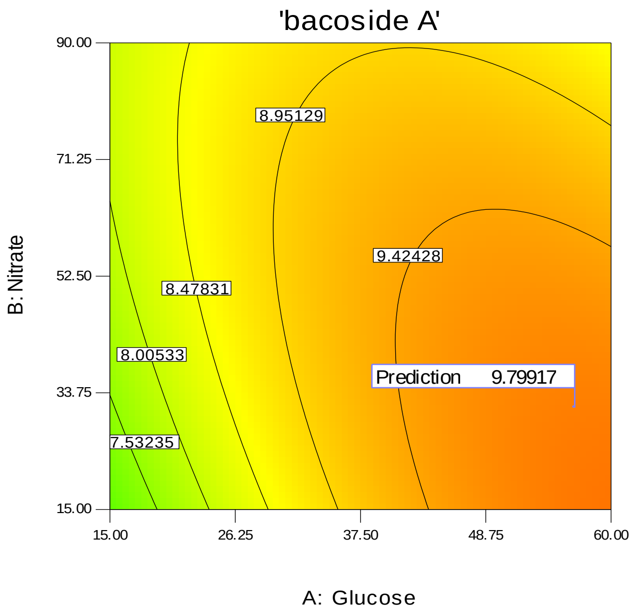


Fig. 6.9 Surface plot showing the effect of (a) glucose and potassium nitrate (b) glucose and potassium dihydrogen ortho-phosphate (c) glucose and inoculum (d) potassium nitrate and potassium dihydrogen ortho-phosphate (e) potassium nitrate and inoculum (f) potassium dihydrogen ortho-phosphate and inoculum on fresh cell weight (FCW)

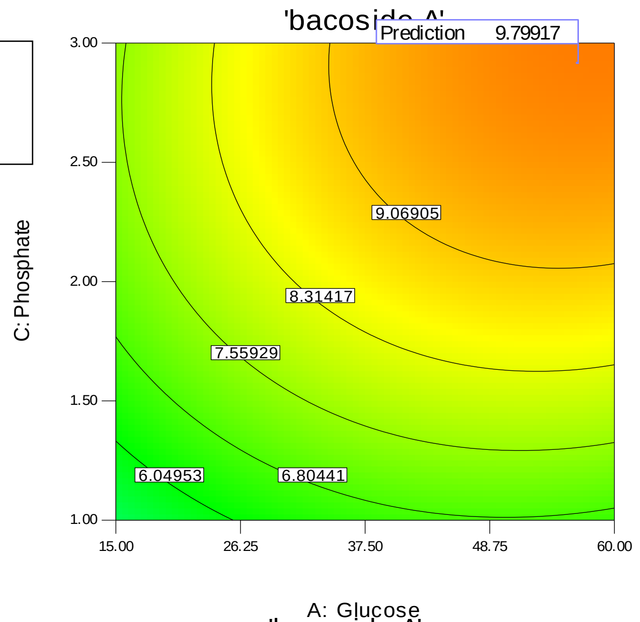
(a)

Actual Factors
C: Phosphate= 2.91
D: Inoculum= 6.60



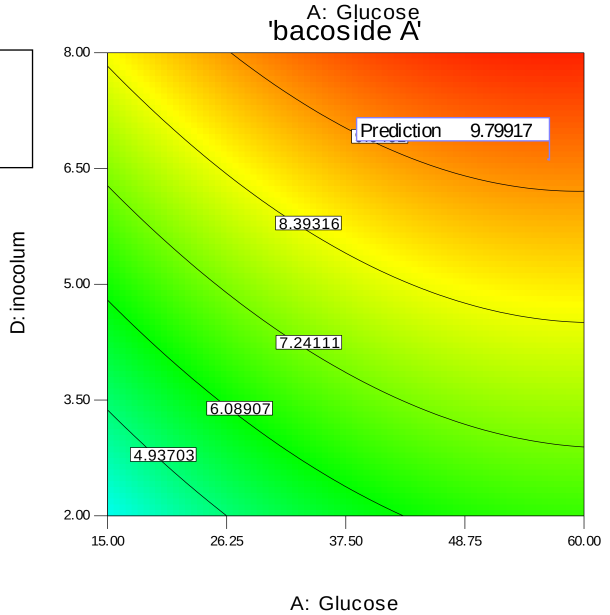
(b)

Actual Factors
B: Nitrate= 31.37
D: Inoculum= 6.60



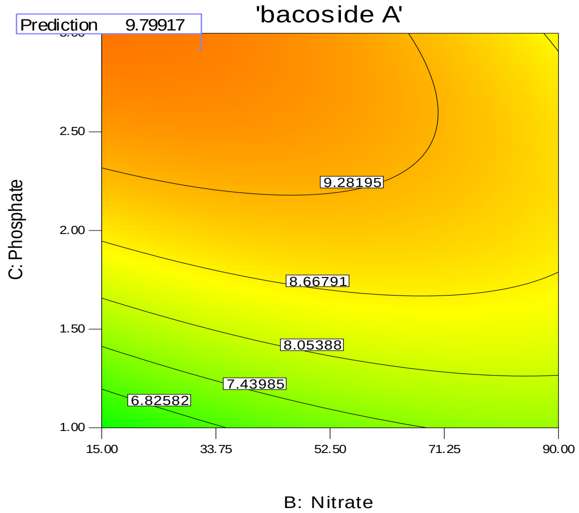
(c)

Actual Factors
B: Nitrate= 31.37
C: Phosphate= 2.91



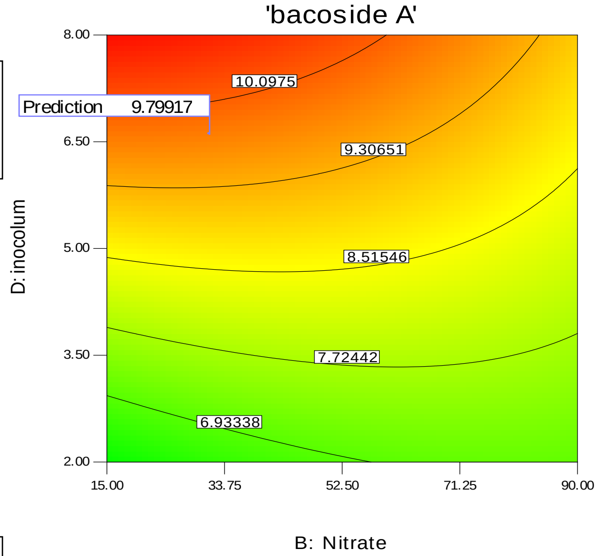
(d)

Actual Factors
A: Glucose= 56.74
D: Inoculum= 6.60



(e)

Actual Factors
A: Glucose= 56.74
C: Phosphate= 2.91



(f)

Actual Factors
A: Glucose= 56.74
B: Nitrate= 31.37

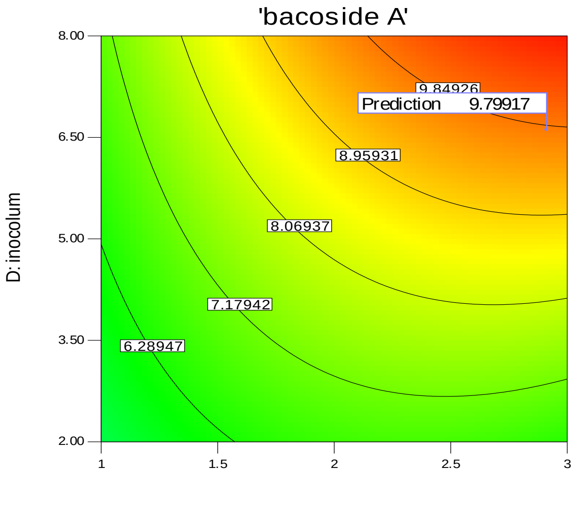
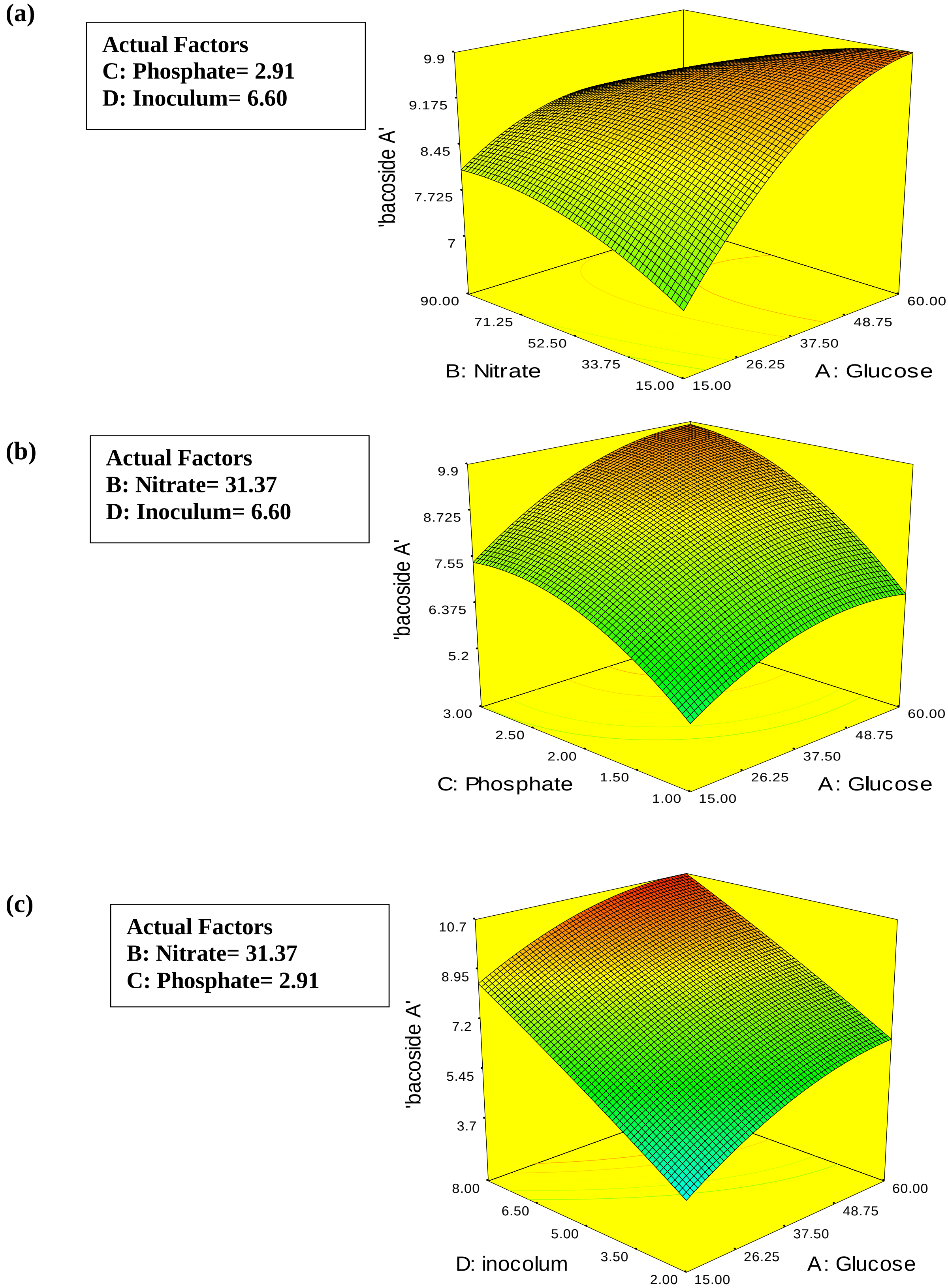


Fig. 6.10 Contour plot between effect of (a) glucose and potassium nitrate (b) glucose and potassium dihydrogen ortho-phosphate (c) glucose and inoculum (d) potassium nitrate and potassium dihydrogen ortho-phosphate (e) potassium nitrate and inoculum (f) potassium dihydrogen ortho-phosphate and inoculum on 'bacoside A' production


(a)
Actual Factors
C: Phosphate= 2.91
D: Inoculum= 6.60
'bacoside A'
9.9
9.175
8.45
7.725
7
90.00
71.25
52.50
33.75
15.00
B: Nitrate
15.00
26.25
37.50
48.75
60.00
A: Glucose
(b)
Actual Factors
B: Nitrate= 31.37
D: Inoculum= 6.60
'bacoside A'
9.9
8.725
7.55
6.375
5.2
3.00
2.50
2.00
1.50
1.00
C: Phosphate
15.00
26.25
37.50
48.75
60.00
A: Glucose
(c)
Actual Factors
B: Nitrate= 31.37
C: Phosphate= 2.91
'bacoside A'
10.7
8.95
7.2
5.45
3.7
8.00
6.50
5.00
3.50
2.00
D: inocolum
15.00
26.25
37.50
48.75
60.00
A: Glucose

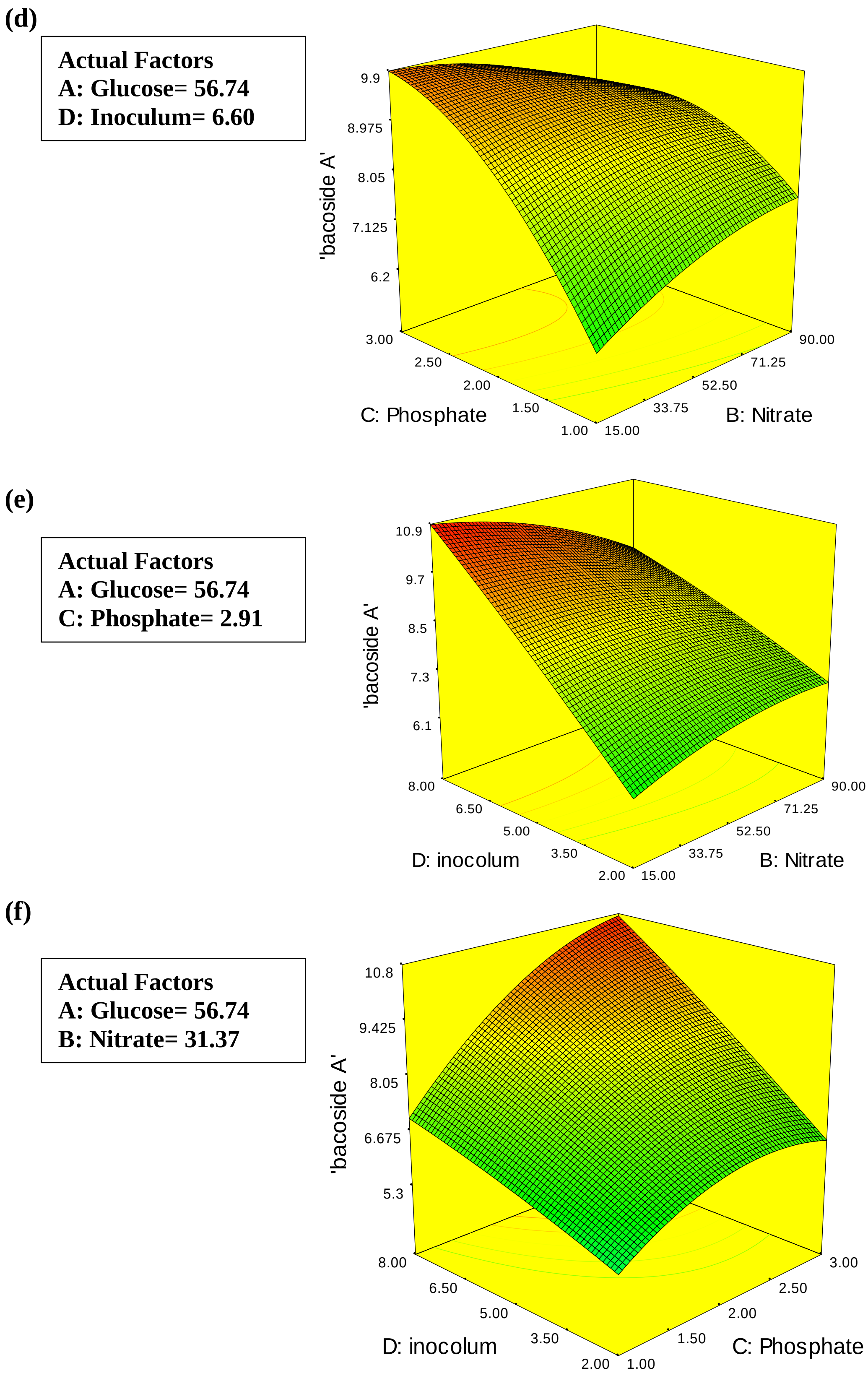


Fig. 6.11 Surface plot showing the effect of (a) glucose and potassium nitrate (b) glucose and potassium dihydrogen ortho-phosphate (c) glucose and inoculum (d) potassium nitrate and potassium dihydrogen ortho-phosphate (e) potassium nitrate and inoculum (f) potassium dihydrogen ortho-phosphate and inoculum on 'bacoside A' production

This work has highlighted the application of Response surface methodology by shedding light on fact that this process allows rapid screening of most important factors for FCW and 'bacoside A' production and reduces the number of experiments significantly. Combination of Plackett- Burman and central composite design proved as an effective and reliable tool to study statistically significant factors and evaluating their optimal concentration within the medium for maximum FCW and 'bacoside A' production.

