Chapter 6

Summary and Conclusions

SUMMARY

*Lawsonia inermis* shrub growing up to 2.6-5.0 m in height with quadrangular branches and grayish brown bark is of immense commercial importance. Leaves are greenish brown to dull green, sub sessile 1.5-5.0 cm long and 0.5-2.0 cm wide with an entire margin. Young bark is grayish brown, older plants having spine tipped branchlets. Fruit is small brownish capsule 4-8 mm in diameter with 32-49 seeds and opens irregularly into four splits. Seeds have pyramidal hard and thick seed coat with brownish coloration. Objectives of present study were to optimize efficient protocol of micropropagation of *Lawsonia* and induction of lawsone content. Further the biochemical estimation of lawsone content of mother plant, *in vitro* raised plant and callus induced from leaf was carried out.

Establishment of *Lawsonia inermis* cultures was achieved using shoot tip and nodal explants on MS medium supplemented with variable concentrations of different plant growth regulators (BAP, KIN, PUT, SPD, TDZ and Z). Protocol for Direct plant regeneration of *Lawsonia inermis* was optimized. During the establishment shoot tips resulted in shoot growth, while nodal segment explants sprouted after 3 weeks of inoculation. Nodal segments proved better explants as compared to shoot tips. Maximum (100%) establishment and multiplication rate of shoots was observed using nodal explants on MS medium supplemented with 0.25 mg l\(^{-1}\) BAP + 0.25 mg l\(^{-1}\) KIN. Multiplication was significantly enhanced when MS medium supplemented with additive 0.25 mg l\(^{-1}\) BAP + 0.25 mg l\(^{-1}\) KIN + 30 mg l\(^{-1}\) AdSO\(_4\) was used. Well developed shoots induced roots on half strength MS medium fortified with 0.5 mg l\(^{-1}\) AdSO\(_4\) within 10 days of inoculation. Regenerated plants were acclimatized, hardened, transferred to poly bags and pots in green house and then transferred to soil with high success rates. Genomic DNA was isolated from mother plant and nine randomly selected *in vitro* tissue culture raised plant lines (Li\(_1\)-Li\(_9\)) were subjected to the genetic fidelity. PCR profiling was prepared using twelve ISSR primers produced 29 distinct and scorable bands, with an average of 2.4 bands per primer. Each primer generated a unique set of amplification products ranging from 100 to 1000 bps. Amplicons in all...
the primers were monomorphic and no polymorphism was detected during the ISSR analysis of *in vitro* raised plant, indicating thereby by true-to-type nature of micropropagated plants. All *in vitro* raised plants were found genetically identical with homogenous DNA profiles when compared with mother plant.

The lawsone content in *in vitro* raised plants leaves regenerated on 0.25 mg l\(^{-1}\) BAP + 0.25 mg l\(^{-1}\) KIN + 30 mg l\(^{-1}\) AdSO\(_4\) medium was significantly high and 10 folds higher as compared to mother plant. Young leaves of *Lawsonia inermis* were used for callus induction on MS medium supplemented with 3% sucrose and various concentrations of 2, 4-D, BAP and NAA alone or in combinations. The highest percentage (100%) of callus formation was observed from young leaves cultured on the MS medium supplemented with 0.5 mg l\(^{-1}\) and 1.00 mg l\(^{-1}\) 2,4-D. Studies on leaf callus induction in *Lawsonia inermis* proved that the lower concentration (0.5 and 1.0 mg l\(^{-1}\)) of 2,4-D is more effective and further increase in 2,4-D concentrations resulted in poor callusing. The callus induction of *Lawsonia inermis* opens new avenues that could facilitate production and extraction of secondary metabolites without harvesting the complete plant itself. High Performance Liquid Chromatography (HPLC) analysis revealed that lawsone concentration enhanced ten folds in *in vitro* raised plants and 3-9 folds in callus as compared to mother plant. MS media supplemented with adenine sulphate enhanced lawsone content both *in vitro* raised plants and in callus.

CONCLUSIONS

- Amongst the different sterilizing treatments, the maximum (100%) survival and asepsis was recorded when shoot tip explants were treated with HgCl\(_2\) (0.1%) for 3 min along with Bavistin (0.2%) and Streptocyclin (0.2%) for 90 min
- Nodal explants treated with HgCl\(_2\) (0.1%) for 4 min along with Bavistin (0.2%) and Streptocyclin (0.2%) for 90 min
- Leaf sterilization, treatment of 0.1% HgCl\(_2\) for 2.5 min along with Bavistin (0.2%) and Streptocyclin (0.2%) for 120 min was found to be best with 100% survival and asepsis amongst the various treatments used
- 33 different media combinations MSLE\(_0\) to MSLE\(_{32}\) were employed for establishment of shoot tip and nodal explants. Amongst all media tested BAP showed good direct regeneration response using shoot tip explants.
KIN showed good to moderate regeneration response using shoot tips

TDZ showed negative burnt effect on the shoot tips establishment

MS medium supplemented with polyamines SPD and PUT employed the maximum percentage (100%) of shoot induction using shoots tip explants within minimum days

