Plant cell and tissue culture technologies can be established routinely under aseptic conditions from explants, such as plant leaves, stems, roots, and meristems for multiplication and extraction of secondary metabolites (Hussain et al. 2012). Plant cells are biosynthetically totipotent, which means that each cell in culture retains complete genetic information and hence is able to produce a range of chemicals found in the parent plant. Plants serve as tremendous source for the discovery of new products with medicinal importance for drug development. Today several distinct chemicals derived from plants are important drugs, which are currently used in one or more formulation throughout the world. Secondary metabolites are economically important constituents for drugs, flavor and fragrances, dye, pigments, pesticides and food additives. Many of the drugs sold today are simple synthetic modifications or copies of the naturally occurring substances. Increasing commercial importance of secondary metabolites is of great interest in the production of bioactive plant metabolites by means of tissue culture technology.

*Lawsonia inermis* being an important medicinal plant was selected for present investigation for the development and optimization of various experimental conditions to secure efficient direct plant regeneration, induction and quantification of the lawsone content in *Lawsonia* spp. The present investigation of *in vitro* culture and induction of lawsone production in *Lawsonia inermis* was undertaken at the Centre for Plant Biotechnology, Chaudhary Charan Singh Haryana Agricultural University Campus, Hisar (Haryana) and Department of Bio and Nano Technology, GJU S & T, Hisar, Haryana.

### 3.1 Materials

#### 3.1.1 Collection of plant material

The plant material microcutting of *Lawsonia inermis* were procured from the trees planted at North-East location of Plant Pathology Department, CCS Haryana Agricultural University, Hisar; East location near polyhouse and from plant grown at West position of Centre for Plant Biotechnology building, CCS Haryana Agricultural...
Materials and Methods

University Campus, Hisar. Stem segments 10-15 cm in length containing 3-4 axillary buds with young leaves were excised from adult plants (4-5 years old of approx. 3-4 feet height) during the months of April to November in the years 2011-2013. The microcuttings collected from twigs were neither hard nor very soft. For high frequency direct plant regeneration and callus induction explants shoots tips 2-3 cm, nodal segments of 2-3 cm and young leaves were excised from field grown plants as shown in Fig 3.1. These explants served as the basic starting material for initiating the in vitro cultures.

Fig 3.1 Selection of various explants from mother plant of Lawsonia inermis employed in present investigation (A) mother plant, (B) shoot tips, (C) nodal segments, (D) leaf explants
3.1.2 Glassware and plasticwares

Glassware employed during course of investigation was of borosilicate quality and procured from Borosil, Mumbai, India Limited and Corning Glass Company. Disposable Petri dishes were procured from Tarson products Private Limited, Kolkata, India (Petri dishes 4”x1”) and conical flask of specified volume 100 ml, 150 ml and 250 ml were also used for culturing explants. The test tubes were of size 25x150 mm and Jam bottles (475ml) with screw caps were used for culturing the explants.

3.1.3 Chemicals

Throughout the investigation, precautions were taken to use chemicals of high purity only. Inorganic salts used were of analytical grade of Hi-Media Laboratories Limited Mumbai, India. The chemicals viz. plant growth hormones, vitamins, myo-inositol, glycine, chelating agents, sucrose, agar-agar (EDTA sodium salt) were procured from Sigma Chemical Co. St. Louis, MO, U.S.A and Hi-Media Laboratories Limited Mumbai, India. Plant tissue culture media powder viz. Murashige and Skoog (PT099), Gamborg B5 (PT016), Nitsch (PT012), Linsmaier and Skoog (PT095), Schenk and Hildebrandt (PT116) and White medium (PT014) were procured from Hi-Media Laboratories Limited, Mumbai, India. Taq DNA polymerase, magnesium chloride and PCR buffer (10X) were obtained from Bangalore Genei, Bangalore India. The dNTPs were obtained from Fermentas USA. For quantitative estimation of lawsone standard (H46805) was procured from Sigma Chemical, Co. St. Louis, MO, U.S.A. HPLC grade solvents (methanol, chloroform, water, NaOH, HCl) were obtained from Sigma Chemical, Co. St. Louis, MO, U.S.A; Hi media Laboratories Limited Mumbai, India; Fisher Scientific and Qualigen. All other chemicals used in the present investigation were of analytical grade and procured from Hi-Media Laboratories Limited Mumbai, India; Fermentas, Bangalore Genei, Bangalore, India; Sigma Chemical, Co. St. Louis, MO, U.S.A; BDH Merck Limited, Mumbai India; E. Merck Mumbai, India; SBS Gentech and SISCO Research Laboratories, India.