Discussion

In this study, parameters affecting the cell growth and 'bacoside A' production were studied. Both chemical as well as physical parameters were studied using the OVAT approach. Response surface methodology was then used to select and optimize the concentration of the nutrients which were having major effect on the cell growth and 'bacoside A' production. These studies will be helpful in increasing the yield of 'bacoside A' in culture and may prove to be a step to achieve a overcome constraints associated with regular supply of secondary metabolites.

Sugars have been recognised as molecules that act as energy sources and as well as signalling molecules that affects growth, development and metabolism of cultured cells (Rolland et al., 2006; Wang and Weathers, 2007). Glucose at concentration of 2 % was found to be an ideal carbon source for cell growth and production of 'bacoside A' in cell suspension cultures of *B. monnieri* (Table 6.1; Fig. 6.1). These variations might be due to differential signalling of sugars. Earlier reports by Weather et al. (2004) have shown the beneficial effect of glucose over sucrose and fructose on the production of artemisinin from *Artemisia annua*. Maximum production of artemisinin was found when hairy roots were grown in the medium supplemented with glucose. These authors also concluded that sugars not only acts as carbon source but also serve as signal molecules that affect the production of secondary metabolites. A similar result was observed by Prakash and Srivastava (2005) in *Azadirachta indica* cell suspension cultures, where glucose supplemented medium resulted in maximum cell growth and azadirachtin production. However, Naik et al. (2010) studied the effect of sucrose concentration on the biomass accumulation and 'bacoside A' production in the shoot cultures of *B. monnieri* and sucrose at 2 % concentration was found to be suitable. This could be due to the preferential utilization of sucrose over glucose by the shoot cultures. Moreover, sucrose is only sugar that can be translocated in plants (Hammond and White, 2008), thus whole plant can effectively use only sucrose. Wang and Weathers (2007) compared the effect of glucose, fructose and sucrose in axenic seedling cultures of *A. annua*. Maximum growth was observed when cultured on Gamborg's B5 medium supplemented with glucose. Also, maximum artemisinin production was reported in glucose supplemented medium while fructose showed significant inhibition. Although hairy roots showed maximum artemisinin production when grown in glucose supplemented medium, the level produced in fructose was twice that in sucrose (Weathers et al., 2004). Thus, reported the differential effects of whole plants from those observed in cell and hairy root cultures.

Nitrogen is an essential component for cell growth and secondary metabolite production because it is used in the synthesis of protein and nucleic acid (Neill et al., 2003, 2006). It acts as a limiting nutrient to a critical growth concentration in the culture medium however its high concentration also acts as inhibitor for cell growth and secondary metabolite production. In the present study, the effect of NH_4^+/NO_3^- ratio on cell growth and 'bacoside A' production in cell suspension cultures was evaluated. It was observed that when nitrate was used as sole source of nitrogen high cell growth and secondary metabolite levels were achieved (Table 6.2; Fig. 6.2). It was also found that high concentration NO_3^- over NH_4^+ was suitable for maximum biomass and ‘bacoside A’ content in shoot cultures of *B. monnieri* (Naik et al., 2011). The adverse effect of high ammonium concentration on cell growth and secondary metabolite production has been established in several plants (Zhong and Wang, 1998; Zhao et al., 2001; Sivakumar et al., 2005; Prakash and Srivastava, 2005). Sivakumar et al. (2005) suggested that NO_3^- is the most important source of nitrogen for the growth. Nitrate efflux and influx are dependent process and nitrate can be reduced to nitrite and then ammonia via nitrate reductase and nitrite reductase. Zhao et al. (2001) studied the relation between the nitrogen source and jaceosidin production in the suspension cultures. These authors also reported that the ratio of NH^+_4 NO^-_3 influenced the jaceosidin production. Cell growth and jaceosidin production increased with an increase of NO^-_3 concentration. The NH^+_4 NO^-_3 ratio of 1:2 in MS medium resulted in maximum cell growth and jaceosidin production. High NH_4^+ concentration inhibited the cell growth and jaceosidin production. Yamakawa et al. (1983) also found that by varying the ratio of ammonium ion – nitrate in the MS medium significant increase in the content of anthocyanin was noticed.

Agitation speed is also another important parameter, as proper agitation speed promotes better growth and secondary metabolites synthesis by enhancing the transfer of nutrients from medium to cells (Chattopadhyay et al., 2002). In the present study, different agitation speeds were tested and the highest cell biomass and ‘bacoside A’ content were observed in cell suspension culture kept at an agitation of 120 rpm (Table 6.3; Fig. 6.3). At higher agitation (140–160 rpm), cell growth and ‘bacoside A’ production were highly disturbed, whereas at lower agitation (80–100 rpm), the cells aggregated into harder clumps. At an agitation of 80 rpm cell death was observed; at 100 rpm the cells were loosely attached in the clump. It might be due to the toxic effect to the cells and may strip nutrients such as CO_2 from the culture (Chattopadhyay et al., 2002). The similar trend was documented by Singh and Chaturvedi (2012) in a cell suspension culture of *Spilanthes acmella*.

In the present study, the effect of light radiation on the cell growth and 'bacoside A' production in the cell suspension cultures was also assessed. It was observed that significant variations were observed in growth and 'bacoside A' content. The maximum growth and 'bacoside A' levels were observed when cultures were incubated in 24h dark period (Table 6.4; Fig. 6.4). The enhancement of growth and secondary metabolite production was observed when cultures were incubated in dark, which can be explained on the basis that light affects the expression of enzymes involved in growth and secondary metabolite pathways (Matsumoto et al., 1973; Zhao et al., 2001). The inhibitory effect of light on growth and secondary metabolite production has previously been reported in cultures of *Helianthus tuberosus.* These authors reported that light influenced the size of dividing cells thus affecting growth (Yeoman and Davidson, 1971). Seibert and Kadkade (1980) also found the decrease in active gibberellin level in the tobbaco calli when grown under light conditions. Fett- Neto et al. (1995) reported the effect of light on taxol and baccatin III accumulation in cell cultures of *Taxus cuspidate.* The inhibitory effect of light on the growth and secondary metabolite production has also been reported earlier by many workers (Sakamoto et al., 1993; Nakamura et al., 1999; Konczak- Islam et al., 2000). Prakash et al. (2005) have also studied the effect of illumination on the cell growth and azadirachtin production. They reported 19 % increase in cell growth and azadirachtin production when *A. indica* cells were cultivated in dark for 24h.

In this study, the growth and production of 'bacoside A' in cell suspension cultures was studied upto 24 days in MS medium (Fig. 6.5). The growth curve showed that cell biomass and 'bacoside A' starts increasing on the third day following inoculation and reaches maximum on the 21st day; thereafter the cell fresh weight and 'bacoside A' content decreased. The decline in ‘bacoside A’ content after 21st day of cultivation could be explained by a low production rate and by initiation of degradation process. These results are in line with the earlier reports on secondary metabolite production by many workers (Zhao et al., 2001; Prakash and Srivastava 2005).

Medium pH was found to play a very important role in affecting the growth and production of a secondary metabolite. The pH of the medium is known to affect the availability of nutrients to the plant, thus affecting the growth and production of secondary metabolites (Mc Donald and Jackman, 1989; Bhatia and Ashwath, 2005). The pH level in regulating the cytoplasmic activity that affects cell division and the growth (Brown et al., 1979). In the present study, the growth and ‘bacoside A’ content in the cell suspension cultures of *B. monnieri* was tested at varying pH (Fig. 6.6; Table 6.5). Growth and ‘bacoside A’ content was observed to increase from pH 4.0 to 6.0. Beyond pH 6.0, there was decline in the growth and ‘bacoside A’ levels. Medium pH is known to decrease ammonia assimilation and increase the nitrogen uptake by the cells (Mc Donald and Jackman, 1989). The effect of medium pH on the growth and secondary metabolite production has also been reported in many other plants (Zhang and Furusaki, 1997; Karim et al., 2007; Wang et al., 2009; Murthy and Praveen, 2013). Chen et al. (2006) studied the effect of medium pH on root growth and tanshinone II A and protocatechuic aldehyde synthesis. They found medium pH of 6.0 was favourable for adventitious root growth and synthesis of secondary metabolite respectively. Naik et al. (2010) reported that the medium pH of 4.5 was suitable for biomass and ‘bacoside A’ production in the shoot cultures of *B. monnieri.* Whereas in *Withania somnifera* cell suspension cultures, initial medium pH set as 6.0 was found suitable for dry cell weight and ‘withanolide A’ content (Nagella and Murthy, 2010).

Culture medium plays an important role in supporting the growth and secondary metabolite production. Culture medium comprises of variety of nutrients as energy sources. In the present study, effect of medium strength on the growth and 'bacoside A' content was tested. It was observed that maximum cell growth and 'bacoside A' production occurred in full strength MS medium (Table 6.6; Fig. 6.7). Though different research groups have tested variety of media but full strength and half strength MS medium has been found to be widely accepted for the production of secondary metabolite by plants (Chattopadyay et al., 2002; Jeong et al., 2008). Lian et al. (2002) studied the effect of medium strength on growth and secondary metabolite production and reported that half and full strength medium were equally suitable for cell growth and secondary metabolite production in cell suspension cultures of *Panax ginseng.* Nagella et al. (2010) examined the effect of four different strength medium on growth and production of secondary metabolites and full strength medium was found to be better for cell suspension cultures of *Withania somnifera.*

B. monnieri contains many triterpenoid saponins, of which 'bacoside A' are of particular significance to the pharmaceutical industry. Plackett - Burman design is a well-established and widely used statistical design for the screening of the medium components (Myers and Montgomery, 2002). The design screens important variables that affect secondary metabolite production as well as their significant levels but does not consider the interaction effects among the variables. Among the variables tested in the present study using Plackett - Burman design glucose, potassium nitrate, potassium dihydrogen ortho-phosphate and cell inoculum were found to have significant influence on FCW and 'bacoside A' production by *B. monnieri* cell suspension cultures (Table 6.8; 6.9). Therefore, these variables were further optimized by RSM. A high similarity was observed between the predicted and experimental results, which reflected the accuracy and applicability of RSM to optimize the process for ‘bacoside A’ production. This resulted in a 2.5 -fold increase in FCW and 1.7 -fold increase in ‘bacoside A’ production (Table 6.10). Glucose, potassium nitrate, potassium dihydrogen ortho-phosphate and inoculum at a concentration of 56.74 g/l, 31.37mM, 2.91 g/l and 6.60 g/l influenced the FCW and ‘bacoside A’ production (Fig. 6.8; 6.9; 6.10; 6.11). Prakash and Srivastava (2005) optimized the medium components influencing growth and azadirachtin accumulation in suspension cultures of *A. indica* using response surface methodology. They found glucose, potassium nitrate, potassium dihydrogen ortho-phosphate and inoculum level at a concentration of 37.50 g/l, 56.25 mM, 1.0 mM and 5 g/l resulted a three-fold increase in azadirachtin accumulation. Pavlov et al. (2000) studied statistical medium optimization for biomass and rosmarinic acid production in *Lavandula vera* cell cultures. Optimization using RSM yielded twenty seven times higher biomass and rosmarinic acid compared with the cultivation in the standard Linsmayer - Skoog medium. Das et al. (2001) reported efficient process for the production of somatic embryos in sandalwood using statistical optimization of important medium constituents (viz. sugar, inorganic nitrogen and abscisic acid). Maximum embryogenesis efficiency of 57.35 % was achieved after optimization using RSM. The optimized medium predicted by using RSM enhanced the lycopene yield by three-fold in the cell culture of Tomato (Lu et al., 2008). Medium optimization using response surface methodology increased the β-carotene production in the suspension cultures of *Daucus carota* by two-fold (Hanchinal et al., 2008).

Thus, Plackett – Burman and RSM has helped in minimizing the cost of ‘bacoside A’ production in shake flasks by enhancing the production and thus, making the process more economical.

Conclusion

The present work has resulted in enhanced cell growth and 'bacoside A' production under optimized conditions using Response surface methodology. The physical and chemical factors which affect the cell growth and 'bacoside A' in cell suspension cultures of *B. monnieri* were firstly varied using one-variable-at-a-time stratergy and after that, plackett - burman design was used to screen multiple variables which affect the cell growth and 'bacoside A' production. Chemical (media strength, carbon source, nitrogen source) as well as physical (pH, agitation and temperature) parameters affected the cell growth and 'bacoside A' production significantly with change in any of the above cited parameters therefore, they need to be optimized. Further optimization using statistical method resulted in three-fold improvement in FCW and two-fold improvement in 'bacoside A' content

Salient findings

- Glucose (2 % w/v) was found to be better carbon source for cell growth and production of 'bacoside A' as compared to sucrose.
- Nitrate as sole nitrogen source was found to be effective for high cell growth and secondary metabolite
- The pH 6 and complete dark was found to be more optimum physical conditions for cell growth and 'bacoside A' accumulation.
- The growth profile showed that cell growth and 'bacoside A' production reaches maximum on the 21st day,

- Full strength MS medium was found to be better for growth and ‘bacoside A’ production.
- Agitation speed (120 rpm) was found optimum for high cell growth and ‘bacoside A’ content.
- Concentration of glucose, potassium nitrate, potassium dihydrogen ortho-phosphate and inoculum were found to have significant effect on cell growth and ‘bacoside A’ production.
- Based on results of central composite design, the optimized media and conditions for maximum cell growth and ‘bacoside A’ production comprised glucose = 56.74 g/l, KNO_3 = 31.37mM, KH_2PO_4 = 2.91 g/l and inoculum = 6.60 g/l.
- The optimized medium resulted in three-fold improvement in FCW and two-fold improvement in ‘bacoside A’ content.

Chapter 7

Optimization of medium components for enhancing root biomass and 'bacoside A' production using hairy root cultures

As it was found that hairy root cultures are very efficient and economical way for large production of secondary metabolites including 'bacoside A'. An attempt was made to enhance the production of 'bacoside A' by optimizing the media components using one variable at a time approach (OVAT) and using statistical method Response Surface Methodology (RSM).

7.1. Selection of constituents of growth media

The constituents of the growth media and process variables play an important role in the growth of tissue, organ and production of secondary metabolites. Among these, the major ones are: medium volume (V_m) to flask volume (V_f) ratio, growth kinetics, medium pH, carbon source, nitrogen source etc. In the present study these physicochemical parameters were optimized by one variable at a time (OVAT) approach. The two carbon sources tested were: glucose and sucrose while nitrogen sources tested were ammonium nitrate and potassium nitrate. The medium volume (V_m) to flask volume (V_f) ratio were also investigated from range 0.06 to 0.18, medium pH range from 4.0 to 7.0.

7.1.1. Optimization of the medium volume (V_m) to flask volume (V_f) ratio

In the present investigation, effect of medium volume to flask volume (V_m/V_f) ratio on the biomass accumulation and 'bacoside A' content was studied. While optimizing the ratio of medium volume to flask volume (V_m/V_f), it was observed that the biomass increased from 4.77 g/l FW at a V_m/V_f ratio of 0.06 to a reasonably high value of 7.4 g/l FW at a V_m/V_f ratio of 0.12, which thereafter decreased to 6.55 g/l FW at V_m/V_f ratio of 0.18 (Fig. 7.1). The V_m/V_f ratio for maximum 'bacoside A' production of 10.25 mg/g DW ('bacoside A3' 1.98 mg/g DW, 'bacopaside II' 5.32 mg/g DW, 'bacopasaponin C' 2.38 mg/g DW and 'bacopaside X' 0.57 mg/g DW) was also worked out to be at ratio of 0.12 over the 40 d culture period (Table 7.1), equivalent to an overall 'bacoside A' productivity of 0.25 mg g^{-1} d^{-1}. Hence, the optimum value of V_m/V_f for maximum biomass accumulation and production of 'bacoside A' was selected at 0.12, which was used for further experimentation.

