Chapter 6

Summary and Conclusion
SUMMARY AND CONCLUSIONS

The continued production of enough food, medicine and energy has become a basic challenge because of the world population scaling-up. Moreover, rapid agricultural development, climate change, use of land for intensive farming and indiscriminate collection of medicinal plants from the wild have resulted in over-exploitation of natural resources. Hence, conservation of genetic diversity of medicinal plants has become an issue of concern. It is therefore of paramount importance to develop techniques ensuring rapid multiplication of such species, optimal storage and conservation. One of the approaches to avoid the limited production of food and medicine is the use of in vitro techniques for plant propagation. These are important biotechnological tools for massive plant production.

*Cassia alata* L. is an important ornamental and medicinal plant, commonly grown for a treat against ringworm and skin diseases. The leaves contain many active substances such as alkaloids and flavonoids which directly activate the antimicrobial activity against pathogenic bacteria and fungi. The extracts from *C. alata* thus have long been realized for their successful antimicrobial activities against several food borne and human pathogenic bacteria and fungi.

Despite a valuable medicinal plant, limited work has been done on in vitro regeneration of *C. alata*. In this study, an in vitro regeneration system using various explants has been developed. Histological analysis was also performed to validate the direct development of shoot buds without intervening callus. Changes in the photosynthetic machinery and antioxidant enzymatic system were also measured during acclimatization of micropropagated plantlets. Genetic fidelity analysis was confirmed among the regenerants (mature nodal derived plantlets) with the donor plants by using DNA-based molecular markers (RAPD and ISSR primers).

The core findings are summed up as under this section.

Full-strength MS medium was best for seed germination. Explants obtained from aseptic seedling and mature plant was tried for in vitro plant regeneration. Direct multiple shoot regeneration was achieved on MS medium supplemented with various concentrations of BA, Kn, 2-iP, TDZ, IAA, IBA and NAA either singly or in
combinations. Among the different explants (seedling derived explants, cotyledonary node (CN), Nodal (N), shoot tip (ST) and mature nodal segments) tried, CN explants obtained from 15 days old aseptic seedlings yielded highest number of shoots (9.6 ± 0.3) with maximum shoot length (1.7 ± 0.1) cm on MS medium augmented with 7.5 μM BA after 6 weeks of culture. Furthermore, addition of auxins at lower concentration to cytokinin containing medium enhanced the shoot multiplication rate. The highest number of shoots per CN explant (25.2 ± 0.9) with mean shoot length of (4.0 ± 0.1) cm was achieved on MS medium augmented with 7.5 μM BA and 0.5 μM NAA after 12 weeks in 90% cultures.

After the standardization of reliable protocol for in vitro regeneration, the effect of different basal media, sucrose concentrations and pH values were also examined on shoot morphogenesis from aseptic seedling derived CN explants with optimal concentrations of BA and NAA. The full-strength MS medium was found to be best basal medium followed by WPM and B₅ media containing BA (7.5 μM) and NAA (0.5 μM) after 12 weeks of incubation. At the same plant growth regulator regime, medium amended with 3% sucrose at pH 5.8 showed the finest performance on the shoot induction and proliferation from CN explants after 12 weeks of culture.

In another approach, attempts were made to develop synthetic seeds by encapsulating the nodal segments obtained from in vitro cultures of mature nodal explants using sodium alginate and CaCl₂. A 3% Na₂-alginate with 100 mM CaCl₂ was found to be the optimum concentration for the production of uniform synthetic seeds. The maximum frequency (78.4%) for conversion of encapsulated beads into plantlets was achieved on MS medium containing BA (7.5 μM) and NAA (0.5 μM) after 6 weeks of culture. Storage of encapsulated axillary buds at 4 °C resulted in high frequency of shoot proliferation (78.4%) with 6.3 ± 0.2 shoots/bead. Successful plant retrieval from encapsulated nodal segments following their storage at low temperature indicate that the standardize method could be potentially used to preserve desirable elite genotype of C. alata over a short period.

Adventitious root induction among regenerated shoots was achieved with MS medium supplemented with various auxins (IBA, NAA and IAA). Better rhizogenesis was obtained on full-strength MS medium containing IBA as compared to other auxins.
The maximum frequency of root formation (100%), number of roots (7.2 ± 0.1) per shoot and root length (2.6 ± 0.1) cm was achieved on full strength MS medium containing after 4 weeks.

