Summary

Conclusion
SUMMARY AND CONCLUSIONS

India is one of the richest country for medicinal and aromatic plant genetic resources in the world. The genetic diversity of medicinal plants in the world is getting endangered due to ruinous harvesting practices and over harvesting for production of medicines. Conservation of genetic diversity in plant species used in traditional medicine and health care has become increasingly important both in terms of adding economic value for biological resources and creating an economic stake for future domestication and cultivation. Advanced biotechnological methods of culturing plant cells and tissues have provided new means for conserving and rapidly propagating valuable, rare and endangered medicinal plants.

The objectives of the present study was to develop an efficient and rapid in vitro propagation protocols of two medicinally and aromatically important plants species viz., Mucuna pruriens and Ocimum basilicum using tissue culture techniques. Changes in photosynthetic parameters and antioxidative enzymes were also measured during the ex vitro acclimatization of micropropagated plantlets.

6.1. Mucuna pruriens L. (DC.)

Mucuna pruriens (Fabaceae) commonly known as velvet bean is an important tropical legume found in bushes and hedges at damp places, ravines and scrap jungles throughout the plain of India. All parts of M. pruriens possess valuable medicinal properties. Roots are useful to relieve constipation, dropsy, ulcers and fever. The report on the occurrence of the catecholic amino acid 3-(3,4-dihydroxy phenyl)-l-alanine (L-Dopa) attracted attention for utilization of the plant for L-Dopa production. L-Dopa, a neurotransmitter precursor, has
found wide application for symptomatic relief of Parkinson’s disease and mental disorder.

In nature, this species propagate only through seeds. However, propagation via seed poses problems due to high allergic properties of pods that cause uncontrolled itching while handling the seeds, and low germinability with poor viability. Thus, conventional propagation through seeds is not an adequate solution to meet the demand for this plant. Germination percentage was significantly enhanced (82.5%) under in vitro conditions on half strength MS basal medium. Addition of GA\textsubscript{3} could not improve the germination percentage (Data not shown).

Direct multiple shoot regeneration was achieved from cotyledonary node (CN) and nodal segment explants cultured on BA, Kin, TDZ and 2-iP either alone or in combination with auxins (IAA and NAA). CN and nodal explants were excised from 7 and 15 days old aseptic seedlings, respectively. Among the different concentration of cytokinins tested, BA (5.0 μM) showed the highest shoot regeneration frequency (81%) and number of shoots (8.5 ± 0.57) from nodal explants after 4 weeks of culture. Superiority of BA over other cytokinins was observed. Addition of NAA (0.5 μM) enhanced the shoot regeneration and MS medium supplemented with BA (5.0 μM) and NAA (0.5 μM) produced the highest frequency of shoot regeneration (91%), highest number (16.8 ± 1.50) and longest shoots (5.3 ± 0.32 cm) from nodal explants.

The effect of thidiazuron (TDZ) was also investigated on multiple shoot induction from both CN and nodal explants. The highest shoot regeneration frequency (77%) and number of shoots (5.7 ± 0.28) with 3.4 ± 0.43 cm shoot length was recorded on MS medium amended with 0.8 μM TDZ from nodal explants after 4 weeks of culture. However, the cultures grown continuously on TDZ containing media formed fasciated and distorted shoots. Therefore, the shoots induced from TDZ were subcultured on TDZ free MS medium.
number of shoots and shoot length increased after every subculture passage and showed no sign of decline even after fifth passage.

Nodal explants were also cultured on liquid MS medium containing TDZ at higher concentrations for different durations followed by their transfer to semi solid MS basal medium. The optimal level of TDZ supplementation to the culture medium was 50 μM for 8 days induction period followed by their transfer to MS semisolid medium devoid of TDZ where maximum regeneration frequency (80%), number of shoots (12.0 ± 1.76) and shoot length (4.6 ± 0.43 cm) per explant was recorded.