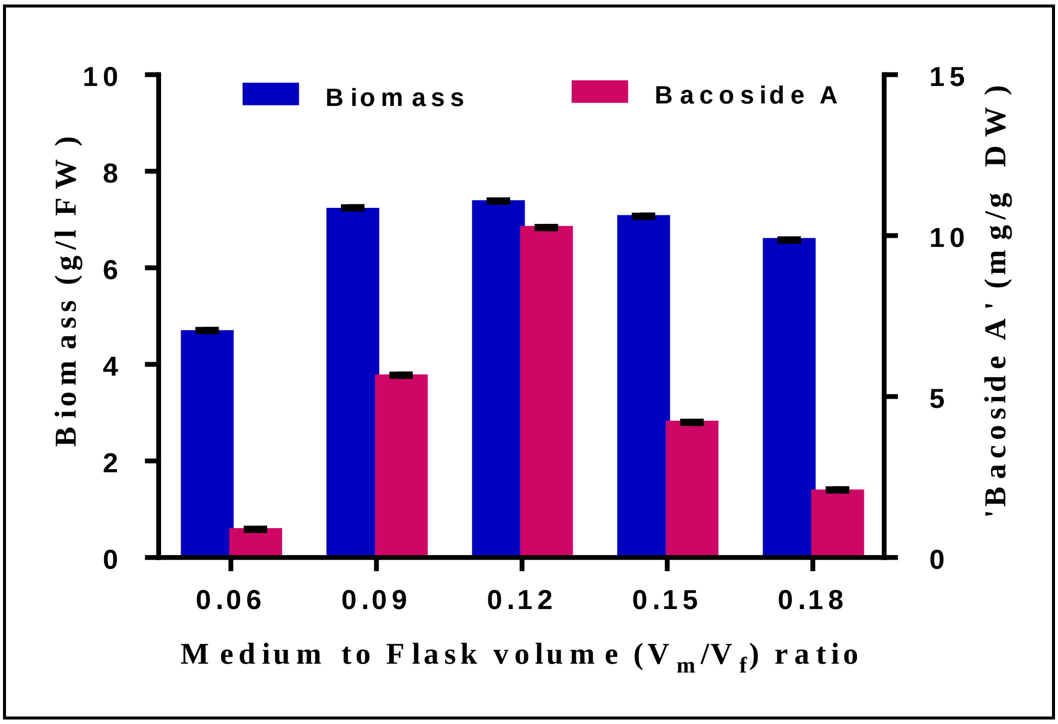


Fig. 7.1 Effect of medium to flask volume ratio on biomass accumulation and production of 'bacoside A' in root cultures of *B. monnieri*. Bars represents mean ± SD (n =3)

Table 7.1 Effect of medium to flask volume ratio on biomass accumulation (g/l) and production of 'bacoside A' (mg/g DW) in root cultures of *B. monnieri*

Medium to flask volume ratio (V_m/V_f)	FW	DW	Total bacoside A	Bacoside A3	Bacopaside II	Bacopasaponin C	Bacopaside X
0.06	4.67±0.12[e]	0.57±0.00[e]	0.88±0.21[e]	0.11±0.11[e]	0.49±0.21[e]	0.24±0.15[e]	0.04±0.09[e]
0.09	7.14±0.27[b]	0.81±0.01[b]	5.62±0.15[b]	0.52±0.07[b]	3.05±0.18[b]	1.75±0.11[b]	0.30±0.11[b]
0.12	7.37±0.27[a]	0.89±0.04[a]	10.25±0.12[a]	1.98±0.01[a]	5.32±0.12[a]	2.38±0.19[a]	0.57±0.01[a]
0.15	7.07±0.14[c]	0.72±0.01[c]	4.15±0.23[c]	0.37±0.04[c]	2.74±0.01[c]	0.86±0.02[c]	0.18±0.04[c]
0.18	6.57±0.23[d]	0.66±0.01[d]	2.06±0.19[d]	0.22±0.01[d]	1.31±0.21[d]	0.43±0.01[d]	0.10±0.06[d]

Values bearing different letters in the same columns are significant at $P<0.05$. Values are mean ± SD (n =3).

7.1.2. Growth kinetics of the root culture

The growth profile of *B. monnieri* hairy roots is depicted in Fig. 7.2. The biomass increased slowly and reached a maximum of 7.70 g/l FW and 0.94 g/l DW at the end of 25 days of culture. A twelve-fold increase in root biomass was found when compared with initial dry weight of roots. The 'bacoside A' content increased gradually during the culture period (Table 7.2). Maximum 'bacoside A' content 10.31 mg/g DW ('bacoside A3' 1.94 mg/g DW, 'bacopaside II' 4.84 mg/g DW, 'bacopasaponin C' 2.80 mg/g DW and 'bacopaside X' 0.73 mg/g DW) was found at the end of 25 days of culture, the content of 'bacoside A' decreased thereafter. Thus, incubation of hairy root cultures for 25 days was found optimal for both biomass accumulation and production of 'bacoside A'.

Table 7.2 Growth study of biomass accumulation (g/l) and production of 'bacoside A' (mg/g DW) in the root culture of *B. monnieri* on basal MS medium

Time (days)	FW	DW	Total bacoside A	Bacoside A3	Bacopaside II	Bacopasaponin C	Bacopaside X
0	2.17±0.07^{i}	0.19±0.00^{i}	0.76±0.32^{h}	0.10±0.08^{g}	0.41±0.01^{e}	0.19±0.04^{f}	0.06±0.01^{f}
5	2.24±0.31^{h}	0.28±0.01^{h}	0.93±0.41^{g}	0.12±0.09^{f}	0.52±0.24^{d}	0.22±0.14^{e}	0.07±0.02^{f}
10	2.68±0.33^{g}	0.37±0.01^{g}	1.48±0.35^{f}	0.18±0.14^{e}	0.91±0.30^{c}	0.29±0.27^{d}	0.10±0.07^{e}
15	3.80±0.37^{f}	0.46±0.02^{f}	4.86±0.37^{e}	1.08±0.29^{d}	2.19±0.27^{b}	1.42±0.27^{c}	0.17±0.01^{d}
20	5.60±0.22^{e}	0.53±0.00^{e}	8.73±0.15^{d}	1.67±0.06^{c}	4.14±0.15^{d}	2.33±0.02^{b}	0.59±0.04^{c}
25	7.70±0.26^{a}	0.94±0.01^{a}	10.31±0.27^{a}	1.94±0.15^{a}	4.84±0.33^{a}	2.80±0.11^{a}	0.73±0.01^{a}
30	7.57±0.12^{a}	0.89±0.03^{b}	10.30±0.21^{a}	1.97±0.23^{a}	4.81±0.18^{a}	2.82±0.1^{a}	0.70±0.27^{a}
35	7.36±0.32^{c}	0.82±0.01^{c}	10.25±0.11^{b}	1.95±0.12^{a}	4.79±0.12^{a}	2.82±0.19^{a}	0.69±0.14^{a}
40	7.30±0.02^{d}	0.73±0.1^{d}	10.18±0.16^{c}	1.91±0.15^{b}	4.83±0.01^{a}	2.80±0.02^{a}	0.64±0.23^{b}

Values bearing different letters in the same columns are significant at $P<0.05$. Values are mean ± SD (n =3).

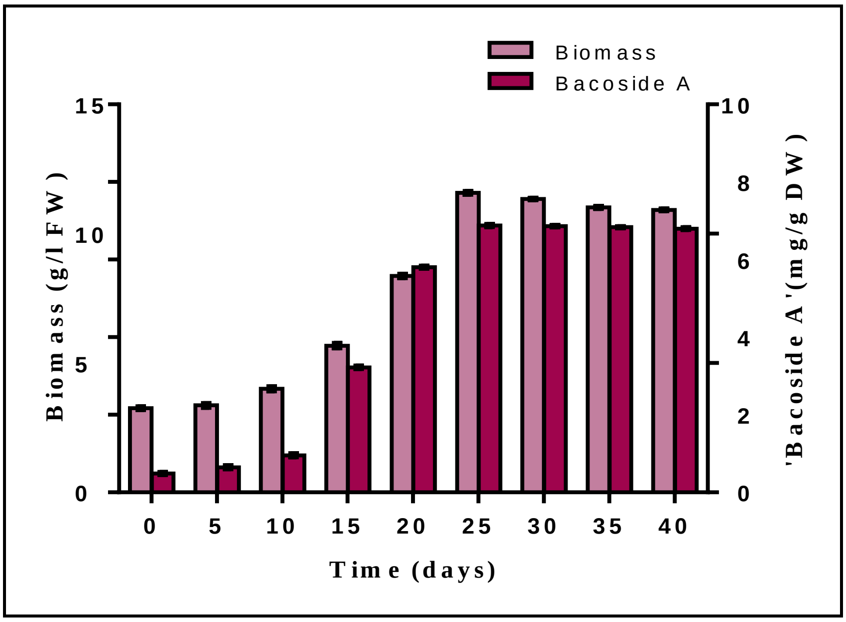


Fig. 7.2 Growth study of biomass accumulation and production of 'bacoside A' in the root culture of *B. monnieri* on basal MS medium. Bars represents mean ± SD (n =3)

7.1.3. Optimization of the medium pH

The effect of medium pH on biomass accumulation and 'bacoside A' production in hairy root cultures is depicted in Fig. 7.3 and Table 7.3. Of the different pH tested, the highest biomass accumulation (7.8 g/l FW and 1.03 g/l DW) was observed in hairy root cultures grown on at a medium with pH of 6.0. In addition, the quantities of 'bacoside A' production were also considerably higher from 0.77 mg/g DW to 10.33 mg/g DW with 'bacoside A3' 1.56 mg/g DW, 'bacopaside II' 5.74 mg/g DW, 'bacopasaponin C' 2.38 mg/g DW and 'bacopaside X' 0.65 mg/g DW, respectively in medium with pH of 6.0. Next to pH 6.0, maximum biomass accumulation (6.9 g/l FW and 0.79 g/l DW) and 'bacoside A' production (7.37 mg/g DW) was found in medium pH of 5.5. The lowest accumulation of biomass and 'bacoside A' production were recorded in the medium pH at 4.0 (Table 7.3).

Table 7.3 Effect of pH on biomass accumulation (g/l) and production of 'bacoside A' (mg/g DW) in the root cultures of *B. monnieri* on basal MS medium

Medium pH	FW	DW	Total bacoside A	Bacoside A3	Bacopaside II	Bacopasaponin C	Bacopaside X
4.0	4.5±0.37^{g}	0.31±0.22^{g}	0.77±0.32^{g}	0.10±0.02^{g}	0.43±0.21^{g}	0.17±0.16^{g}	0.07±0.02^{e}
4.5	4.7±0.26^{f}	0.35±0.17^{f}	0.95±0.11^{f}	0.15±0.09^{f}	0.50±0.32^{f}	0.22±0.02^{f}	0.08±0.04^{e}
5.0	6.1±0.18^{d}	0.61±0.09^{d}	2.87±0.31^{d}	0.41±0.10^{d}	1.62±0.29^{d}	0.59±0.22^{d}	0.25±0.07^{c}
5.5	6.9±0.33^{b}	0.79±0.21^{b}	7.37±0.27^{b}	1.21±0.13^{b}	4.06±0.14^{b}	1.47±0.17^{c}	0.63±0.01^{a}
6.0	7.8±0.24^{a}	1.03±0.16^{a}	10.33±0.19^{a}	1.56±0.03^{a}	5.74±0.35^{a}	2.38±0.12^{a}	0.65±0.01^{a}
6.5	6.4±0.14^{c}	0.67±0.12^{c}	6.50±0.35^{c}	0.96±0.15^{c}	3.44±0.23^{c}	1.61±0.01^{b}	0.49±0.09^{b}
7.0	5.1±0.26^{e}	0.47±0.04^{e}	1.14±0.21^{e}	0.19±0.22^{e}	0.58±0.27^{e}	0.27±0.17^{e}	0.10±0.04^{d}

Values bearing different letters in the same columns are significant at P<0.05. Values are mean ± SD (n =3).

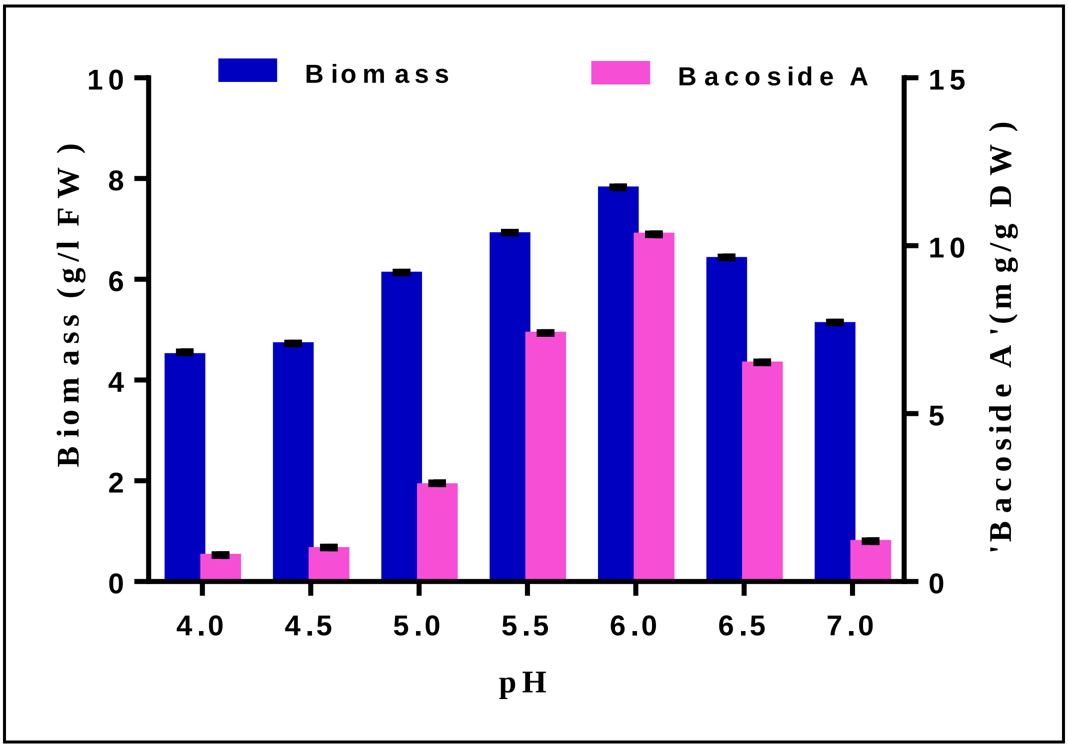


Fig. 7.3 Effect of pH on biomass accumulation and production of 'bacoside A' in the root cultures of *B. monnieri* on basal MS medium. Bars represents mean ± SD (n =3)

7.1.4. Effect of the sucrose and glucose on biomass accumulation and 'bacoside A' production

In the present investigation, effect of sucrose and glucose was evaluated on biomass accumulation and 'bacoside A' production (Table 7.4 and Fig. 7.4). Carbon source had significant effect on growth and levels of 'bacoside A'. Glucose was found to be better carbon source for the biomass accumulation (8.08 g/l FW and 1.13 g/l DW) and 'bacoside A' synthesis (10.58 mg/g DW). The higher levels of various components of 'bacoside A' viz., 'bacoside A3' 1.76 mg/g DW, 'bacopaside II' 5.73 mg/g DW, 'bacopasaponin C' 2.45 mg/g DW and 'bacopaside X' 0.64 mg/g DW respectively were detected on glucose supplemented medium (Fig. 7.4; Table 7.4).

Table 7.4 Effect of carbon sources on biomass accumulation (g/l) and 'bacoside A' production (mg/g DW) in hairy root cultures of *B. monnieri*

Carbon source	Conc.	FW	DW	Total bacoside A	Bacoside A3	Bacopaside II	Bacopasaponin C	Bacopaside X
Glucose	30	8.08±0.21[a]	1.13±0.14[a]	10.58±0.18[a]	1.76±0.02[a]	5.73±0.21[a]	2.45±0.16[a]	0.64±0.02[a]
Sucrose	30	7.75±0.07[b]	0.97±0.27[b]	10.34±0.04[b]	1.69±0.09[b]	5.69±0.32[b]	2.38±0.02[b]	0.58±0.04[b]

Values bearing different letters in the same columns are significant at P<0.05. Values are mean ± SD (n =3).

