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™ 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

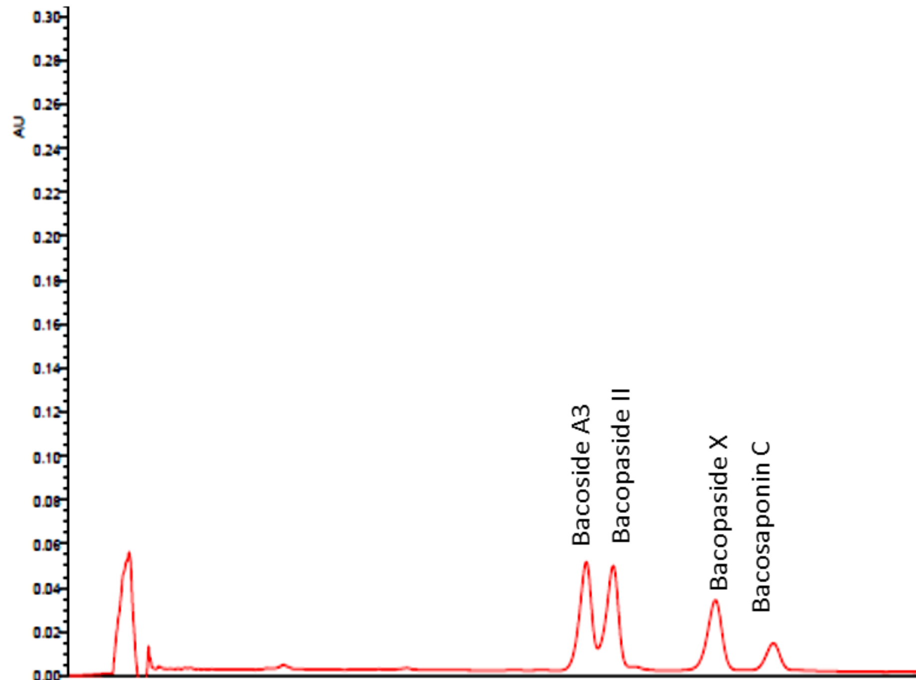
Plants from different populations were grouped based on bacosides content by principal component analysis (PCA) using loading plots (SPSS 16, IBM, Chicago, USA) to reveal pattern of relatedness within a matrix coordinates in two dimensions for each accession.

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.

a)



b)

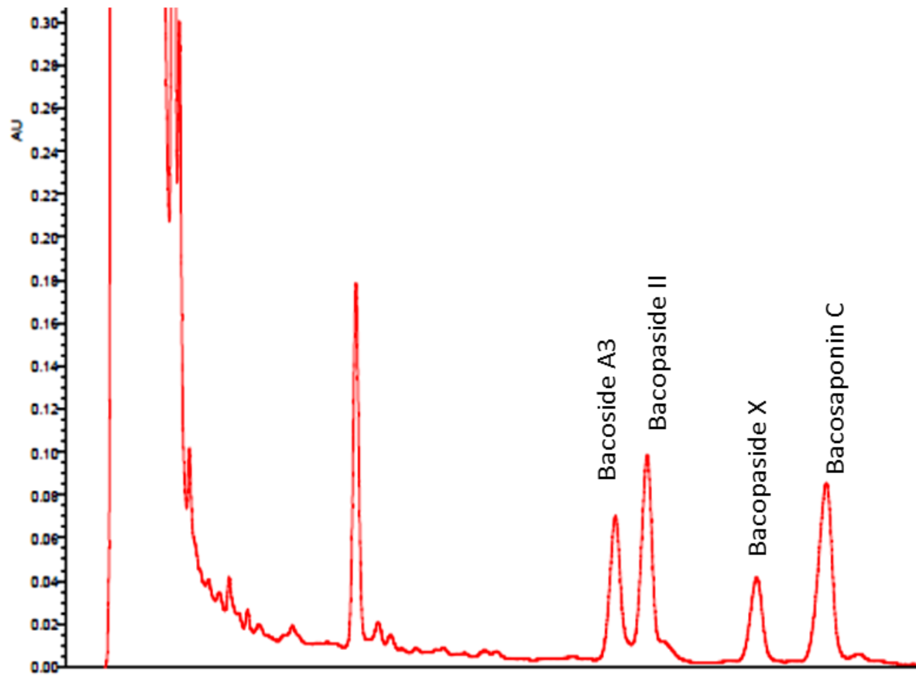


Fig. 3.2 HPLC chromatographs showing elution of 'bacoside A' components i.e. 'bacoside A3', 'bacopaside II', 'bacopaside X' and 'bacopasaponin C' in (a) standard (b) plant extract

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	$(GC)_8$ AT
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	$(GA)_8$ T
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 °C 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 °C 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 $P \leq 0.05$.