CHAPTER 5

SUMMARY

Medicinal plants are commonly utilized for the production of Ayurveda, Siddha and Unani medicines. As stated by the Government of India (GoI), traditional medicines are being used till date as sole means of health care for about 65% of the population (Srivastava, 2002). It is also reported that almost 85% of traditional formulations uses plants or plant extracts (Vieira and Skorupa, 1993), and their demand is enhancing rapidly throughout the world (Nalawade et al., 2003; Cole et al., 2007). World Health Organization (WHO) has postulated that the requirement of medicinal plants is approximately US $ 14 billion per year which will increase at the rate 15-25% annually, and will reach to more than US $ 5 trillion by 2050 (Sharma and Thokchom, 2014).

According to the International Union for Conservation of Nature (IUCN) and the World Wild Fund for Nature (WWF), 50,000 - 80,000 flowering plant species are used for medicinal purposes worldwide. These plant resources are being harvested in high volumes from natural habitats and in the recent decades it has nearly increased by 8-15% per year in different parts of the world (Ross, 2005; Bently, 2010). Thus, conservation of these important medicinal plants is imperative, and this can achieved through plant tissue culture technique. It is one of the most important tool which offers great potential for rapid propagation utilizing small plant parts as the starting material for cultures. These cultures play a vital role in the different areas such as breeding and genetic transformations (Sahai et al., 2010). *In vitro* plants can be obtained throughout the year irrespective of season through different pathways like indirect/direct oraganogenesis, somatic embryogenesis etc.

In vitro cultures are also an attractive alternative source to whole plant for the production of high-value secondary metabolites (DiCosmo and Misawa, 1995; Stockigt et al., 1995; Dornenburg and Knorr, 1997; Karuppusamy, 2009). Plants are known to synthesize secondary metabolites as a defense mechanism which have medicinal properties and thus are harvested in large amounts endangering and threatening these natural resources (Fujita et al. 2006; Sharifzadeh Naeini et al. 2020). Shoot cultures, callus cultures etc. have proved to synthesize similar secondary metabolites present in the mother plants but usually their content is low. Treating *in vitro* cultures with different precursors, elicitors etc. have been documented to be effective in enhancing secondary metabolite (Verpoorte et al., 1999; Smetanska, 2008).

Thus, in the present study as *in vitro* cultures of *L. reticulata* and *T. indica* were established using leaf and nodal explants by treating them with different PGRs. These cultures were further utilized for phytochemical analysis, in which qualitative analysis was done using

HPTLC fingerprint and quantification of the targeted metabolite *p*-coumaric acid in *L. reticulata* and lupeol in *T. indica* was carried out. The cultures were elicited using different elicitors treatments for these metabolites in respective plants. The conclusions of the present study are summarized below.

5.1 Development of *In vitro* Cultures for *L. reticulata*

In vitro cultures were established by placing leaf and nodal explants on MS media supplemented with 3% sucrose and different concentrations of individual and combinations of cytokinins and auxins.

5.1.1 Leaf Explants

Initially when explants were tried for the development of cultures, bacterial contamination hindered the growth of callus/shoots. Therefore, an antibacterial assay was performed using disk diffusion method and it was identified that tetracycline and streptomycin antibiotic showed clear zone of inhibition. When leaf explants were placed on the medium supplemented with individual cytokinins like BA and Kn showed that BA was beneficial for callus differentiation as compared to Kn. Whereas individual auxins i.e. IAA, NAA and 2,4-D gave better response in terms of callus formation in 60-70% cultures when compared to cytokinins.

When synergistic combinations of cytokinins and cytokinins with auxins were evaluated, they formed only callus and somatic embryos. Combinations like BA (20 µM) with NAA (0.5 µM) and BA (15 µM) with 2,4-D (1.0 µM) showed significant callus proliferation with high percent response. Synergistic combination of BA (15 µM) with Kn (5 µM) differentiated 20.90 ± 2.86 SEs in 90% cultures by end of eight weeks. Whereas another combination BA (15 µM) and $AdSO_4$ (15 µM) formed a maximum 21.70 ± 2.06 embryos in 100% cultures and BA-IAA together also formed SEs but the frequency was less.

Mature SEs were transferred to static and liquid media supplemented with different PGRs for further development. Among all PGRs tried, MS static medium fortified with 1 µM GA_3 proved to be beneficial for the plantlet development and elongation and in the same medium simultaneously secondary somatic embryos were formed directly as well as indirectly from the hypocotyls of in vitro plantlet.

