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.