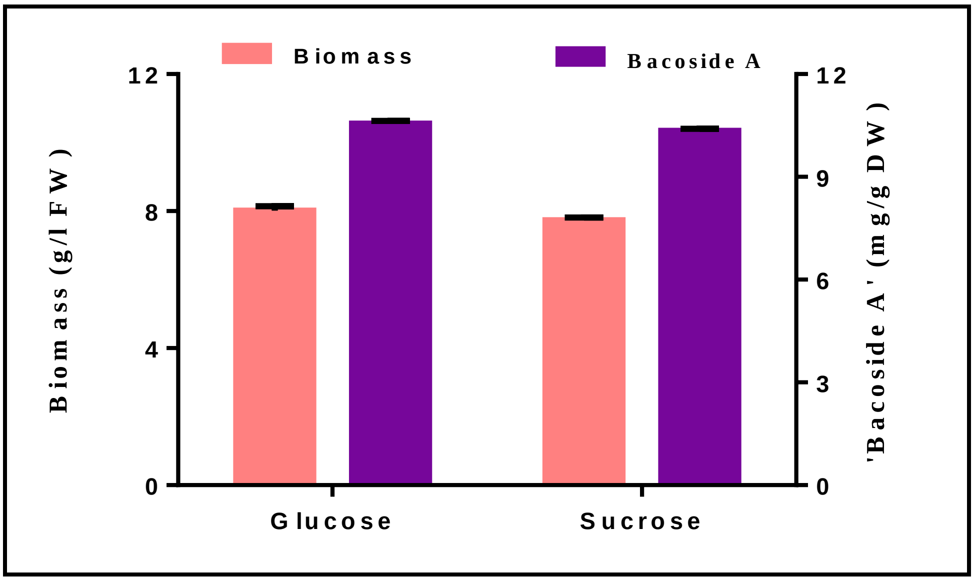


Fig. 7.4 Effect of glucose and sucrose on biomass accumulation and 'bacoside A' production. Bars represents mean ± SD (n =3)

7.1.5. Effect of nitrogen source on biomass accumulation and 'bacoside A' production

In the present study, hairy root cultures responded differently to nitrogen sources i.e. potassium nitrate (KNO_3) and ammonium nitrate (NH_4NO_3). Biomass accumulation and 'bacoside A' levels varied with nitrogen source tested (Table 7.5). Among the two nitrogen sources tested, maximum biomass accumulation (8.17 g/l FW and 1.17 g/l DW) was recorded in MS liquid medium fortified with 2 g/l KNO_3 (Fig. 7.5). In this medium, higher biomass accumulation as well as 'bacoside A' levels were recorded. In medium supplemented with KNO_3 at a conc. of 2 g/l, higher levels of 'bacoside A' (10.72 mg/g DW) were recorded. The levels of different components of 'bacoside A' were found higher in KNO_3 supplemented medium (Table 7.5 and Fig. 7.5)

Table 7.5 Effect of nitrogen source on biomass accumulation (g/l) and production of 'bacoside A' (mg/g DW) in hairy root cultures of *B. monnieri*

Nitrogen source	Conc. (g/l)	FW	DW	Total bacoside A	Bacoside A3	Bacopaside II	Bacopasaponin C	Bacopaside X
KNO_3	2	8.17±0.12[a]	1.17±0.02[a]	10.72±0.21[a]	1.83±0.02[a]	5.79±0.21[a]	2.51±0.16[a]	0.59±0.02[a]
NH_4NO_3	2	5.03±0.14[b]	0.46±0.00[b]	2.97±0.21[b]	0.51±0.09[b]	1.27±0.32[b]	0.78±0.02[b]	0.41±0.04[b]

Values bearing different letters in the same columns are significant at P<0.05. Values are mean ± SD (n =3).

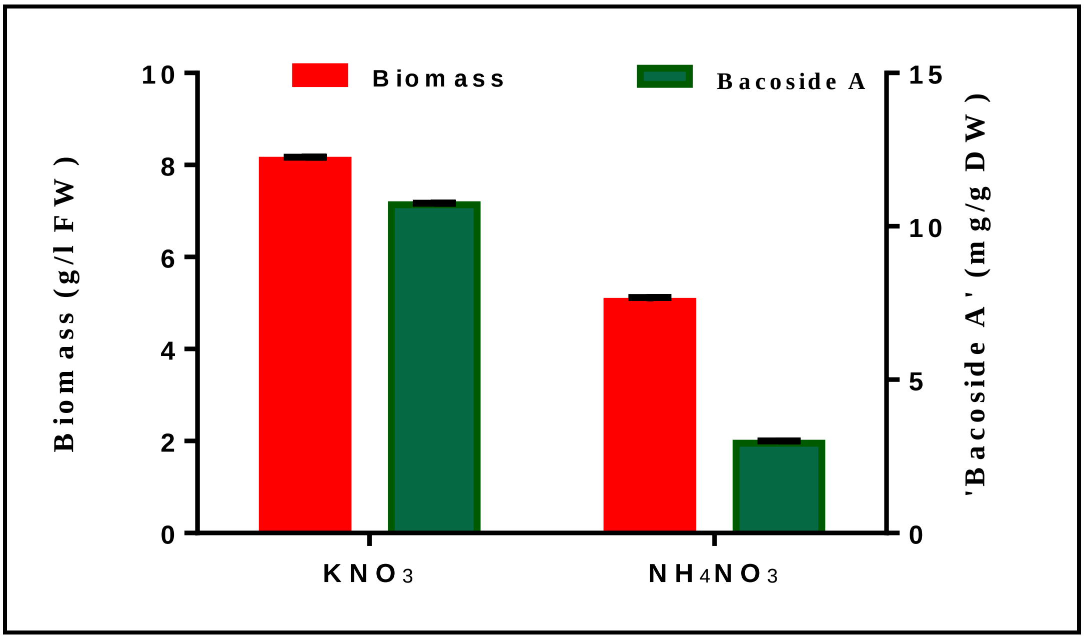


Fig. 7.5 Effect of nitrogen sources on biomass accumulation and production of 'bacoside A'. Bars represents mean ± SD (n =3)

7.2. Optimization of medium components for enhancing biomass and 'bacoside A' production using RSM

7.2.1. Plackett - Burman design

Plackett - Burman design was used to investigate the most significant factors for biomass accumulation and 'bacoside A' production in the media constituents at two levels in total of 12 experiments. The variables and their selected levels are given in table 7.6.

Table 7.6 The two levels of medium components used in the Plackett- Burman design

Components (g/l)	Symbol	Low (-1)level	High (+1)level
Glucose	A	15.00	60.00
Potassium nitrate	B	2.00	8.00
Ammonium sulphate	C	0.60	2.70
Potassium dihydrogen ortho-phosphate	D	0.08	0.34
Magnesium sulphate	E	0.18	0.74
Calcium chloride	F	0.22	0.88

The responses (biomass accumulation and 'bacoside A' production) obtained after 25 days in each of the experiments (Table 7.7) were subjected to analysis by the Design-Expert software.

Table 7.7 Experimental design matrix with response obtained for biomass accumulation and 'bacoside A' production using Plackett – Burman design for medium optimization

Run order	A	B	C	D	E	F	Biomass (g/l)	Bacoside A (mg/g)
1	1	1	1	1	-1	-1	7.02	5.25
2	1	-1	1	-1	-1	-1	7.77	4.05
3	-1	1	1	-1	1	-1	8.16	5.58
4	-1	-1	-1	1	-1	1	4.88	1.52
5	1	1	-1	-1	1	1	14.12	13.44
6	1	1	-1	-1	-1	1	12.27	9.15
7	-1	1	1	1	-1	1	6.71	4.03
8	-1	-1	1	-1	1	1	6.08	3.78
9	-1	1	-1	1	1	-1	6.07	4.73
10	-1	-1	-1	-1	-1	-1	5.37	2.52
11	1	-1	-1	1	1	-1	9.97	4.69
12	1	-1	1	1	1	1	10.07	9.85

Table 7.8 shows the *t* value (*t* coefficient) contribution and *p* value of the Plackett-Burman analysis. Value of 'Prob > F' less than 0.05 indicate model terms are significant and values greater than 0.1000 indicate model terms are not significant. Value of "Model Prob > F" is 0.0075 and 0.0200 for biomass accumulation and 'bacoside A' production which implies the model are significant (Plackett and Burman, 1946). In this case glucose, potassium nitrate, potassium dihydrogen ortho-phosphate and magnesium sulphate were found to have significant effect on biomass accumulation and 'bacoside A' production.

Table 7.8 ANOVA table for the determination of variables playing most significant role for biomass accumulation and 'bacoside A' production using Plackett - Burman design.

Symbol	Components	t Coefficient		Studied Effect		Contribution (%)		Prob > F (p-value)*#	
		Biomass	Bacoside A	Biomass	Bacoside A	Biomass	Bacoside A	Biomass	Bacoside A
A	**Glucose**	6.21	4.27	3.99	4.05	53.05	38.35.87	0.0014	0.0067
B	**KNO_3**	2.57	2.78	1.63	2.45	8.83	14.55	0.0472	0.0409
C	**$(NH_4)_2SO_4$**	-2.01	-1.47	-1.51	-1.41	7.57	4.65	0.0597	0.1825
D	**KH_2PO_4**	3.07	3.12	1.74	2.59	10.10	15.74	0.380	0.359
E	**$MgSO_4.7H_20$**	2.92	3.12	1.70	2.63	9.64	16.19	0.0410	0.0343
F	**$CaCl_2.2H_20$**	-1.42	-0.57	-1.14	-0.59	4.37	0.80	0.1250	0.5487
	Model	-	-	-	-	-	-	0.0075	0.0200

Glucose, KNO_3, KH_2PO_4 and $MgSO_4$ were identified as important components influencing on biomass accumulation and 'bacoside A' production. The effect for the above mentioned four variables was confirmed from the high positive *t* values (or regression coefficients) calculated for these components in the ANOVA test of the data analysis done by the Design-Expert software (Table 7.8). $CaCl_2$ and $(NH_4)_2SO_4$ had a negative effect as indicated by their negative *t* values calculated in the ANOVA test of the data analysis.

Out of the six variables studied under PB design; $CaCl_2$ and $(NH_4)_2SO_4$ had a negative effect on biomass accumulation and 'bacoside A' production (as indicated by the negative *t* value and studied effect) and with "Prob>F" (*p* value) more than 0.1. These two variables were included at a fixed concentration level in the optimized medium. Although, the PB design could be successfully used for the reasonable prediction of the significance level of different variables affecting the response (biomass accumulation and 'bacoside A' production), some of the significant interactive effects of the chosen variables (two-factor interaction) were confounded in its complex structure. Because of this reason, the actual main effects of these variables may have been influenced. So, the significant components (glucose, KNO_3, KH_2PO_4 and $MgSO_4$) whose interactive effects were demonstrated by PB design were further modelled more precisely by RSM.

7.2.2. Response surface methodology for optimizing concentrations of medium components

Statistical designs serve as effective tools for determining main as well as interactive influences of different parameters on production of secondary metabolites. Response surface methodology (RSM) is a collection of certain statistical techniques for designing the experiments, building models, evaluating effect of factors and searching for optimal conditions for desirable responses (Myers and Montgomery, 2002).

The four identified important and effective variables (glucose, KNO_3, KH_2PO_4 and $MgSO_4$) were optimized using response surface methodology. In the present study, Central composite design (CCD) was used to obtain the combination of values for optimizing the response. Table 7.9 shows various combinations used and corresponding biomass accumulation and production of 'bacoside A' (experimental and predicted). The amounts of remaining components in all assemblies were the same as those in basal medium.

Table 7.9 Central composite experimental design matrix with experimental and predicted value for medium optimization

Trials	Actual values (g/l)				Responses			
	Glucose	KNO_3	KH_2PO_4	$MgSO_4$	Experimental		Predicted	
					Biomass	Bacoside A	Biomass	Bacoside A
1	60.00	2.00	0.08	0.74	7.77	8.63	7.71	9.50
2	37.50	11.00	0.21	0.46	10.57	10.99	12.09	14.93
3	37.50	5.00	0.21	0.46	12.62	16.13	12.53	16.11
4	15.00	2.00	0.08	0.18	6.57	8.83	5.13	9.26
5	37.50	5.00	0.21	0.46	12.12	16.13	12.53	16.11
6	60.00	2.00	0.34	0.74	6.57	10.93	8.06	10.77
7	82.50	5.00	0.21	0.46	9.07	10.38	12.12	14.56
8	37.50	0.00	0.21	0.46	3.87	10.4	5.54	10.19
9	60.00	2.00	0.08	0.18	8.67	12.69	8.47	12.28
10	37.50	5.00	0.21	1.02	14.02	16.04	12.15	15.26
11	15.00	2.00	0.34	0.18	5.37	10.93	5.83	9.79
12	60.00	2.00	0.34	0.18	9.47	10.99	8.62	12.42
13	0.00	5.00	0.21	0.46	3.07	8.58	3.68	9.44
14	15.00	2.00	0.34	0.74	7.57	9.13	6.77	10.05
15	37.50	5.00	0.21	0.46	12.82	16.13	12.53	16.11
16	37.50	5.00	0.47	0.46	14.02	13.04	12.44	12.12
17	60.00	8.00	0.08	0.74	11.17	10.13	11.93	11.09
18	15.00	2.00	0.08	0.74	5.87	9.13	5.87	8.40
19	37.50	5.00	0.21	0.46	12.62	16.13	12.53	16.11
20	60.00	8.00	0.34	0.18	10.67	11.04	11.89	11.59
21	15.00	8.00	0.34	0.18	6.77	10.34	6.80	10.57
22	37.50	5.00	0.00	0.46	11.47	12.44	12.27	14.54
23	60.00	8.00	0.08	0.18	11.37	11.54	12.14	11.72
24	37.50	5.00	0.21	0.00	10.77	16.05	11.73	15.81
25	60.00	8.00	0.34	0.74	10.47	11.42	11.88	12.08
26	37.50	8.00	0.21	0.46	13.72	16.13	12.53	16.11
27	15.00	8.00	0.08	0.18	6.77	10.35	6.50	10.33
28	15.00	8.00	0.08	0.74	6.97	11.95	7.79	11.61
29	15.00	8.00	0.34	0.74	6.87	12.75	8.29	12.98
30	37.50	5.00	0.21	0.46	12.52	16.13	12.56	16.11

The response (biomass accumulation and production of 'bacoside A') for each experiment were analysed by linear multiple regression and graphical analysis using Design-Expert software. The mathematical models incorporating the interactive effect of these nutrients were proposed for biomass accumulation (Model 1) and 'bacoside A' production (Model 2) and the following equation was obtained:

Model 1:

$$\textbf{Biomass} = +12.54 + 1.73\,A + 1.30\,B + 0.16\,C + 0.18\,D + 0.58\,AB - 0.14\,AC - 0.38\,AD - 0.10\,BC + 0.14\,BD + 0.050\,CD - 2.15\,A^2 - 1.74\,B^2 - 0.10\,C^2 - 0.19\,D^2$$

Model 2:

$$\textbf{`Bacoside A'} = +16.11 + 0.53A + 0.59B + 0.38C - 0.092\,D - 0.41AB - 0.094\,AC - 0.48\,AD - 0.070\,BC + 0.54\,BD + 0.28\,CD - 2.08\,A^2 - 1.77\,B^2 - 1.18\,C^2 - 0.17\,D^2$$

where A is glucose, B is potassium nitrate, C is potassium dihydrogen ortho-phosphate, and D is magnesium sulphate, respectively.

The statistical analysis of the model equations was performed by ANOVA. The ANOVA of linear regression model equation demonstrated that model equation was highly significant as evident from value of "Model Prob > F" less than 0.0001 (Table 7.10). In this case A, B, A^2, B^2 are significant model terms.

The goodness of fit of the models was checked by the determination correlation coefficient (R^2). Normally, a regression model with R^2 value of higher than 0.8 is considered as having a very high correlation. R^2 value for biomass and 'bacoside A' production is 0.95 and 0.93 respectively, so it is reasonable to use the regression model to analyse the trends in the response. The values of the determination coefficients indicated that only 5 % and 7 % of the total variation for each response was not explained by the respective models.