5.1.2 Nodal Explants

Nodes were inoculated in media fortified with individual cytokinins (BA and Kn), 5 µM of BA formed 2.20 ± 0.28 shoots along with length of 2.95 ± 0.25 cm in 100% cultures by end of eight weeks. Whereas 2.33 ± 0.19 shoots achieved a length of 3.43 ± 0.36 cm in 60% cultures at 20 µM Kn. BA proved better for regenerating shoots as it showed higher percent response as compared to Kn, but in presence of Kn significantly shoot elongation was observed.

When synergistic combinations were tried, BA with Kn failed to improve number of shoots but this increased when BA was combined with $AdSO_4$. Both PGRs at 10 µM formed optimum number of shoots i.e. 5.10 ± 0.33 in 100% cultures having 3.74 ± 0.60 cm length at the end of eight weeks. Whereas Kn with $AdSO_4$ failed to increase the shoot number but they significantly improved the length in almost all concentrations and it became maximum (4.12 ± 0.68 cm) at 10 µM Kn with 5 µM $AdSO_4$. Shoot elongation was achieved by subculturing in medium supplemented with CW (4%).

5.1.3 Rooting of Shoots

Shoots regenerated from nodal explants were placed for root development in liquid full and half strength MS medium supplemented with IBA (2 and 4 µM). Full strength MS medium, with 4 µM IBA induced maximum number of roots (1.67 ± 0.27) which grew to 1.9 ± 0.05 cm length in 30% cultures by end of four weeks. Whereas half strength MS medium failed to increase the number of roots.

5.2 Phytochemical Analysis

In vitro callus/shoots regenerated from leaf/nodal explants were analyzed qualitatively and quantitatively. Further the cultures were treated with YE and SA for eliciting the marker compound *p*-coumaric acid in the culture.

5.2.1 HPTLC Fingerprint

HPTLC fingerprint was developed using three extracts i.e. hexane, ethyl acetate and methanol and observations revealed variation among all the samples (L1-L6). The biosynthetic potency of *in vitro* shoot cultures was similar with *in vivo* shoots, whereas callus cultures gave comparatively less number of bands. Thus, from this experiment it was

concluded that *in vitro* shoot cultures derived from nodal explants were chemically similar to *in vivo* shoots.

5.2.2 Quantitative Analysis

Standard curve for *p*-coumaric acid was prepared and HPTLC method validation was done according to ICH guidelines. Quantification was done for *p*-coumaric acid in different shoot and callus cultures using HPTLC, and highest quantity 78.08 ± 2.20 mg/gm was obtained in leaf derived callus culture developed in presence of BA (20 µM) + NAA (0.5 µM). In *in vivo* shoot sample, *p*-coumaric acid was not quantifiable.

5.2.3 Elicitation of *p*-Coumaric Acid

Cultures in which highest quantity of *p*-coumaric acid was obtained were utilized for elicitation by treating them with different concentrations of YE and SA. It could be concluded from the elicitation experiment that YE was beneficial for callus biomass as well as eliciting the *p*-coumaric acid content to 88.17 ± 2.72 mg/gm within four weeks in presence of 200 mg/l of YE. But in presence of SA at all concentrations, cultures failed to survive and died within two weeks.

5.3 Development of *In vitro* Cultures for *T. indica*

Leaf and nodal explants were utilized for the development of *in vitro* cultures with the help of MS media fortifying different PGRs in individual and combinations.

5.3.1 Leaf Explants

Individual cytokinins and auxins when tried, only callus was differentiated in presence of BA whereas Kn only induced swelling of explants. When cytokinins were replaced with auxins, IAA and NAA showed only swelling, whereas 2,4-D was able to form callus in all concentrations. Few synergistic combinations of PGRs i.e. BA/Kn with 2,4-D formed significant callus.

Other few combinations formed indirect shoots, like BA (10 µM) with Kn (15 µM) induced optimum of 22.50 ± 0.81 shoots in 80% cultures by the end of eight weeks. Whereas in BA (5 µM) with $AdSO_4$ (20 µM) formed 8.00 ± 0.63 shoots in 50% cultures and in BA-IAA almost similar number of shoots were recorded.

