CHAPTER 5

SUMMARY

Since the beginning of the mankind, plants have been used as a source for medicines and World Health Organization (WHO) reported that around 80% of the world's population still relies on plant based medicines. Worldwide requirement of herbal medicines is around $ 120 billion which estimated to reach $ 5 trillion by the year 2050 as reported by WHO. Reports suggested that more than 75% of the requirement is fulfilled by wild populations and this leads to overexploitation of threat to populations. India is one of the 12 mega biodiversity countries and around 7000-7500 medicinal plants are being used for treatment and better livelihood. But still 90% of the plants are collected from the wild and this indiscriminate harvesting creates gap between demand and supply of the plant material. National Medicinal Plant Boards (NMPB) listed total 178 species which are being consumed in volume exceeding 100 MT/year of which *Hemidesmus indicus* (L.) R. Br. (Asclepiadaceae) is one of the plant. It is commonly known as anantamula, sariva or Indian sarsaparilla, is widely used in Ayurvedic, Unani and Homoeopathic medicines and is also an official drug in Indian and British Pharmacopoeia. However the main source of supply is from wild populations as the seed viability and germination is poor and also the success rate of vegetative propagation is less. This increased threat on its natural population and reports documented that it has depleted and is now becoming rare in different parts of India. This leads to scarcity of raw materials for the industries which can be fulfilled by biotechnological technique like plant tissue culture.

Plants regenerate under *in vitro* conditions due to their totipotent nature and thus they aid in conservation and can also synthesize metabolites as mother plant. They are considered to be a potential alternative to wild plants for extraction of metabolites as they have less variation in metabolite content as compared to wild plants. However sometimes the *in vitro* synthesis of the metabolites is less which can be increased by treating the cultures with elicitors. Elicitation is an effective strategy for enhancement of secondary metabolites and amongst different types of elicitors, YE is commonly used biotic whereas SA and MJ are commonly used abiotic elicitors.

The present study has covered these aspects and shoot regeneration was achieved utilizing leaf as well as nodal explant of *H. indicus*. Simultaneously regeneration potential of *in vitro* nodes were also evaluated in cytokinin fortified medium. Further studies were carried out on shoot cultures regenerated from nodal explant as they were analyzed qualitatively using HPTLC fingerprint. These shoot cultures were also analyzed for lupeol quantity followed by elicitation of same using YE, SA and MJ was tried. Shoot cultures were also analyzed for rutin synthesis in control and treated shoots as well as gene

expression analysis for the same was also studied. Following conclusions were derived on the results of present study.

5.1 REGENERATION STUDIES

In vitro regeneration was achieved by placing leaf and nodal explants on MS media fortified with sucrose (3%) and different concentrations of cytokinins and auxins (individually and in combinations).

5.1.1 Regeneration using Leaf Explant

Individual cytokinins like BA and Kn when added individually in the medium which evoked only callus formation within eight weeks. Addition of individual auxins like IAA, NAA and 2,4-D also differentiated callus in 100% cultures as compared to cytokinin fortified media. After individual PGRs, combination of BA and Kn was tried but all the combinations induced meagre callusing. As these combinations induced only callus from leaf, the synergistic effect of cytokinins (BA and Kn) and auxins (IAA and NAA) was evaluated.

Combination of BA and IAA when tried, indirect organogenesis was observed at high level of BA (25 µM) with IAA (0.1-2 µM) within eight weeks. Optimum number shoots i.e. 19.67±0.81 with 100% response was observed in medium fortified BA (20 µM) and IAA (1 µM). Further increasing the concentrations failed to increase shoot number. Replacing IAA with NAA failed to differentiate shoots and only callus was formed in all the combinations. Replacing BA with Kn along with auxins also induced only callus within eight weeks and none of the combinations induced shoot buds.

5.1.2 Regeneration using Nodal Explant

Regeneration studies utilising nodal explants to establish shoot cultures in MS medium fortified with cytokinins (BA and Kn) and auxins (IAA and NAA) was also tried. In presence of individual cytokinins in the range of 5-20 µM induced only 2.00±0.20 shoots at 10 µM BA with 100% response. This shoot number decreased to 1.67±0.14 when Kn was used at same concentration. Similarly when auxins were used individually they failed to induce multiple shoots and only 2.17±0.31 shoots (83.33%) were formed in presence of IAA at 2 µM. Whereas NAA at 1 µM was able to form only 1.00±0.31 shoots in 50% cultures.

As both the cytokinins and auxins individually failed to induce multiple shoots, synergism of cytokinins as well as cytokinin and auxin were tried. When BA and Kn were combined, 100% cultures responded and optimum regeneration was observed with 11.00±0.24 shoots in medium fortified with BA (10 µM) and Kn (5 µM). This shoot number reduced to 5.17±0.88 (75%) in BA (10 µM) and IAA (0.5 µM). A further decrease in shoot number to 3.67±0.77 was observed in combination of BA (10 µM) and NAA (0.5 µM). When Kn with IAA and NAA were tried it induced poor response and a total of 4.25±0.65 shoots (83.33% response) were formed with Kn (10 µM) and IAA (1 µM), whereas only 3.25±0.45 shoots (83.33% response) were formed in Kn (20 µM) and IAA (0.5 µM).