Table 7.10 Regression coefficients and their significance for response surface model

Source	Sum of squares		df		Mean square		F- value		Prob> F	
	Biomass	Bacoside A	Biomass	Bacoside A	Biomass	Bacoside A	Biomass	Bacoside A	Biomass	Bacoside A
Model	242.52	192.92	14	14	17.32	13.78	7.67	15.80	<0.0001	<0.0001
A	67.10	6.27	1	1	67.10	6.27	29.72	7.19	<0.0001	0.0171
B	37.64	7.87	1	1	37.64	7.87	16.67	9.02	0.0010	0.0089
C	0.58	3.15	1	1	0.58	3.15	0.26	3.62	0.6184	0.0766
D	0.74	0.19	1	1	0.74	0.19	0.33	0.22	0.5760	0.6485
AB	5.29	2.62	1	1	5.29	2.62	2.34	3.01	0.1466	0.1033
AC	0.30	0.14	1	1	0.30	0.14	0.13	0.16	0.7194	0.6936
AD	2.25	3.67	1	1	2.25	3.67	1.00	4.21	0.3339	0.0582
BC	0.16	0.078	1	1	0.16	0.078	0.071	0.09	0.7937	0.7684
BD	0.30	4.62	1	1	0.30	4.62	0.13	5.3	0.7194	0.0361
CD	0.040	1.27	1	1	0.040	1.27	0.018	1.45	0.8959	0.2470
A^2	100.27	94.33	1	1	100.27	94.33	44.42	108.18	<0.0001	<0.0001
B^2	65.72	68.55	1	1	65.27	68.55	39.11	78.62	<0.0001	<0.0001
C^2	0.22	29.54	1	1	0.22	29.54	0.099	33.88	0.7572	<0.0001
D^2	0.75	0.59	1	1	0.75	0.59	0.33	0.67	0.5716	0.4251
Lack of fit	32.43	13.08	10	10	3.24	1.31	11.35	78.87	0.0076	<0.0001

The value of the adjusted determination coefficient (adjusted R^2 =0.82 and 0.87) was also reasonably high, supporting a high significance of the model. Predicted R^2 of 0.58 and 0.56

demonstrated reasonable predictability of the model for biomass and 'bacoside A' production.

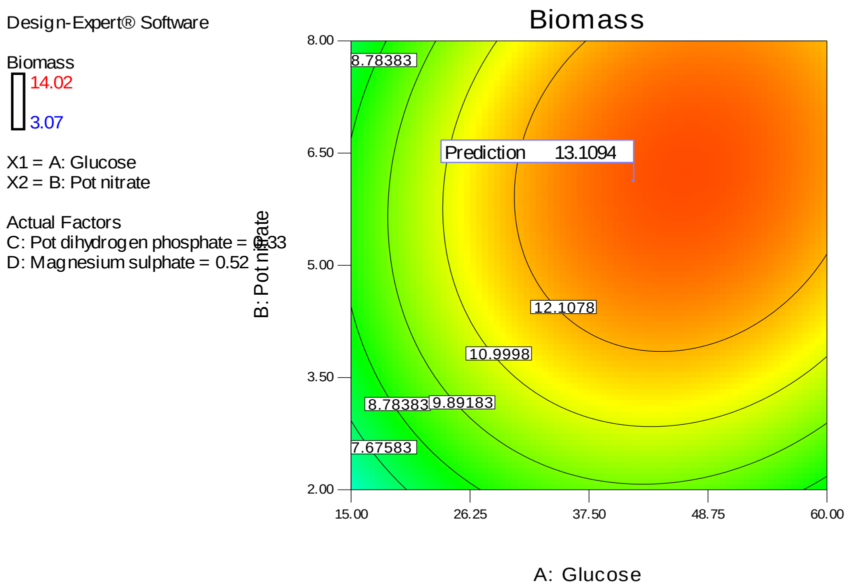
At the same time, the relatively low values of the coefficient of variation (CV=13.54 and 7.66 for biomass accumulation and 'bacoside A' production) with adequate precision values 15.46 and 11.67, indicated an acceptable precision and reliability of the experiments.

The 3D response surface and the 2D contour plots generated during the data analysis are graphical representations of the regression equations. It provides a method to visualise the relationship between the response and experimental levels of each variable and the type of interactions between the test variables in order to deduce the optimal conditions (Fig 7.6, 7.7, 7.8 and 7.9). Each contour curve represents an infinite number of combinations of two test variables, another variable being at a optimal level of concentration range.

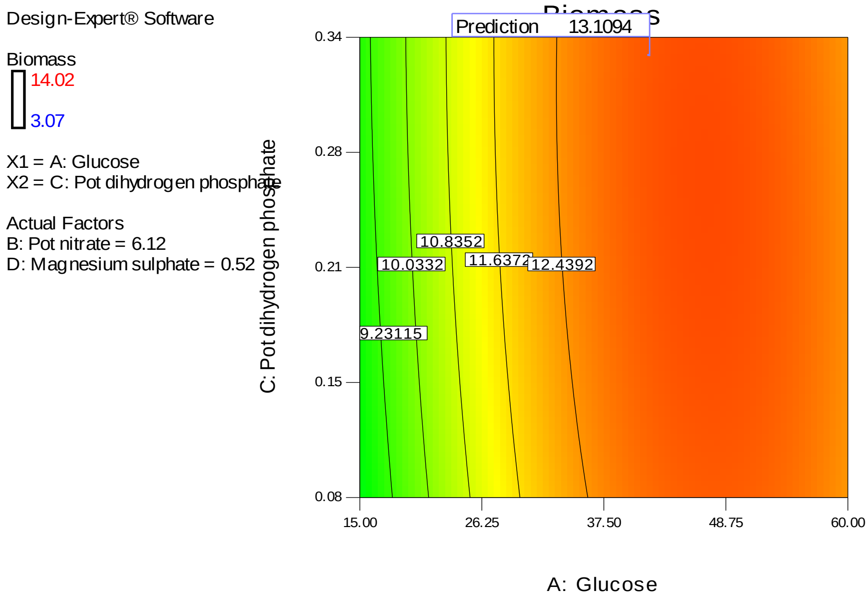
From the study of response surface contour plots, the maximum biomass accumulation and 'bacoside A' production was obtained when glucose, potassium nitrate, potassium dihydrogen ortho-phosphate and magnesium sulphate, were at a concentration of 41.72 g/l, 6.12 g/l, 0.33 g/l and 0.52 g/l, respectively. The developed model predicted a biomass of 13.02 g/l FW and 'bacoside A' content of 15.54 mg/g DW which was experimentally verified and yielded a biomass accumulation of 13.01 g/l FW and 'bacoside A' production of 15.44 mg/g DW. Thus, indicated a strong agreement between predicted and experimental value. In comparison with the pre-optimized media where biomass accumulation and 'bacoside A' production was 7.4 g/l FW and 10.23 mg/g DW, optimized media had enhanced biomass accumulation and 'bacoside A' production.

So, the optimal conditions for biomass accumulation and production of 'bacoside A' contained glucose= 41.72 g/l, potassium nitrate= 6.12 g/l, potassium dihydrogen ortho-phosphate = 0.33 g/l and magnesium sulphate= 0.52 g/l at pH 6.0, V_m/V_f of 0.12 and temperature of 25°C for incubation of 25 days. It was verified that optimized media led to 1.6 times fold increase in the biomass accumulation and 'bacoside A' production and has been undoubtedly proved that with the application of RSM, biomass accumulation and 'bacoside A' production were enhanced significantly.

(a)



(b)



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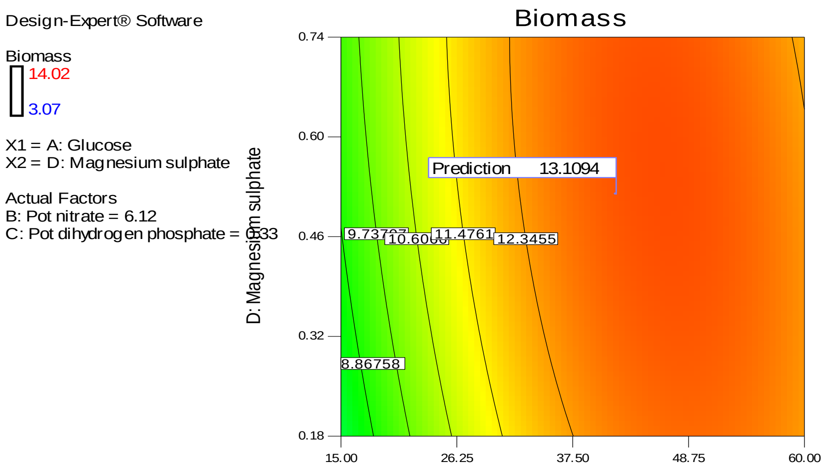


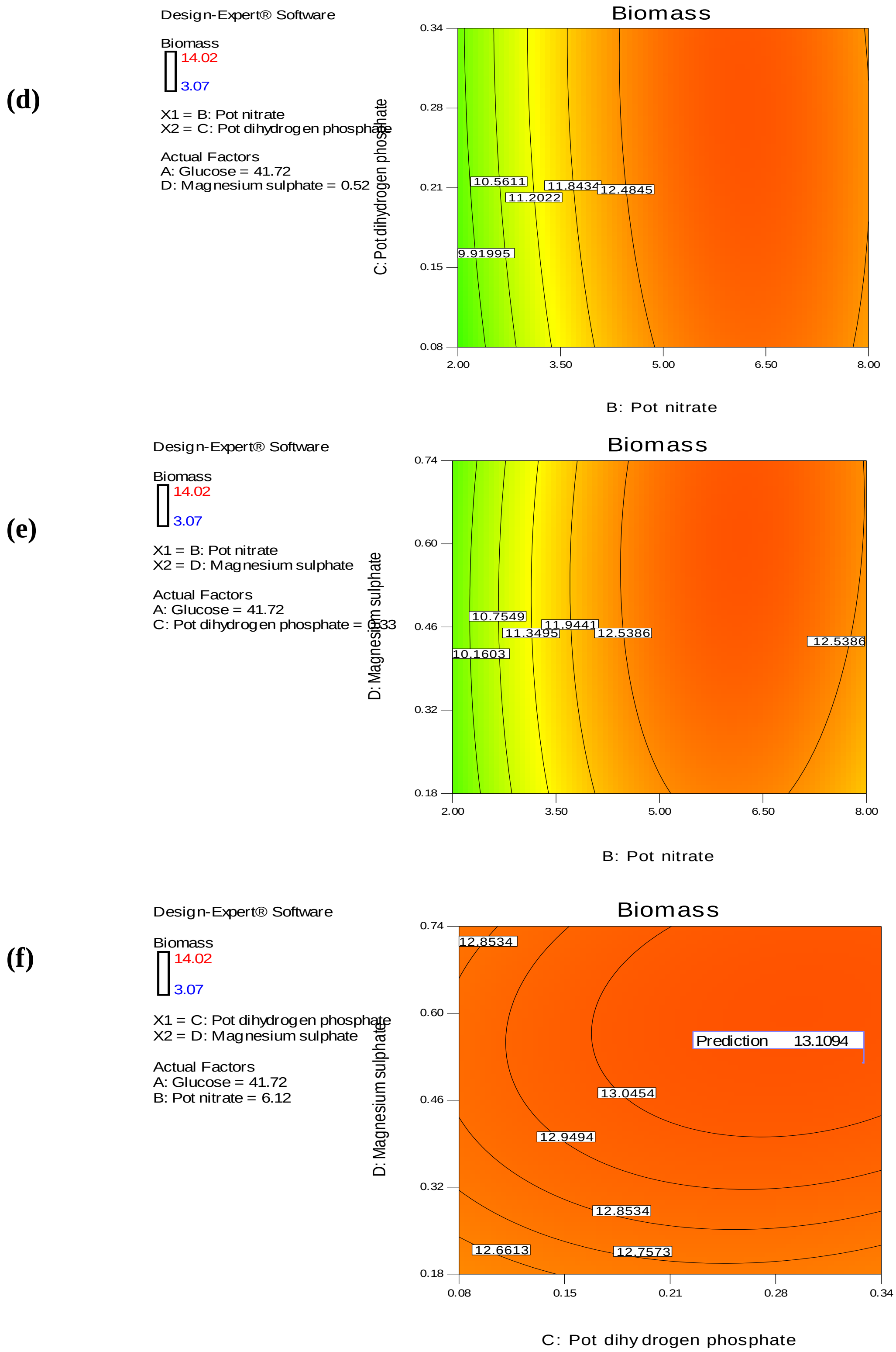
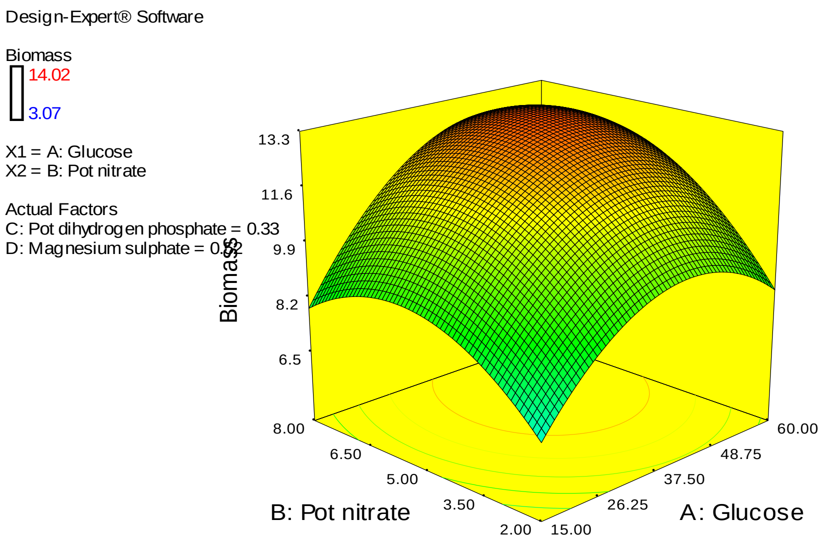
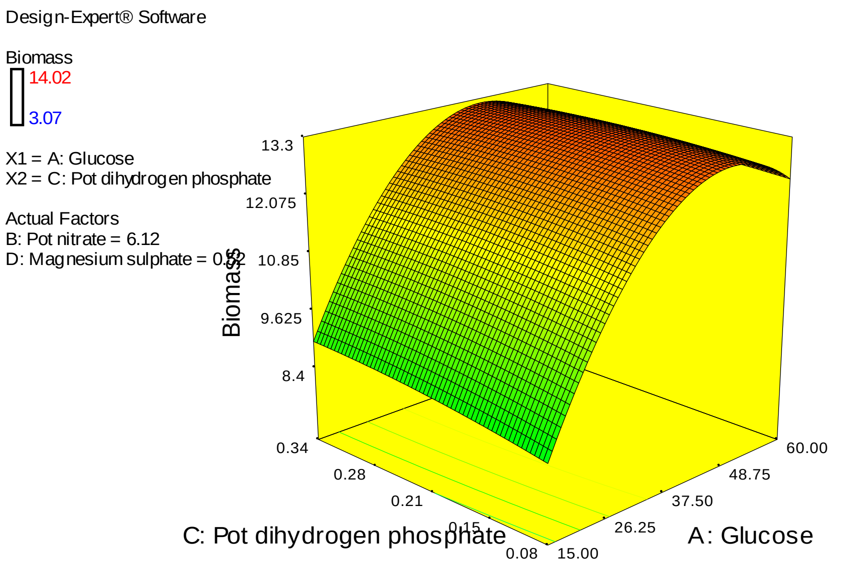


Fig.7.6 Contour plot between effect of (a) glucose and potassium nitrate (b) glucose and potassium dihydrogen ortho-phosphate (c) glucose and magnesium sulphate (d) potassium nitrate and potassium dihydrogen ortho-phosphate (e) potassium nitrate and magnesium sulphate (f) potassium dihydrogen ortho-phosphate and magnesium sulphate on biomass accumulation

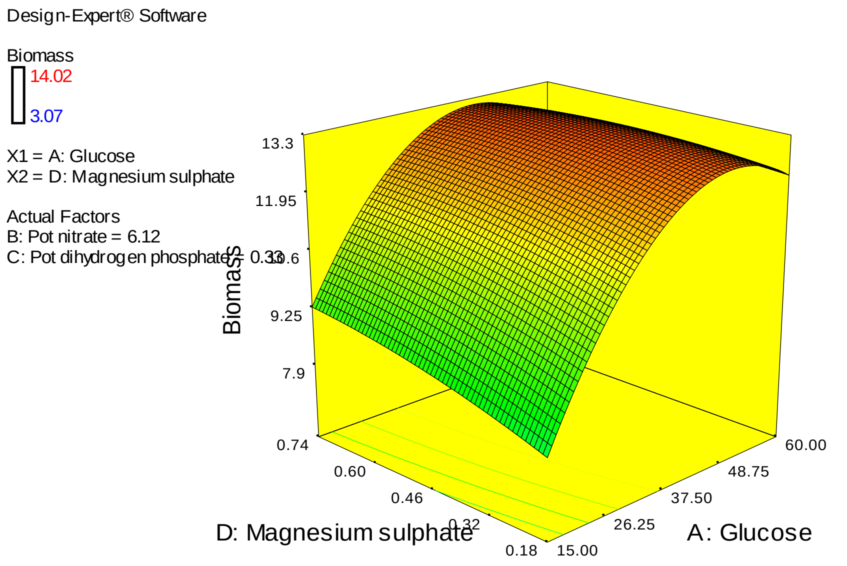
(a)