The effect of different basal medium (MS, ½MS; B5, ½B5; L2 and ½L2) and different pH levels (4.5, 5.0, 5.4, 5.8, 6.2 and 6.5) was also examined with optimal concentration of BA (5.0 μM) and NAA (0.5 μM). The half strength MS medium and 5.8 pH was found to be most suitable for maximum shoot induction and proliferation as it produced 23.3 ± 1.50 shoots per explant with maximum shoot length of 6.2 ± 0.37 cm from nodal explants after 8 weeks of culture.

The in vitro regenerated shoots produced roots when transferred to full, half and half strength MS medium supplemented with different concentrations of IAA, IBA and NAA. The best condition for rooting was half strength MS medium supplemented with 2.0 μM IBA. Rooting was also carried out by ex vitro method. The best results for rooting was recorded when shoots were dipped in IBA (200 μM) as it gave the maximum frequency of rooting (84%), number of roots (5.4 ± 0.51) and root length (4.6 ± 0.40 cm) after 4 weeks of transplantation. The in vitro raised plantlets with well developed shoots and roots were successfully acclimatized in plastic pots filled with sterile soilrite with 100% survival rate and about 90% plantlets survived in greenhouse.

Changes in photosynthetic parameters viz., chlorophyll a, b, carotenoid and net photosynthetic rate and antioxidative enzymes i.e. superoxide
dismutase (SOD) and catalase were evaluated at 0 (control), 7, 14, 21 and 28 days of acclimatization. Content of Chl a and b showed an increasing trend whereas carotenoid and net photosynthetic rate were first decreased and subsequently increased thereafter. SOD activity was increased significantly at day 7 over control plantlets and thereafter showed a decreasing trend whereas catalase activity increased during the whole experiment compared to control plants.

6.2. *Ocimum basilicum* L.

*Ocimum basilicum* (Lamiaceae) commonly known as sweet basil is an evergreen multipurpose herb. In tropical countries, it is often cultivated in homestead gardens and as a pot plant in many countries. It contains volatile oil with eugenol, methyl eugenol, cervacrol and caryophyllin. Dried leaves of basil are used to flavour stew, sauces, salads, soups, meat and tea. *Ocimum* is used as stomachic, antihelminthic, antipyretic, diaphoretic, expectorant, carminative, stimulant and pectoral. It is also used in the bronchitis, hiccough and diseases of heart and brain. The conventional method for propagation is via seeds. However, poor germination potential restricts its multiplication and seedling progeny show variability as a result of cross pollinated nature of plant.

Direct multiple shoot regeneration was achieved from nodal and shoot tip explants excised from 2 year old mature plants. Among the cytokinins (BA, TDZ, Kin and 2-iP) tested as supplements to MS medium, 5.0 µM BA was optimum in inducing bud break in both nodal and shoot tip explants. The highest rate of shoot multiplication (16.4 ± 1.47) was achieved on MS medium supplemented with reduced concentration of BA (2.5 µM) and IAA (0.5 µM) from nodal segments after 8 weeks of culture.

The shoots regenerated from TDZ supplemented medium when subcultured to TDZ free MS medium considerably increased the number of shoots and shoot length by the end of third subculture. Pretreatment of nodal
segment and shoot tip explants to higher concentration of TDZ for different
duration was found beneficial and a maximum of 14.8 ± 1.24 shoots with 5.3 ±
0.32 cm shoot length was recorded on MS medium supplemented with 50 μM
TDZ for 8 days followed by their transfer to MS basal medium for 8 weeks.

The effect of different basal media (MS, %MS; B5, %B5; L2 and %L2) and pH
levels (4.5, 5.0, 5.4, 5.8, 6.2 and 6.5) was also evaluated with optimal
concentration of BA (2.5 μM) + IAA (0.5 μM) for highest shoot proliferation and
elongation. The maximum shoot multiplication and shoot length was achieved
in half strength MS medium at pH 5.8.

Efficient in vitro rooting was achieved on MS medium supplemented with
1.0 μM IBA than in IAA or NAA. To reduce the regeneration cost and time,
rooting ex vitro was also carried out by dipping the basal portion of in vitro
regenerated shoots in IBA (50-300 μM) for half an hour and subsequently
planted in plastic pots containing sterile soilrite. The best results for rooting was
recorded when shoots were dipped in IBA (150 μM) after 4 weeks of
transplantation. In vitro and ex vitro rooted plantlets were successfully
hardened off inside the growth room in soilrite with 95% survival rate followed
by their transfer to greenhouse where all exhibited normal development.

