ABSTRACT

Introduction: Medicinal plants are commonly utilized for the production of Ayurveda, Siddha and Unani medicines. 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 of 15-25% annually, and will reach to more than US $ 5 trillion by 2050. 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. Thus, conservation of these important medicinal plants is imperative, and this can be 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. *In vitro* plants can be obtained throughout the year irrespective of season through different pathways like indirect/direct organogenesis, somatic embryogenesis etc. Shoot and callus cultures 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.

Materials and Methods: The present study focuses on two important medicinal plants of Asclepiadaceae family i.e. *Leptadenia reticulata* (Retz.) Wight and Arn and *Tylophora indica* (Burm. F.) Merrill. The experimental work is divided into two parts: Development of *in vitro* cultures and phytochemical analysis. Firstly to establish regeneration protocol utilizing leaf and nodal explants for both the plants using different cytokinins (5–20 μM) like BA, Kn and AdSO4 and auxins (0.1–1.0 μM) like IAA, NAA, 2,4-D. Then rooting was achived for shoots in different MS media strength with PGRs and were further hardened and acclimatized. The qualitative and quantitative analysis was done for selected secondary metabolites in different *in vitro* cultures using HPTLC. Lastly *in vitro* cultures were treated with different elicitors like yeast extract (25-200 mg/l) and salicylic acid (25-200 μM) for the enhancement of targeted metabolites in both the plants.

Results: In *L. reticulata* development of cultures and regeneration was attempted using leaf and nodal explants in MS medium fortified with sucrose (3%) and different cytokinins and auxins. Through leaf explants total 21.70 ± 2.06 somatic embryos were recorded in 100% cultures in BA (15 μM) with $AdSO_4$ (15 μM). Further these embryos were transferred to static medium augmented with GA_3 (1 μM) which facilitated development of plantlets within eight

weeks. The nodal explants in presence of BA (10 µM) with $AdSO_4$ (10 µM) formed total 5.10 ± 0.33 shoots (100% response). The 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. In phytochemical analysis, callus and shoot cultures derived through leaf and nodal explants respectively were further qualitatively and quantitatively analyzed for their biosynthetic potential. HPTLC fingerprints of the samples confirmed that the *in vitro* shoots showed almost similar banding patterns in comparison with *in vivo* shoots in terms of number of peaks and bands on TLC plate, whereas in callus samples the banding pattern was differed. Further quantification of *p*-coumaric acid revealed that in *in vivo* and *in vitro* shoots were in LOD (10 µg) range but out of LOQ (100 µg). The content of this metabolite was 78.08 ± 2.20 mg/gm in six weeks old callus cultures obtained from BA (20 µM) + NAA (0.5 µM) fortified medium, and it can be a chemical marker for *L. reticulata*. 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 was concluded 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, which was 1.13 fold higher than *in vitro* callus sample of six weeks. But in presence of SA at all concentrations, cultures failed to survive and died within two weeks.

Tylophora indica (Burm. F.) Merrill is an another important medicinal plant of Asclepiadaceae family. Regeneration was attempted using leaf and nodal explants in Murashige and Skoog's (MS) medium fortified with sucrose (3%) and different concentrations of cytokinins and auxins resulted in different modes of regeneration i.e. organogenesis and somatic embryogenesis. In leaf explants an optimum 22.50 ± 0.81 shoots regenerated via indirect organogenesis in presence of BA (10 µM) and Kn (15 µM) in 80% cultures. Many of the synergistic combinations induced embryogenic calli and optimum 26.00 ± 1.13 embryos were recorded in media fortified with Kn (5 µM) and IAA (0.1 µM). Embryos developed into plantlets (9.38 ± 0.35 cm length) when transferred to liquid medium augmented with GA_3 (1 µM) within four weeks. Nodal explants formed multiple shoots (4.10 ± 0.22) with 100% response by eight weeks in a synergistic combination of BA (10 µM) and Kn (5 µM). Simultaneously the nodal derived callus differentiated shoot buds and somatic embryos in presence of BA (15 µM) with $AdSO_4$ (10 µM) and Kn (10 µM) with $AdSO_4$ (15 µM) respectively. An optimum number of roots/shoots (9.75 ± 0.74) were induced in ¼MS

medium supplemented with 4 µM of IBA. Rooting studies revealed that ½MS media fortified with NAA (4 µM) differentiated embryos (15.4 ± 1.82) simultaneously with root formation. Plantlets which developed either through organogenesis or somatic embryogenesis were transferred for hardening in cocopeat:sand (1:1) under laboratory condition, then to greenhouse and later to field with 100% survival. *In vitro* shoots regenerated from leaf and nodal explants were analyzed qualitatively and quantitatively. 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. 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. Quantification was done for lupeol using different samples, and all samples were able to synthesize the same. Maximum content of 254.16 ± 6.90 µg/gm was synthesized by nodal derived shoot cultures developed in presence of Kn (10 µM) + $AdSO_4$ (15 µM). Quantity of lupeol was 10.40 fold higher in *in vitro* nodal derived shoots as compared to *in vivo* shoots showed 24.43 ± 1.28 µg/gm content. Elicitation was performed using cultures developed in medium which had highest lupeol content and treated 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 and YE treated samples. 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 as compared to control of six weeks. This content was highest among all concentrations of SA and YE.