Ex vitro rooting was also attempted as a means to decrease the microropagation cost and also the time from field to laboratory conditions. The best rooting was achieved by dipping the basal portion of shoots in 150 µM IBA solution for 20 min followed by transfer to soilrite. About 90% plants survived well following transfer from soilrite to garden soil in greenhouse conditions. There was no detectable variation among the potted plants with respect to morphological and growth characteristics.

The regenerated plantlets were successfully acclimatized first under culture room in sterile soilrite, followed by their transfer to field conditions with 90% survival rate after 8 weeks of transfer. Changes in pigment content (Chlorophyll a, b and carotenoid) and enzymatic (SOD, CAT, GR, APX) activites were assessed during ex vitro acclimatization of plantlets at 0, 7, 14, 21 and 28 days. Increment in the chlorophyll (a and b) and carotenoids after 07 days of accclimization revealed an autotrophic behavior of plants under ex vitro condition. However, higher level of antioxidant enzyme activities was observed which reflect the plant’s capacity to develop defence mechanisms during acclimatization and determine the ability to survive in oxidative stress.

DNA-based molecular markers (RAPD and ISSR) were used to assess the genetic stability of micropropagated plantlets. The micropropagated plantlets were choosen from clonal collection of shoots that originated from mature nodal explants (mother plant). 38 RAPD and 10 ISSR primers produced monomorphic band suggesting a high level of genetic fidelity among them. The number of bands produced per primer was greater in ISSR than RAPD. A total of 71 with an average of 7.1 bands were amplified per ISSR primer and a total of 82 bands with 4.2 bands from Kit N and 69 bands with an average of 3.8 bands per RAPD primer from Kit L were scored. The monomorphic banding profile obtained among the tissue culture-raised progeny and the donor plant, indicate the mode of regeneration attempted was appropriate for obtaining true-to type plants.
The findings of the present thesis lead to the following conclusions;

1. Seeds of C. alata germinated successfully on full-strength MS basal medium with 90% germination.
2. Among the various explant tried, aseptic cotyledonary node explants responded best in comparison to nodal, shoot tip and mature nodal explants for achieving maximum percentage response, shoot number and shoot length.
3. MS medium amended with a combination of BA (7.5 μM) + NAA (0.5 μM) at pH 5.8 and 3% sucrose was found to be best media for maximum shoot multiplication from CN explants.
4. The encapsulated nodal segments showed a maximum conversion frequency on MS medium supplemented with BA (7.5 μM) and NAA (0.5 μM) and the synseeds retained their viability even after 4 weeks of storage at 4 °C.
5. Maximum root induction with highest rooting efficiency was observed on MS medium containing 0.5 μM IBA. Ex vitro rooting was achieved successfully using 150 μM IBA for root induction in microshoots.
6. The regenerated plantlets were successfully hardened off in soilrite followed by their transfer to greenhouse with 90% survival rate.
7. The successful acclimatization of micropropagated plantlets suggested the development of functional photosynthetic machinery along with auxiliary enzymatic scavenging system.
8. Genetic uniformity of micropropagated plants was confirmed by PCR-based DNA fingerprinting techniques viz., RAPD and ISSR.

Considering all the aspects of my study, I conclude that the experiments conducted in this thesis work would help in the conservation and mass propagation of C. alata. The present protocol demonstrate the successful use of tissue culture techniques in effective establishment of shoot multiplication, rhizogenesis and acclimatization of the plantlets year round, irrespective of seasonal constraints, which possibly will assist unremitting supply of plant material for sustainable utilization, molecular exploration and large scale plantation. The present protocol also provides a promising technique for reducing the dependence on natural stands of plants for pharmaceutical purposes, and will also serve as a means of conservation of other economically and medicinally important tree legume.
The encapsulation approach using vegetative propagules provide an alternative mode of propagation for this important medical plant. The synthetic seed technology described could possibly paves the way for the conservation, short-term germplasm storage, they can be treated like seeds having the additional advantages over micropropagation for handling, transportation, efficient delivery of plants and would minimize the cost of production.

The physiological machinery and biochemical metabolism developed in plants during acclimatization reflected the tolerance capacity of plants to survive in oxidative stress and play an important role for better environmental adaptation of transplanted plantlets from in vitro to ex vitro environment. This approach could be also used as a starting point for acclimatization of other medicinally and economically important plants and their eventual establishment in the field. The high frequency of shoot regeneration and plant survival rates suggest that the protocol not only provides an alternative viable system for rapid clonal multiplication but also explores the possibility of preserving the genetic stability of the selections or promotes true-to-type genotypes for commercial applications and reintroduction into the favourable or natural habitats.