Thus regeneration utilizing nodal explant revealed that medium fortified with BA (10 µM) and Kn (5 µM) (MS-1) induced optimum shoots. However shoots grown in medium fortified with IAA (2 µM) showed maximum leaf broadening and length of shoots. Thus shoots grown in MS-1 medium were also transferred for another eight weeks (MS-2) and phytochemical analysis was done in shoots grown in MS-1 (eight weeks old) and MS-2 (sixteen weeks old) in comparison to *in vivo* shoots.

5.1.3 Regeneration using *In Vitro* Nodes

As the presence of nodal explants was better in cytokinin fortified medium, potency of *in vitro* nodes to regenerate shoots was also evaluated. When BA and Kn were used individually, nodes failed to form multiples and maximum of only 1.83±0.16 shoots were observed in medium fortified with 10 µM of BA. However combining both cytokinins induced multiple shoot formation and optimum 5.42±0.36 shoots with 100% response was observed in BA (10 µM) and Kn (5 µM).

5.1.4 *In vitro* Rooting of Shoots

Regenerated shoots from leaf and nodal explant were utilized for rooting of shoots in three different strengths of MS medium i.e. full, half and quarter along with PGRs like IBA and NAA in the concentration range from 1-25 µM. Observations revealed that IBA is better for root induction in shoots as compared to NAA. IBA concentrations in full and half strength medium induced less roots and the number was increased to 8.83±0.28 with 100% rooting and shoot survival in IBA 8 µM. Changing IBA with NAA induced less roots and maximum 3.42±0.55 roots (91.67% response) with 100% shoot in full strength medium fortified with 8 µM NAA.

5.2 SECONDARY METABOLITE STUDIES

As shoots regenerated without callus intervention when nodes were used as an explants. However the shoots regenerated from optimized medium (MS-1) i.e. BA (10 µM) and Kn (5 µM) were subcultured to medium supplemented with IAA (2 µM) (MS-2) to observe the effect of media on metabolite synthesis.

5.2.1 HPTLC Fingerprinting and Lupeol Quantification in Shoots

HPTLC fingerprinting was done for three extracts viz. hexane, ethyl acetate and methanol and observations revealed that shoot cultures developed in optimized media (MS-1) as well as auxin fortified media (MS-2) showed variation in fingerprint. Lupeol is an important metabolite of *H. indicus* and when it was quantified in *in vivo* shoots it was recorded to be 185±0.00 µg/gm. However the content was increased to 187±0.01 µg/gm in shoots grown in MS-1 media which again reduced to 53±0.00 µg/gm in shoots grown in MS-2 media. Thus for elicitation of lupeol, shoots from medium MS-1 were utilized.

5.2.2 Elicitation of Lupeol

Three different elicitors like yeast extract (YE), salicylic acid (SA) and methyl jasmonate (MJ) were used for elicitation studies. Addition of biotic elicitor YE in the medium increased biomass as well as lupeol content in shoot cultures and 50 mg/l YE increased the lupeol content to 275.56±0.00 µg/gm as compared to control medium (260.58±0.00 µg/gm). Further increasing (100 and 200 mg/l) or decreasing (25 mg/l) the YE level failed to increase the lupeol in shoots. Using SA as an elicitor in the study increased the lupeol content optimally and 50 µM concentration of it enhanced the quantity to 335.40±0.04 µg/gm. Whereas when MJ was added in the media it adversely affected the shoots and they turned brown.

5.2.3 HPTLC Analysis for Rutin in Shoots along with Gene Expression Studies

Rutin is another important metabolite of *H. indicus* but when *in vitro* shoots from MS-1 and MS-2 were assessed it was observed that rutin was not synthesized in both samples. Mass spectra also revealed absence of quercetin in samples and hence the selected cultures were qualitatively analyzed for kaempferol which is precursor of quercetin. Thus elicitation studies were carried out using YE and SA and TLC plates showed present of band at same Rf as standard rutin at 254 nm. However change in spectra was observed in

in vivo as well as *in vitro* cultures when spectral analysis was done. This confirmed that rutin was not synthesized in *in vivo* as well as *in vitro* shoots even after treating the cultures with elicitor. However when qualitative analysis of upstream metabolite i.e. kaempferol was done, it was observed in all samples i.e. *in vivo* and *in vitro* shoots (MS-1) as well as shoots treated for three weeks with 50 mg/l YE and 50 µM SA.

Hence further analysis was done for expression of genes converting kaempferol to quercetin i.e. Flavonoid 3',5'-hydroxylase (*F3'5'H*) and flavonoid 3'-monooxygenase (*F3'H*), β-actin was used as a housekeeping gene. The analysis revealed amplification of β-actin gene however none of the primers amplify bands which may be due to change in gene sequence or absence of gene in *H. indicus*.