Design-Expert® Software
Biomass
14.02
3.07
X1 = A: Glucose
X2 = B: Pot nitrate
Actual Factors
C: Pot dihydrogen phosphate = 0.33
D: Magnesium sulphate = 0.52
Biomass
13.3
11.6
9.9
8.2
6.5
8.00
6.50
5.00
3.50
2.00
B: Pot nitrate
15.00
26.25
37.50
48.75
60.00
A: Glucose

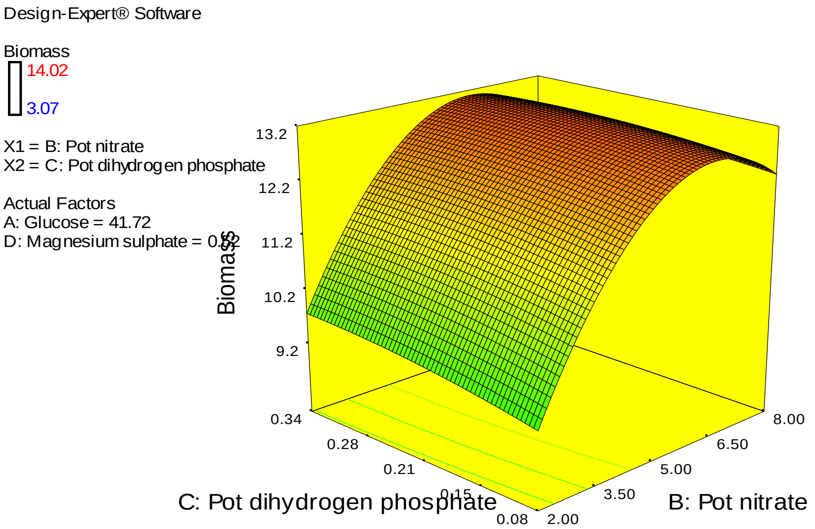
(b)


Design-Expert® Software
Biomass
14.02
3.07
X1 = A: Glucose
X2 = C: Pot dihydrogen phosphate
Actual Factors
B: Pot nitrate = 6.12
D: Magnesium sulphate = 0.52
Biomass
13.3
12.075
10.85
9.625
8.4
0.34
0.28
0.21
0.15
0.08
C: Pot dihydrogen phosphate
15.00
26.25
37.50
48.75
60.00
A: Glucose

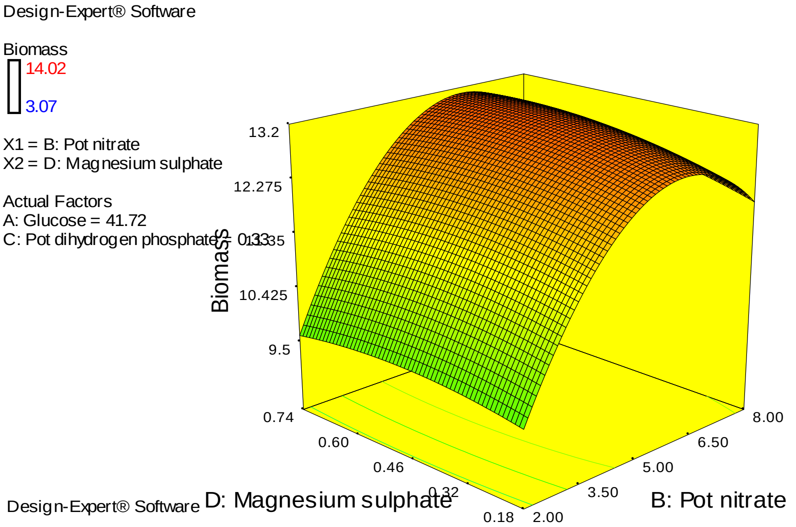
(c)


Design-Expert® Software
Biomass
14.02
3.07
X1 = A: Glucose
X2 = D: Magnesium sulphate
Actual Factors
B: Pot nitrate = 6.12
C: Pot dihydrogen phosphate = 0.33
Biomass
13.3
11.95
10.6
9.25
7.9
0.74
0.60
0.46
0.32
0.18
D: Magnesium sulphate
15.00
26.25
37.50
48.75
60.00
A: Glucose

(d)



(e)



(f)

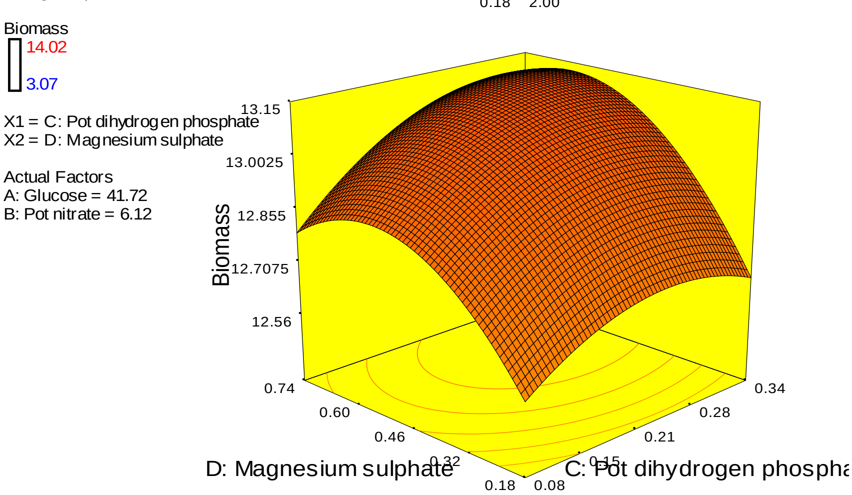
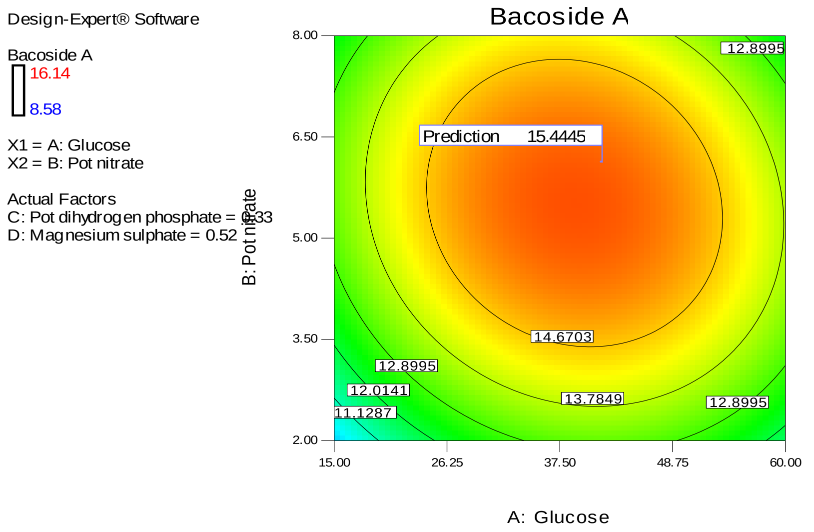
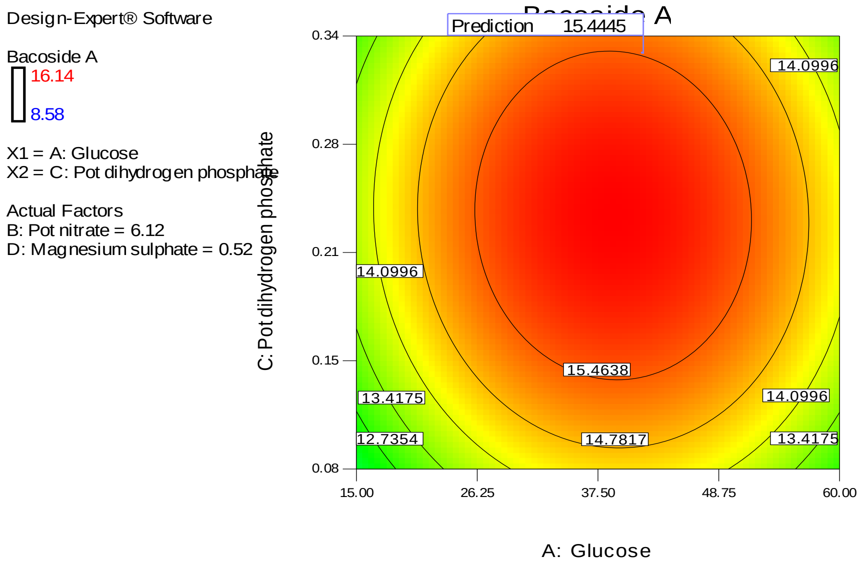


Fig. 7.7 Surface plot showing the effect (a) glucose and potassium nitrate (b) glucose and potassium dihydrogen ortho-phosphate (c) glucose and magnesium sulphate (d) potassium nitrate and potassium dihydrogen ortho-phosphate (e) potassium nitrate and magnesium sulphate (f) potassium dihydrogen ortho-phosphate and magnesium sulphate on biomass accumulation

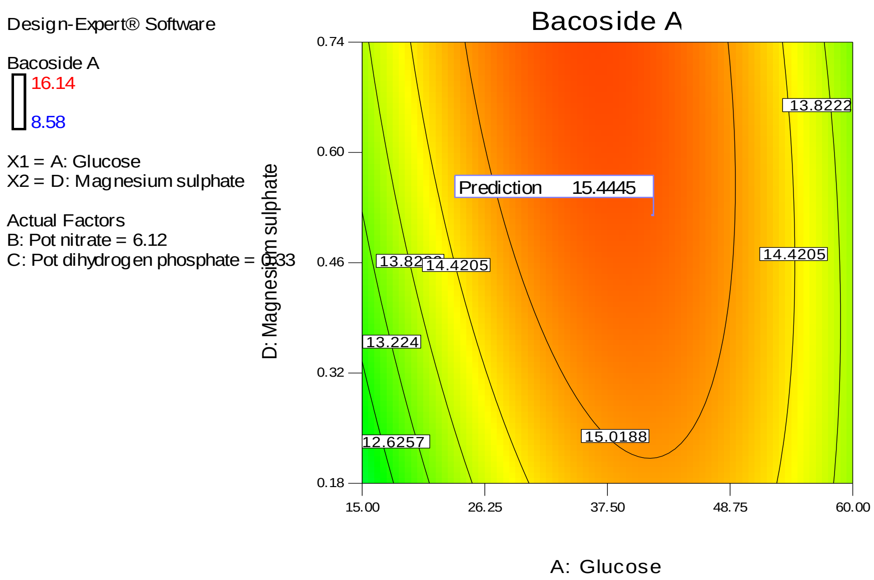
(a)


Design-Expert® Software
Bacoside A
16.14
8.58
X1 = A: Glucose
X2 = B: Pot nitrate
Actual Factors
C: Pot dihydrogen phosphate = 0.33
D: Magnesium sulphate = 0.52
Bacoside A
B: Pot nitrate
A: Glucose
Prediction 15.4445
12.8995
14.6703
13.7849
12.0141
11.1287
8.00
6.50
5.00
3.50
2.00
15.00
26.25
37.50
48.75
60.00

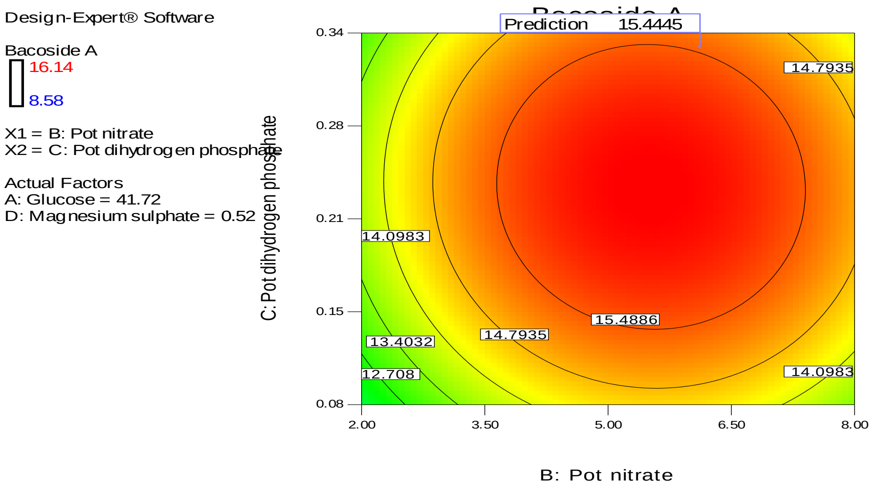
(b)


Design-Expert® Software
Bacoside A
16.14
8.58
X1 = A: Glucose
X2 = C: Pot dihydrogen phosphate
Actual Factors
B: Pot nitrate = 6.12
D: Magnesium sulphate = 0.52
Bacoside A
C: Pot dihydrogen phosphate
A: Glucose
Prediction 15.4445
14.0996
15.4638
13.4175
12.7354
14.7817
0.34
0.28
0.21
0.15
0.08
15.00
26.25
37.50
48.75
60.00

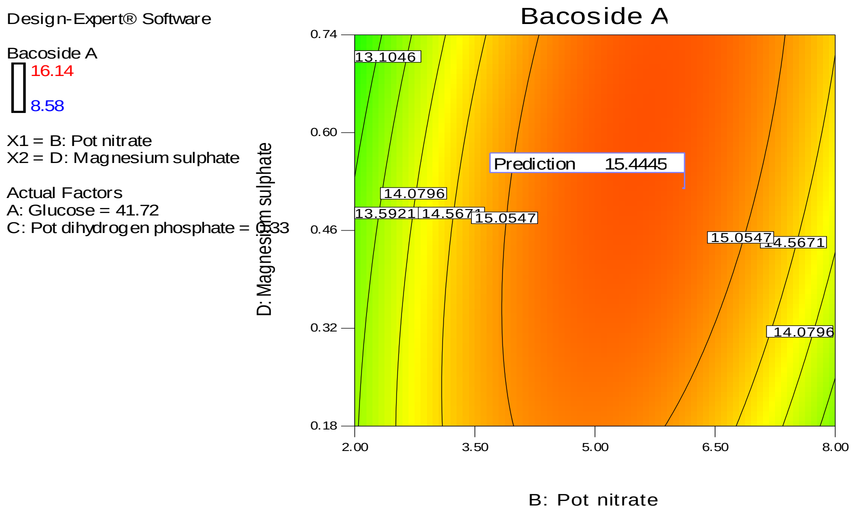
(c)


Design-Expert® Software
Bacoside A
16.14
8.58
X1 = A: Glucose
X2 = D: Magnesium sulphate
Actual Factors
B: Pot nitrate = 6.12
C: Pot dihydrogen phosphate = 0.33
Bacoside A
D: Magnesium sulphate
A: Glucose
Prediction 15.4445
13.8222
14.4205
13.224
12.6257
15.0188
0.74
0.60
0.46
0.32
0.18
15.00
26.25
37.50
48.75
60.00

(d)



(e)



(f)