3.1.4 Sterilization of glassware

All the glassware viz. pipettes, beakers, flasks, measuring cylinders, test tubes, petridishes, jam bottles used during the investigation were washed with liquid detergent followed by sufficient washing under running tap-water to remove the residues of detergent and then rinsed with double distilled water. The petridishes and beakers were
Materials and Methods

3.2 Preparation of Stock Solutions and The Storage

The Basal media used during the course of present investigation included Murashige and Skoog (1962), Gamborg B5 (1968), Nitsch (1969), Linsmaier and Skoog (1965), Schenk and Hildebrandt (1972) and White medium (1943). Compositions of basal media are presented in the Table 3.1. The basal media used for the present study was MS (Murashige and Skoog 1962). All the stock solutions of MS basal medium (Murashige and Skoog 1962) as major, minor, iron, organic hormones, vitamins and amino acids were prepared separately in double distilled water. Stock solutions were prepared as 10X or 100X. Details are given in Appendix1. Stock solutions of growth hormones such as auxins, cytokinins, polyamines and TDZ were prepared by dissolving small volume of NaOH or HCl (as per requirement) and then finally by dissolving in double distilled sterilized water in the ratio 1:1. All stock solutions were stored in borosil reagent bottles with screw caps in a refrigerator at 4 °C. Stock solutions of chelating agent and IAA were kept in amber coloured bottles for protection against light.

3.2.1 Preparation of culture media

Basal MS medium (Murashige and Skoog 1962) was prepared by the mixing the stock solutions as given in Appendix 1 in 500 ml of distilled water by continuous stirring with magnetic stirrer. Myoinositol (100 mg l⁻¹) and sucrose (3%), unless mentioned otherwise, were added to the medium. The final volume was made to one litre by adding distilled water. The pH of the medium was adjusted to 5.8 with pH meter (Elico-digital company) using 1N HCl or 1N KOH/NaOH. Agar (BDH) @ 8g l⁻¹ was melted in microwave (LG Electronics, MG-605AP). The medium was stirred regularly to avoid formation of agar clumps till melted thoroughly. It was allowed to cool for few minutes at room temperature. Twenty millilitres of medium was aliquot into each of the culture tube/flask and plugged with non absorbent cotton wrapped in muslin cloth and covered with aluminium foil. The medium was autoclaved at 121 °C for 20 min (15 lbs inch⁻²) in autoclave (Narang Scientific Works, Pvt. Ltd., New Delhi, India) and allowed to solidify at room temperature. The sterilized medium was stored at room temperature till further use.
Table 3.1 Composition of different types of basal media

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Murashige &amp; Skoog (mg L⁻¹)</th>
<th>Nitsch &amp; White (mg L⁻¹)</th>
<th>Schenk &amp; Hildebrandt (mg L⁻¹)</th>
<th>Gamborg B5 (mg L⁻¹)</th>
<th>Linsmaier &amp; Skoog (mg L⁻¹)</th>
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</table>
3.3 Methods