Syn-seeds of O. basilicum were developed by encapsulation of nodal
segments excised from in vitro cultures to calcium alginate hydrogel. The
presence of 3% sodium alginate and 75 mM CaCl₂.2H₂O were found most
suitable for synthetic seed production. Half strength MS medium supplemented
with BA (5.0 μM) and IAA (0.5 μM) gave the maximum frequency (80%) of
conversion of encapsulated nodal segments into plantlets with maximum of 7.9
± 0.54 shoots after 8 weeks. However, roots were very thin and difficult to
handle. Encapsulated nodal segments demonstrated successful regeneration
after different period (1-6 weeks) of cold storage at 4 °C. The synthetic seeds
stored at 4 °C for a period of 4 weeks resulted in maximum conversion
frequency (90%) after 8 weeks when placed back to regeneration medium. The isolated shoots when cultured on MS medium supplemented with 1.0 μM IBA, produced healthy roots and plantlets with well developed shoot and roots were successfully hardened off in plastic pots containing sterile soilrite inside the growth chamber and gradually transferred to greenhouse where they grew well.

Physiological parameters i.e. Chl a, b, carotenoid and net photosynthetic rate were measured in leaves during ex vitro acclimatization at 0, 7, 14, 21 and 28 days. During initial days, these parameters showed a decreasing trend but subsequently increased after 7 days of acclimatization. However, activities of antioxidative enzymes i.e. SOD and catalase were significantly increased and reached maximum at 28 days of acclimatization.

The findings of my investigation lead to the following conclusions;

1. Seeds of *M. pruriens* germinated well on MS basal medium but half strength MS medium proved effective for maximum germination.

2. Direct multiple shoot formation in *M. pruriens* was achieved from cotyledonary node and nodal segment explants using cytokinins (BA, Kin, 2-iP and TDZ) either alone or in combination with auxins (IAA and NAA).

3. Maximum frequency of shoot regeneration was obtained on half strength MS medium supplemented with BA (5.0 μM) and NAA (0.5 μM) at pH 5.8 from nodal segment.

4. Half strength MS medium supplemented with IBA (2.0 μM) showed best rhizogenesis in *M. pruriens*.

5. Direct shoot regeneration in *O. basilicum* was achieved through mature explants (nodal and shoot tip) using cytokinins singly or in combination with auxins.

6. Maximum shoot multiplication was recorded in *O. basilicum* on half strength MS medium supplemented with BA (2.5 μM) + IAA (0.5 μM) from nodal explants at pH 5.8.

7. Nodal explants of both *M. pruriens* and *O. basilicum* pretreated with 50 μM TDZ for 8 days followed by their transfer to MS basal medium devoid of TDZ was found effective for multiple shoot induction.
8. Rooting was best induced in *O. basilicum* on MS medium amended with 1.0 μM IBA.

9. The *in vitro* regenerated plantlets of *M. pruriens* and *O. basilicum* were successfully hardened off in soilrite followed by their transfer to garden soil under full sun.

10. Encapsulated nodal segments of *O. basilicum* showed maximum conversion frequency on half strength MS medium containing BA (5.0 μM) + IAA (0.5 μM) and retain their viability even after 4 weeks of storage at 4 °C.

11. Changes in photosynthetic pigments and antioxidative enzymes in both *M. pruriens* and *O. basilicum* indicated the adaptation of micropropagated plants to *ex vitro* conditions.

To conclude, I could achieve success in developing an efficient, replicable and complete micropropagation protocols for *in vitro* regeneration of two valuable medicinal plants, *M. pruriens* and *O. basilicum*. Changes observed in physiological and biochemical parameters during acclimatization has helped to understand better adaptation process of micropropagated plants to *ex vitro* conditions. The protocols developed could provide a rapid technique for mass propagation and multiplication of these two potential medicinal plants and can further be used in crop improvement using genetic transformation technology.