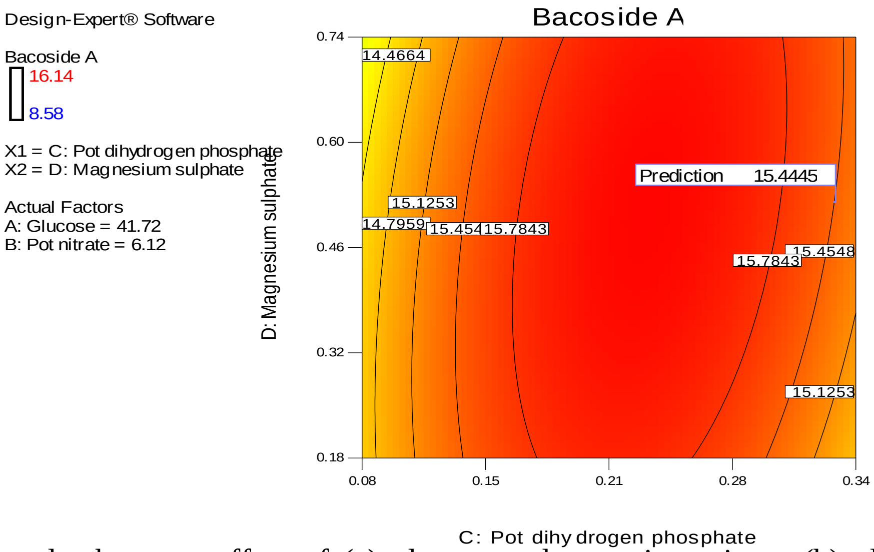
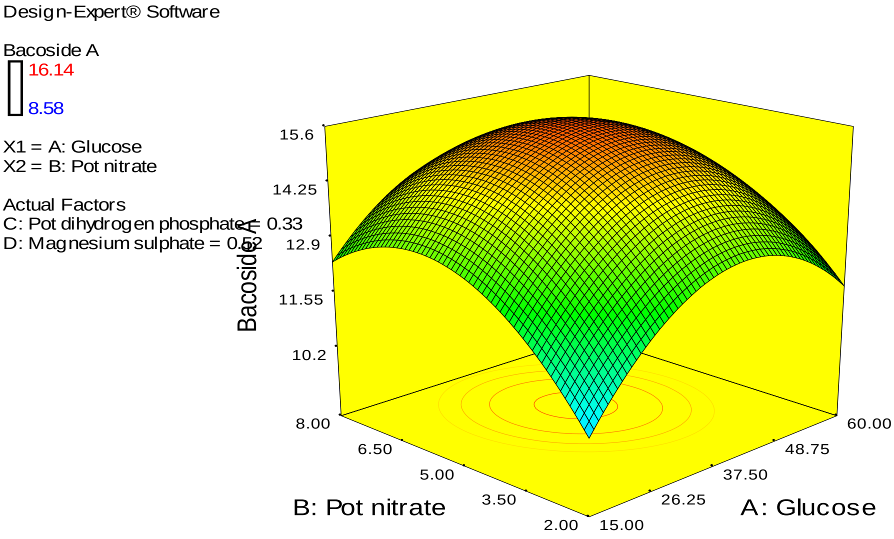
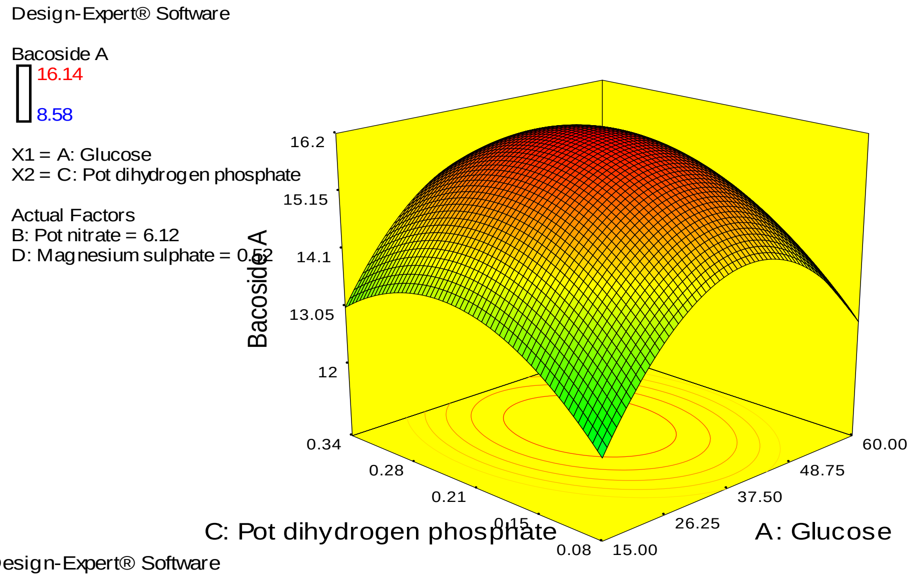


Fig.7.8 Contour plot between effect of (a) glucose and potassium nitrate (b) glucose and potassium dihydrogen ortho-phosphate (c) glucose and magnesium sulphate (d) potassium nitrate and potassium dihydrogen ortho-phosphate (e) potassium nitrate and magnesium sulphate (f) potassium dihydrogen ortho-phosphate and magnesium sulphate on 'bacoside A' production

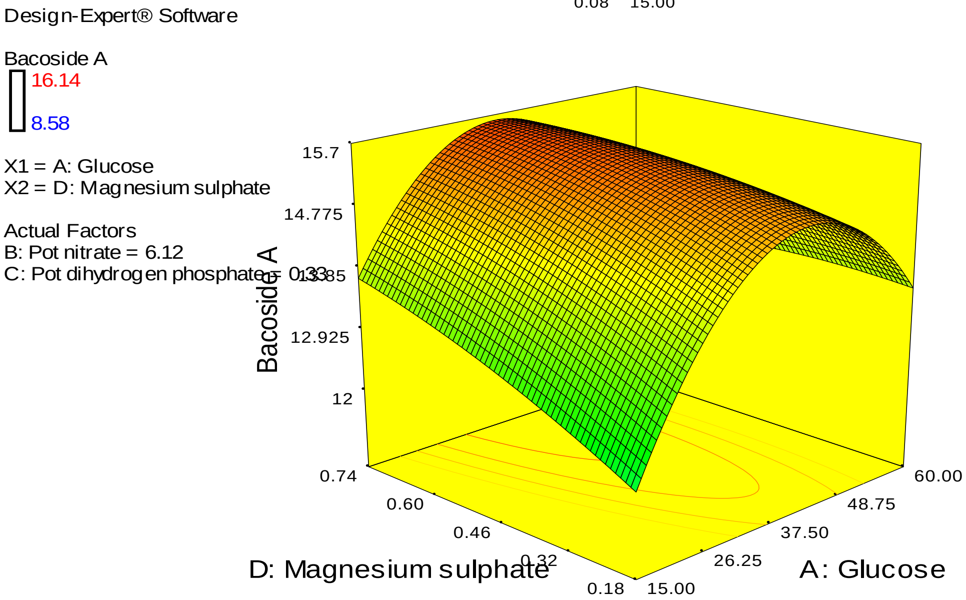
(a)


Design-Expert® Software
Bacoside A
16.14
8.58
X1 = A: Glucose
X2 = B: Pot nitrate
Actual Factors
C: Pot dihydrogen phosphate = 0.33
D: Magnesium sulphate = 0.52
Bacoside A
15.6
14.25
12.9
11.55
10.2
8.00
6.50
5.00
3.50
2.00
B: Pot nitrate
15.00
26.25
37.50
48.75
60.00
A: Glucose

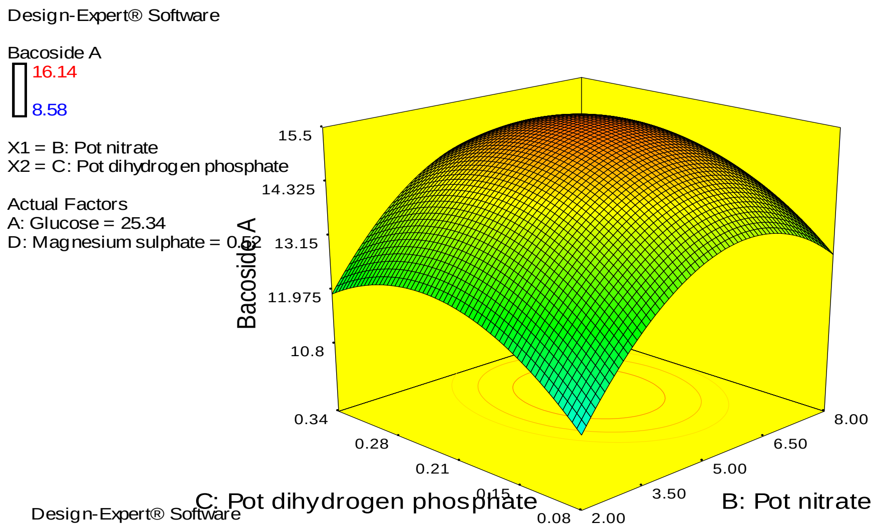
(b)


Design-Expert® Software
Bacoside A
16.14
8.58
X1 = A: Glucose
X2 = C: Pot dihydrogen phosphate
Actual Factors
B: Pot nitrate = 6.12
D: Magnesium sulphate = 0.52
Bacoside A
16.2
15.15
14.1
13.05
12
0.34
0.28
0.21
0.15
0.08
C: Pot dihydrogen phosphate
15.00
26.25
37.50
48.75
60.00
A: Glucose

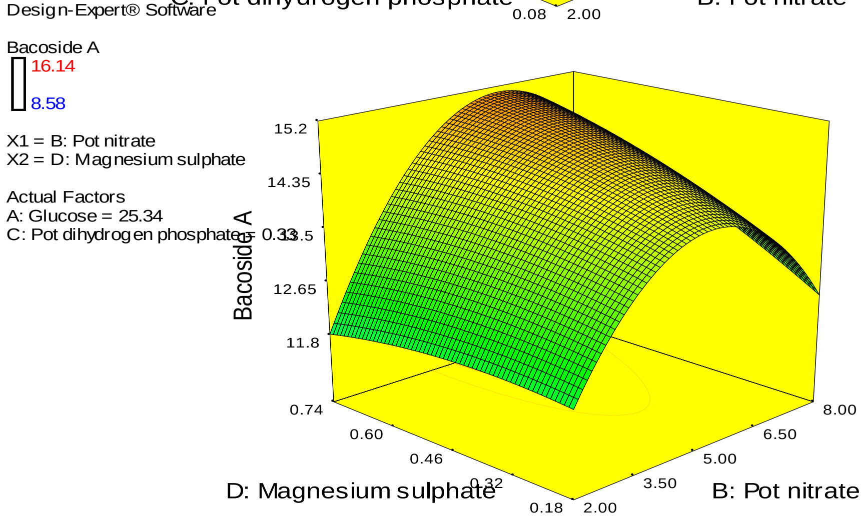
(c)


Design-Expert® Software
Bacoside A
16.14
8.58
X1 = A: Glucose
X2 = D: Magnesium sulphate
Actual Factors
B: Pot nitrate = 6.12
C: Pot dihydrogen phosphate = 0.33
Bacoside A
15.7
14.775
13.85
12.925
12
0.74
0.60
0.46
0.32
0.18
D: Magnesium sulphate
15.00
26.25
37.50
48.75
60.00
A: Glucose

(d)



(e)



(f)

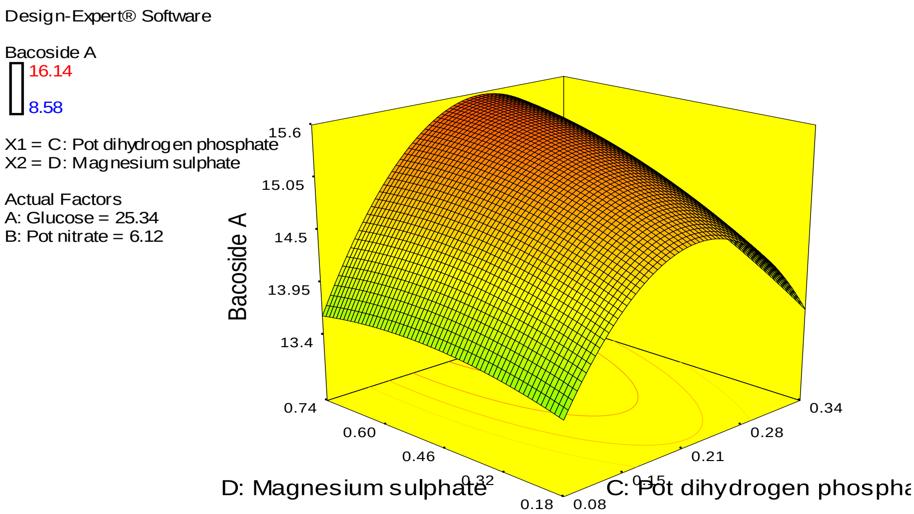


Fig. 7.9 Surface plot showing the effect of (a) glucose and potassium nitrate (b) glucose and potassium dihydrogen ortho-phosphate (c) glucose and magnesium sulphate (d) potassium nitrate and potassium dihydrogen ortho-phosphate (e) potassium nitrate and magnesium sulphate (f) potassium dihydrogen ortho-phosphate and magnesium sulphate on 'bacoside A' production

This work has highlighted the application of Response surface methodology by shedding light on fact that this process allows rapid screening of most important factors for biomass accumulation and 'bacoside A' production and reduces the number of experiments significantly. Combination of placket - burman and central composite design proved as an effective and reliable tool to study statistically significant factors and working out their optimal concentration within the medium for highest biomass accumulation and 'bacoside A' production.

Discussion

In the present study, different parameters affecting the biomass accumulation and levels of 'bacoside A' in the root cultures were studied. These included both chemical (carbon source, nitrogen source) as well as physical (medium volume to flask volume, pH and temperature) parameters. Also, statistical optimization tool response surface methodology (RSM) was used to optimize factors for the enhancement of biomass accumulation and 'bacoside A' content.

The medium volume/flask volume (V_m/V_f) ratio is an important parameter as biomass accumulation and secondary metabolite production depends on system aeration and agitation (Chen et al., 1999). The medium volume to flask volume (V_m/V_f) ratio was assessed for maximum biomass accumulation and 'bacoside A' production. The influence of medium volume/flask volume (V_m/V_f) ratio on the biomass and secondary metabolite production has also been highlighted earlier (Vadehra and Harmon, 1969; Elibol and Ozer, 2000). In the present investigation, increase in biomass and 'bacoside A' production was observed while decreasing the V_m/V_f ratio. It was found that V_m/V_f ratio of 0.12 resulted in maximum biomass accumulation and 'bacoside A' production. The increase in the biomass accumulation and 'bacoside A' production while decreasing the V_m/V_f ratio might be explained by the fact that a reduction in V_m/V_f increases the oxygenation of shake-flask cultures significantly because it increases the gas–liquid interfacial area (Amaral et al., 2007). Earlier, Srivastava and Srivastava (2012) also observed similar results in *Azadirachta indica* hairy root cultures, where reduction of V_m/V_f ratio increased the azadirachtin productivity. They found that a V_m/V_f ratio of 0.15 was optimum for maximum azadirachtin accumulation and production. Proper aeration promotes better uptake of nutrients from the liquid to the plant cells for the growth and secondary metabolite production (Chattopadhyay et al., 2002).

The growth profile of the hairy roots was studied in order to determine the period when maximum biomass accumulation and 'bacoside A' production occurs. The results showed that root biomass and 'bacoside A' content starts increasing on the fifth day of inoculation and reaches maximum on the 25th day. However, after 25th day, the growth rate of the hairy roots decreased gradually and began to fall into the growth plateau. The decline in 'bacoside A' content after 25th day of cultivation could be explained by a low production rate and by initiation of degradation process. The maximum biomass accumulation on the 25th day of culture had a positive effect on the secondary metabolite production and resulted in maximum 'bacoside A' production. Sudha et al. (2013) in their study on *Decalepis arayalpathra* also found that the concentration of MBALD compound was highest when the root growth was at log phase and with decline in the root growth, the production of compound also declined. Growth related production of secondary metabolites has also been reported in many other plants (Tiwari et al., 2007; Triplett et al., 2008). He-Ping et al. (2011) also observed similar result in *Pogostemon cablin* hairy root cultures, where maximum biomass accumulation was observed during log phase of culture, resulted in maximum patchouli essential oil production.

Hydrogen ion concentration (pH) of the medium is another important parameter for the growth of tissues and organ (Murthy and Praveen, 2013). Because the pH affects the uptake of nutrient from the medium to the plant, thus also affects the secondary metabolite accumulation in cultured cells and organs. The effect of initial medium pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) on biomass accumulation and 'bacoside A' production in the root cultures of *B. monnieri* was evaluated. The effect of medium pH on biomass accumulation and secondary metabolite production has been studied by many researchers (Naik et al., 2010; Nagella and Murthy, 2010; Murthy and Praveen, 2013). In the present investigation, highest accumulation of biomass and 'bacoside A' production was observed at pH 6.0. Higher and lower medium pH did not favoured biomass accumulation and 'bacoside A' production. These results are in line with the earlier findings of Praveen and Murthy (2013) on *Withania somnifera* root cultures. These authors reported that initial medium pH at 5.8 favoured maximum biomass accumulation and a medium pH of 5.5 resulted in high 'withanolide A' content. Sivakumar et al. (2005) also reported that the initial medium pH range of 6.0-6.5 favoured maximum root growth and ginsenoside production in *Panax ginseng* hairy root cultures. Whereas, Naik et al. (2010) reported the medium pH at 4.5 favoured maximum biomass accumulation and 'bacoside A' production in the shoot culture of *Bacopa monnieri.*

The effect of different carbon source on biomass accumulation and 'bacoside A' production in root cultures of *B. monnieri* was also studied. Because, sugars molecules are known to induce signals that affect the growth and development and metabolism of plants (Jang and Sheen, 1994; Rolland et al., 2006). Effect of sugars on the growth and development of the cultured cells have earlier been reported (Rolland et al., 2006; Wang and Weathers, 2007). In this study, glucose was found to be an ideal carbon source for high biomass accumulation and 'bacoside A' production in hairy roots of *B. monnieri* (Table 7.4). These findings are also accordant with the earlier findings of Weather et al. (2004) and Wang and Weather (2007) who verified the effect of sugars on artemisinin production. These authors showed maximum production of artemisinin when hairy roots were grown in medium supplemented with glucose.