Many combinations differentiated somatic embryos in which BA (10 µM) with $AdSO_4$ (10 µM) formed 9.50 ± 0.35 embryos by end of eight weeks. When BA was combined with IAA it failed to improve the number of embryos. Frequency of embryos increased to 16.75 ± 2.53 in presence of BA (10 µM) with NAA (0.1 µM). Maximum (26.00 ± 1.13) SEs was differentiated in 100% cultures when Kn (5 µM) was combined with IAA (0.1 µM). When IAA was replaced with NAA, it also gave significant number of embryos almost in all concentrations and formed 24.38 ± 2.14 embryos with 80% response in presence of Kn (5 µM) with NAA (1.0 µM).

SEs which germinated into plantlet were transferred to static and liquid media containing different PGRs combinations and for further development. Among them liquid medium containing 1 µM GA_3 proved to be beneficial for development of healthy plantlets at the end of four weeks.

5.3.2 Nodal Explants

When nodal explants were placed in the medium supplemented with individual cytokinins, BA (5 µM) was able to form only 1.40 ± 0.15 shoots and number remained similar in all the concentrations. Whereas in Kn (15 µM) there was slight increase in number (1.60 ± 0.15) but significant increase in length (5.92 ± 1.44 cm).

Synergistic combinations of cytokinins when evaluated, effect of BA (10 µM) with Kn (5 µM) resulted in multiple shoots (4.10 ± 0.22) in 100% cultures at the end of eight weeks. BA and $AdSO_4$ failed to improve the number of shoots but it showed highest length 7.05 ± 1.16 cm of shoot in presence of 10 µM of BA and $AdSO_4$. Along with this the combination also induced indirect shoot regeneration from the basal callus formed at the nodes. When Kn was combined with $AdSO_4$, it differentiated embryogenic callus which further appeared with number of SEs.

5.3.3 Rooting of Shoots and Hardening

Rooting was induced in shoots obtained from leaf/nodal explants using two different strength of liquid MS medium fortified with different concentrations of IBA and NAA. Quarter strength of medium with 4 µM of IBA formed an optimum of 9.75 ± 0.74 roots (80% response) with 3.11 ± 0.33 cm length at the end of four weeks. NAA formed less number of roots as compared to IBA but it simultaneously differentiated SEs (15.4 ± 1.82) from the intermodal portions of shoot in half strength.

Plantlets derived through SEs and rooted shoots were transferred to cups filled with mixture of cocopeat:sand (1:1) for hardening. Healthy plants grown in the field formed flowers and fruits after 90 days.

5.4 Phytochemical analysis for *T. indica*

In vitro shoots regenerated from leaf and nodal explants were analyzed qualitatively and quantitatively. These cultures were treated with YE and SA for the eliciting the marker compound lupeol in the culture.

5.4.1 HPTLC Fingerprint

Fingerprint was developed using three different extracts i.e. hexane, ethyl acetate and methanol for different samples, and densitometric analysis showed variation in number of bands and peak areas in all samples (T1-T7). The chemical profile exhibited that *in vitro* samples had the potency to synthesize similar metabolites like *in vivo* plants. Chromatogram also showed variation in peaks in different samples from which unique peaks were identified with respect to particular sample.

5.4.2 Quantitative Analysis

Quantification was done for lupeol using different samples (T1-T7), all samples were able to synthesize the same. Maximum content of 254.16 ± 6.90 µg/gm was synthesized by nodal derived shoot cultures (T7) developed in presence of Kn (10 µM) + $AdSO_4$ (15 µM). Quantity of lupeol was 10.40 fold higher in T7 as compared to *in vivo* plants which showed 24.43 ± 1.28 µg/gm content. SEs derived hardened plant (T4) were also able to synthesize a high content (73.77 ± 4.14 µg/gm) when compared to *in vivo* plant.

5.4.3 Elicitation of Lupeol

Elicitation was performed using cultures developed in MS medium fortified with Kn (10 µM) + $AdSO_4$ (15 µM) and with different concentrations of YE and SA. Higher biomass was obtained in 200 mg/l of YE treated cultures of six weeks. Lupeol yield was 1.65 fold higher in 25 mg/l YE compared to control after four weeks. Rest all concentrations also showed higher content in four weeks as compared to control.

When SA was tried, the biomass obtained was less as compared to control. But the lupeol yield was maximum in 25 µM of SA at six weeks i.e. 584.26 ± 8.14 µg/gm which was

1.28 fold higher compared to control. This content was high among all concentrations of SA and YE.