Zeatin showed poor regeneration response for shoot tip explants

Amongst all media tested BAP & KIN showed good regeneration response using nodal segments

MS medium supplemented with polyamines SPD and PUT employed the maximum percentage (100%) of shoot induction using nodal explants within minimum day

TDZ was also found lethal for establishment of nodal explants

Zeatin showed good regeneration response using nodal segments

The nodal explants were found more responsive than shoot tip explants for multiplication

33 different media combinations MSLM₀ to MSLM₃₂ were used for shoot multiplication from nodal explants. KIN alone showed moderate multiplication response

BAP alone showed poor shoot multiplication response

Combinations of BAP+ KIN showed good shoot multiplication response using nodal segment. MS basal medium fortified with 0.25 mg l⁻¹ BAP + 0.25 mg l⁻¹ KIN was found to be the most effective with maximum (27.9 ± 0.41) number of shoots per explant after 6 weeks of culture

Combinations of IAA with BAP or KIN found less efficient for shoot multiplication

15 different media combinations MSLM₃₃ to MSLM₄₇ supplemented with varying concentration of additives AdSO₄, CH, CA+AA were employed for improving shoot multiplication using MS basal medium supplemented with 0.25 mg l⁻¹ BAP + 0.25 mg l⁻¹. Amongst 30 mg l⁻¹ AdSO₄ was found to be most
effective with maximum (32.5 ± 0.43) shoots per explants after 6 weeks of culture

- 22 different media combinations MSLR₀ to MSLR₂₁ supplemented with varying concentration of auxins (IAA, IBA, NAA) employed for in vitro rooting. Highest percentage (77.8 ± 0.08%) of rooting was observed on the half MS medium fortified with 0.5 IAA mg l⁻¹ with maximum 14.8 roots/shoot in minimum 9.3 days. IAA was found to be more useful instead of NAA and IBA

- IBA showed moderate rooting response

- NAA was not an auxin of choice for root induction except low concentration.

- The survival of in vitro raised plants was 100% in potting mixture containing Sand + Soil + FYM (1:1:1) and Sand + Soil + Vermi compost (1:1:1) under greenhouse conditions

- Total genomic DNA was isolated from young leaves of mother plant (MP) and nine tissue culture raised (TCL) lines (L₁₋₁₀) of Lawsonia inermis by using modified CTAB method

- 12 ISSR primers produced 29 distinct and scorable bands ranging between 100 to 1000 bps with an average number of 2.4 bands per primer, indicating genetic homogeneity among the regenrants and mother plant

- DNA profiling of in vitro raised and mother plants revealed that all the raised micropropagated plants were true-to-type & phenotypically normal

- Rooted plants were acclimatized, hardened, transferred to polybags and in pots in greenhouse and successfully transferred to soil under field conditions. A 100% survival of tissue culture raised plants was observed under the field conditions

- 61 different media combinations MSLC₀ to MSLC₆₀ supplemented with varying concentration of BAP, 2,4-D and NAA were employed for inducing callus from leaf explants of Lawsonia inermis. Auxin 2,4-D alone and its combination with BAP give better callus induction as compared auxin NAA alone or its combination with BAP
Amongst all the media tested MS basal medium supplemented with 0.50 mg l\(^{-1}\) 2, 4-D and 1.00 mg l\(^{-1}\) 2, 4-D was found to be the best with 100 % callusing response

NAA alone failed to induce any callus from leaf explants

An efficient protocol for lawsone extraction from dried leaf and callus samples was developed using soxhlet

HPLC conditions were optimized for estimation of lawsone in extracted samples of *Lawsonia inermis*

HPLC analysis revealed that highest concentration of lawsone was found in TCL. Lawsone content in TCL was found ten folds higher as compared to field grown plants of *Lawsonia inermis*

Effect of Elicitors: CH, AdSO\(_4\), and YE (10, 20, 30, 40 mg l\(^{-1}\)) was studied using MS medium supplemented with 0.5 mg l\(^{-1}\) 2,4-D. Among the different elicitors used 30 mg l\(^{-1}\) AdSO\(_4\) was found to be best for induction of lawsone content in leaf derived callus samples of *Lawsonia inermis*

Thus, micropropogation protocols have been developed for induction, establishment, multiplication from shoot tip and nodal explants of *Lawsonia inermis*. Evidence has been presented of true-to-type of regenerated plant through genetic fidelity and regenerated plants have been successfully hardened, acclimatized & transferred to soil. Protocols have been developed for establishment of callus from leaf segments. Conditions have also been optimized by employing elicitors such as CH, AdSO\(_4\) and YE for enhanced induction of lawsone production from leaf derived calli of *Lawsonia inermis*. Protocols have also been developed for biochemical estimation of lawsone from leaf segments of field grown plants, *in vitro* regenerated plants & leaf derived calli. In the present investigation effect of polyamines: PUT, SPD; Additives: AdSO\(_4\), CH, CA+AA on direct plant regeneration and effect of Elicitors: AdSO\(_4\), CH & YE on induction of lawsone production in *Lawsonia inermis* have been reported for first time. Protocols so developed will help to bridge the gap between ever increasing demand and supply of lawsone necessary for dying, cosmetic and pharmaceutical industry.