The effect of nitrogen sources on the growth of hairy roots and production of 'bacoside A' was also studied. Nitrogen is reposted to play an important role in affecting the growth and production of a secondary metabolite (Forde and Clarkson, 1999; Wilson et al., 2008). Because, nitrogen induces nitrate reductase activity and nitrate transport, thus acts as a signaling molecule for the growth and production of metabolite (Coruzzi and Zhou, 2001). Therefore, two nitrogen sources namely, potassium nitrate (KNO_3) and ammonium nitrate (NH_4NO_3) was tested to find better nitrogen source. In the present investigation maximum biomass accumulation and 'bacoside A' production were achieved when KNO_3 was used as sole nitrogen source. The positive effect of KNO_3 in the induction of nitrate uptake and its reduction is well known (Forde and Clarkson, 1999). As, nitrate down regulates the key enzyme involved in starch biosynthesis pathway, thus diverting the carbon pool towards nitrogen assimilation pathway (Scheible et al., 1997). Earlier, Sivakumar et al. (2005) in their study on *P. ginseng* hairy roots reported the importance of KNO_3 as the nitrogen source for the root growth and ginseng production. Similarly, *Panax quinquefolium* showed inhibition of saponin and polysaccharide when ammonium was used as sole nitrogen source (Zhong, 1998).

RSM employed in this investigation suggested the importance of nutrient variables in biomass accumulation and 'bacoside A' production. The accuracy and applicability of RSM to optimize the biomass accumulation and 'bacoside A' production was observed with high similarity between the predicted and experimental results. The utility of RSM can be judged by 35 % increase in production of antibiotic was reported from *Streptomyces coelicolor* by using RSM (Elibol et al., 2004). Among six variables tested in this investigation, glucose, potassium nitrate, potassium dihydrogen ortho-phosphate and magnesium sulphate, at a concentration of 41.72 g/l, 6.12 g/l, 0.33 g/l and 0.52 g/l, were the major factors that influenced the biomass accumulation and 'bacoside A' production. The biomass and 'bacoside A' yield attained were higher than that at concentrations considered to be optimal by one variable at a time approach.

A higher level of 'bacoside A' was attained following optimization with RSM; this enhancement could be attributed to improved carbon and nitrogen source, maintenance of pH, medium to flask volume ration. Improvement in product yields could be due to better control of process parameters in the present study. A 1.6 times fold increase in the biomass accumulation and 'bacoside A' production in hairy roots of *B. monnieri* were achieved in shake flasks as a result of optimization of variables by RSM. Earlier, Satdive et al. (2007) reported a maximum increase of azadirachtin accumulation by nine-fold in the *A. indica* hairy roots by optimizing the growth medium and elicitation. However, in the present investigation elicitors were not used. Srivastava and Srivastava (2012) reported a 68 % enhancement in the azadirachtin production after medium optimization using RSM. Thus, application of response surface methodology has proved as an effective and reliable tool to study statistically significant factors and evaluating their optimal concentration within the medium for highest biomass accumulation and 'bacoside A' production.

Conclusion

The present work has culminated in production of higher biomass and ‘bacoside A’ content by the application of Response surface methodology. The superior root line induced by strain MTCC 2364 was used for optimization study. The physical and chemical factors which affected the biomass accumulation and ‘bacoside A’ production in root line of *B. monnieri* were firstly varied one at a time. Subsequently, plackett - burman design was used to screen multiple variables which affected biomass accumulation and ‘bacoside A’ production. Glucose, potassium nitrate, potassium dihydrogen ortho-phosphate and magnesium sulphate, were found to be most significant factors for the biomass accumulation and ‘bacoside A’ production. Based on results of central composite design, the optimized media and conditions for maximum biomass accumulation and ‘bacoside A’ production comprised glucose= 41.72 g/l, potassium nitrate = 6.12 g/l, potassium dihydrogen ortho-phosphate = 0.33 g/l and magnesium sulphate = 0.52 g/l at pH 6.0, V_m/V_f of 0.12 and temperature of 25 °C for incubation of 25 days which resulted in 60 % increase in the biomass accumulation and ‘bacoside A’ production in the optimized media.

Salient findings

- The V_m/V_f ratio of 0.12 was optimum for maximum biomass and ‘bacoside A’ production.
- The 25 d of culture period resulted in maximum biomass and ‘bacoside A’ production.

- Medium pH at 6.0 resulted in highest biomass accumulation and production of ‘bacoside A’.
- Glucose at a concentration of 3 % was found to be better carbon source.
- Among the different nitrogen source tested, higher biomass accumulation and ‘bacoside A’ production was found in MS liquid medium with 2g/l KNO_3.
- 1.6 folds increase in both the biomass accumulation and ‘bacoside A’ production in the optimized media.
- Glucose, KNO_3, KH_2PO_4 and $MgSO_4$, were found to be most significant factors for the biomass accumulation and ‘bacoside A’ production.

Chapter 8

Summary

The health care of humans via medicinal plants has been known since time immemorial. A considerable part of the traditional medicines has been formulated and documented into organised systems of medicines such as Ayurveda, Yunani, Sidha etc. Apart from the fact that plant with medicinal importance is a natural phenomenon, secondary metabolite production via plant cell culture by way of cell and organ culture, presents a promising approach for the enhancement of secondary metabolites.

B. monnieri herb is considered to be invaluable and irreplaceable medicinal plant with memory enhancing property. This herb is under threat of survival as the rate of use accelerates and regeneration has dropped. Also, natural processes, such as temperature, human activities etc. deteriorates the natural regeneration leading to decrease in 'bacoside A' production. So, there is need for conservation of *B. monnieri* herb through conventional and biotechnological tools which can overcome these problems.

The production of 'bacoside A' using cell and organ culture seems to offer potential for overcoming the growing demand of natural plant medicines. Though there are many biotechnological tools for secondary metabolites, but production of secondary metabolite via cell and organ culture represents a promising tool for various applications. The major factors responsible for making cell and organ culture favourable are: sustainable and assured production of secondary metabolites, easy isolation and purification of secondary metabolites from cell / organ culture, these cultures are biosynthetically totipotent and fast growth. All these benefits broaden the applications of cell/ organ culture for secondary metabolite production.

The present study is divided into two sections. The first part deals with the collection of *B. monnieri* accessions from different locations of India and characterization using biochemical, morphological and molecular markers while the second part focused on the applications of cell and organ culture for production of 'bacoside A' and optimizing the conditions for enhancing the production of 'bacoside A'.

As the quality of the raw material collected from the wild locations has been reported to show considerable variation in the active principle. So, we collected fourteen wild accession of *B. monnieri* from different locations of India for the selection of elite accession with higher growth and 'bacoside A' content. All the accessions were morphologically and biochemically characterized. Significant variation with respect to growth rate, harvest index and 'bacoside A' level was detected amongst accessions. Accession BM1 and BM7 showed maximum growth rate, harvest index and 'bacoside A' level and BM14 accession showed minimum. Time of harvest was also studied to select season with maximum growth and 'bacoside A' level. Summer was found to be appropriate season with maximum biomass and 'bacoside A' content. Accessions were also characterized using molecular markers (RAPD and ISSR primers). Data of the accessions was analyzed by Jaccard's coefficient and dendrogram was constructed by unweighted pair group method with arithmetic means (UPGMA) using MVSP (v 3.2.1). The Jaccard's similarity coefficient of 14 accessions of *B. monnieri* based on RAPD and ISSR revealed that similarity value among accessions ranged from 0.758 to 0.871, indicating moderate levels of genetic similarity. Maximum similarity value of 0.871 was recorded among BM9 and BM13. Dendrogram constructed using Jaccard's similarity coefficient, grouped accessions into two major clusters. Accession BM14 was placed separately as an out group. Two-component PCA analysis showed 55.0 and 8.7 % variation and BM14 was again separated out.

The shoot organogenesis and rooting capability of microshoots of all the accessions was compared. Maximum regeneration potential was recorded in case of accession BM6, where 96 % explants showed shoot organogenesis with an average of 39.8 shoots per explant. In case of accession BM3, only 36.3% explants showed shoot organogenesis. A minimum rooting efficiency of microshoots (93%) was recorded for accession BM5 and 100 % rooting of shoots was recorded in accessions BM 1, BM2, BM7, BM10 and BM14.

After characterizing all the accessions for growth and 'bacoside A' production, the most efficient accession with high growth rate and 'bacoside A' level, BM6 was selected for establishment of cell and organ culture to investigate the production of 'bacoside A'.

The next section targeted the production of 'bacoside A' using cell suspension cultures. Calli cultures were established on MS media supplemented with different combination of NAA and KIN. These calli cultures were used for the establishment of cell suspension cultures and were then investigated for the cell growth and 'bacoside A' production. Cell suspension cultures established on MS medium supplemented with NAA (5.0 µM) and KIN (1.15 µM) was found to be appropriate which harboured the capability of maximum cell growth (5.5 g/l FW and 1.78 g/l DW) and 'bacoside A' level (5.56 mg/g DW). The production of 'bacoside A' in higher amount than plants showed the potential of cell cultures. Though calli cultures of *B. monnieri* has been earlier reported by some workers, but our work is the first step in establishing the cell suspension cultures for production of 'bacoside A'.

This section targeted the establishment of hairy roots using different strains of *A. rhizogenes* strain and evaluates the 'bacoside A' production. Leaf was found to be better explant than the internodal segment. Also, factors influencing the root induction were also studied. Bacterial density of 0.6, 100µM acetosyringone in bacterial medium, infection time of 10 mins and co-cultivation period of 2 days enhanced the root induction frequency. Recognizably hairy roots induced by different *A. rhizogenes* strain under similar conditions were found to be morphologically as well as biochemically distinct. Biomass accumulation of roots induced by different bacterial strains was also found to be different. Roots induced by strain MTCC 2364 showed maximum biomass accumulation (6.8 g/l FW) and 'bacoside A' production (10.02 mg/g DW). This higher secondary metabolite production and fast-growth of hairy roots offers enormous possibilities for large-scale production of biomass in bioreactors and stable production for pharmaceutical and other important components.

'Bacoside A' production using cell suspension cultures is looked-for because these cultures are biosynthetically totipotent and are fast growing. Biotechnological tools were used to enhance the cell growth and 'bacoside A' production of cell suspension cultures. The effect of glucose and sucrose as carbon source on cell growth and 'bacoside A' production was studied. It was found that cell suspension cultures with glucose (at a concentration of 20g/l) showed maximum cell growth and levels of 'bacoside A'. The study showed that there was significant increase in levels of 'bacoside A' in glucose containing media. Also, the effect of different NO_3^-/ NH_4^+ ratio on cell growth and level of 'bacoside A' was evaluated. Amongst the different NO_3^-/ NH_4^+ ratio tested, NO_3^- alone (at a concentration of 60mM) as nitrogen source showed maximum cell growth and levels of 'bacoside A'. Various other physical and chemical parameters such as agitation speed, light illumination, medium pH and medium strength were found to influence cell growth and 'bacoside A' production. This study demonstrated the positive potential of these physical and chemical parameters in improving the cell growth and levels of 'bacoside A'. Agitation speed at 120 rpm, cultures under complete darkness, medium pH at 6.0 and full strength medium significantly enhanced the cell growth and levels of 'bacoside A'.

After investigating the physical and chemical parameters affecting the cell growth and 'bacoside A' production, the next aim was to optimize the medium components affecting cell growth and 'bacoside A' production by the application of Response surface methodology. In this part, four significant factors such as glucose, potassium nitrate, potassium dihydrogen ortho-phosphate and cell inoculum which effect the cell growth and 'bacoside A' production were determined by Plackett - Burman design. The optimal concentration of these most important factors governing the cell growth and 'bacoside A' production worked out using RSM and was found to be 56.74 g glucose, 31.37 mM potassium nitrate, 2.91 g potassium dihydrogen ortho-phosphate and 6.60 g cell inoculum which led to 2.5 -fold improvement in FCW and 1.7 -fold improvement in levels of 'bacoside A'.

Most of the studies on hairy root cultures in *B. monnieri* were carried out to evaluate the 'bacoside A' production in the root cultures. The evaluation of physical and chemical parameters in enhancing the biomass accumulation and 'bacoside A' production has not been studied. Thus, the effect of various physical and chemical parameters was studied so as to enhance the biomass accumulation and 'bacoside A' production in *B. monnieri*. The effect of medium volume to flask volume on biomass accumulation and levels of 'bacoside A' was studied. It was observed that biomass and levels of 'bacoside A' increased from 4.77 g/l FW and 0.88 mg/g DW to 7.4 g/l FW and 10.25 mg/g DW respectively, at a V_m/V_f ratio of 0.12. The effect of medium pH on biomass accumulation and 'bacoside A' production was also evaluated. The study showed that there was significant variation in biomass and levels of 'bacoside A' on medium with different pH. It was found that hairy root cultures cultured on medium at pH of 6.0 showed maximum biomass accumulation and 'bacoside A' production.

The carbon and nitrogen sources were tested on biomass accumulation and levels of 'bacoside A'. It was found that glucose as carbon source and potassium nitrate as nitrogen source significantly enhanced the biomass accumulation and 'bacoside A' production.

Finally a successful attempt was made to optimize medium nutrients for enhancing biomass and levels of 'bacoside A' by the application of Response surface methodology on MTCC 2364 induced hairy roots. Plackett – Burman design was used to select the medium components affecting response. In this part, four medium components which effect the biomass and 'bacoside A' production were found to be glucose, potassium nitrate, potassium dihydrogen ortho-phosphate and magnesium sulphate. The concentration of these medium nutrients was determined using central composite design. The concentration of these most important factors governing the maximum biomass accumualtion and 'bacoside A' production was found to be 41.72 g glucose, 6.12 g potassium nitrate, 0.33 g potassium dihydrogen ortho-phosphate and 0.52 g magnesium sulphate. The optimized media led to 1.6 fold increase in both the biomass and 'bacoside A' production.

'Bacoside A' has numerous applications in the management of a range of mental conditions including anti-cancer property. The application of cell suspension and hairy root cultures offered higher biomass accumulation and 'bacoside A' production. Present study has undoubtedly demonstrated the potential of callus and hairy root cultures in improving the 'bacoside A' production. This study has also shed light on importance of physical and chemical parameters on growth and 'bacoside A' production. This is also the first published study to achieve enhancement in the biomass and 'bacoside A' production in cell suspension and hairy root cultures via the application of Response surface methodology.

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Appendix I

(A) Luria broth

Ingredient	Quantity (g/l)
Tryptone	10.0
Yeast Extract	5.0
Sodium Chloride (NaCl)	10.0

Boiled to dissolve the medium completely, sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min, pH 7.0 ± 0.2

(B) Yeast mannitol broth

Ingredient	Quantity (g/l)
Yeast extract	1.0
Mannitol	10.0
Dipotassium phosphate	0.5
Magnesium sulphate	0.2
Sodium chloride	0.1
Calcium carbonate	1.0

Boiled to dissolve the medium completely, sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min, pH 6.8 ± 0.2

(C) CTAB Buffer

Ingredient	Quantity
CTAB	2 %
EDTA	20mM
Tris - HCl (pH 8)	100mM
NaCl	1.4M
ß-mercaptoethanol	0.2 %

Make up the volume to 1 litre. Sterilize by autoclaving.

(D) 10X TBE Buffer (Tris-borate-EDTA)

Ingredient	Quantity
Tris Base	108.0 g/l
Boric Acid	55.0 g/l
0.5M EDTA	40ml

Make up the volume to 1 litre. Sterilize by autoclaving.

(E) TE Buffer

Ingredient	Quantity
1M Tris-Cl (pH 7.4)	10 ml (10mM)
0.5M EDTA (pH 8)	2 ml (1mM)

Adujsted the volume 1 litre by distilled water.

(F) 3M Sodium Acetate

Ingredient	Quantity
Sodium acetate	408.24 g

Dissolve in 800 ml of distilled water. Adjust to pH 5.2 with glacial acetic acid and make the volume to 1 liter with distilled water.

(G) (1X) STET Buffer

Ingredient	Quantity
Tris - HCl	10mM
EDTA (pH 8)	1mM
NaCl	100mM
Triton X-100	5 % (v/v)

pH adjusted to 8.0 with 1N NaOH, sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

(H) Agarose Gel Loading Dye (6X)

Bromophenol blue	0.25 %
Xylene cyanol FF	0.25 %
Glycerol in water	30.0 %