3.3.1 Surface sterilization and isolation of explants

Actively growing young stems (5-6 cm) of *Lawsonia inermis* were collected from field grown plants and thoroughly washed in running tap water along with a drop of mild detergent (Teepol) for 10 min. After washing with detergent, shoot tip, nodal segment and leaf explants were excised from sterilized tissue and subjected to anti-bacterial treatment (0.2% Streptocyclin) of different duration ranging from 30-150 min to prevent bacterial contamination. Excised explants were also treated with 0.2% Bavistin (systemic antifungal agent) for 30-150 min to check fungal contamination. Subsequently explants were surface sterilized with (0.1% w/v) mercuric chloride (HgCl₂) for 0.5-6.0 min and washed 3-4 times with sterilized double distilled water to remove traces of HgCl₂. The explants: shoot tip, nodal and leaf segment were excised out with sterilized blades under aseptic conditions in Laminar air flow (Webcon Instrument Pvt. Ltd., Varanasi, India). The details of disinfection treatment of shoot tips, nodal segment and leaf explants are given in Table 3.2-3.4.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Code</th>
<th>Bavistin</th>
<th>Streptocyclin</th>
<th>Ethanol (70%)</th>
<th>HgCl₂ (0.1%)</th>
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<td>% Conc.</td>
<td>Time (sec)</td>
<td>Time (min)</td>
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<td>2</td>
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<td>3</td>
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<td>3</td>
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LSTS denotes *Lawsonia inermis* Shoot tip Explants Sterilization
LSTS0 - LSTS17 denotes various treatments for Sterilization of *Lawsonia inermis* Shoot tip Explants
Micropropagation of *Lawsonia inermis* L. and *in vitro* Manipulations

### Table 3.3 Surface sterilization of nodal segments

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<th>S. No.</th>
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<th>Streptocyclin</th>
<th>Ethanol (70%)</th>
<th>HgCl₂ (0.1%)</th>
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<td>% Conc.</td>
<td>Time (min)</td>
<td>% Conc.</td>
<td>Time (sec)</td>
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<tr>
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</tr>
<tr>
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LNPS denotes *Lawsonia inermis* Nodal Segments Sterilization
LNPS₀-LNPS₁₈ denotes various treatments for sterilization of *Lawsonia inermis* Nodal Segments

### Table 3.4 Surface sterilization of leaf explants

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<th>Streptocyclin</th>
<th>HgCl₂ (0.1%)</th>
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<tr>
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<td>60</td>
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<td>90</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
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<td>60</td>
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<td>120</td>
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</tr>
<tr>
<td>14</td>
<td>LLS₁₃</td>
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<td>150</td>
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</tr>
<tr>
<td>15</td>
<td>LLS₁₄</td>
<td>0.2</td>
<td>120</td>
<td>0.1</td>
</tr>
<tr>
<td>16</td>
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<td>0.2</td>
<td>120</td>
<td>0.1</td>
</tr>
<tr>
<td>17</td>
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</tr>
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<td>18</td>
<td>LLS₁₇</td>
<td>0.2</td>
<td>120</td>
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<tr>
<td>19</td>
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<td>0.2</td>
<td>120</td>
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</tr>
<tr>
<td>20</td>
<td>LLS₁₉</td>
<td>0.2</td>
<td>120</td>
<td>0.2</td>
</tr>
</tbody>
</table>

LLS denotes *Lawsonia inermis* Leaf Explant sterilization
LLS₀-LLS₂₀ denotes various treatments for sterilization of *Lawsonia inermis* Leaf Explants
3.3.2 Inoculation and culture conditions of explants

After Surface sterilization, explants were inoculated in jam bottles containing various culture media. The cultures were kept in culture room under aseptic conditions. Temperature of culture room maintained at 25±2 °C with light intensity of 100 µEM^-2 sec^-1 (1000 lux) was provided by florescent tubes. Sixteen hour light and eight hour dark conditions were maintained in the growth chamber.

3.4 Experimental Design for Establishment, Multiplication of In vitro Cultures

Surface sterilized explants viz. Shoots tips, nodal segments and leaf were inoculated on MS basal medium supplemented with different concentrations of growth regulators for shoot induction under aseptic conditions in laminar air flow chamber. Explants were inoculated on MS medium supplemented with different combinations of auxin and cytokinin coded as MSLE_0 to MSLE_32 as shown in Table 3.5 for establishment. Regenerated shoots of Lawsonia inermis were sub cultured on various shoot multiplication media having MS basal salts supplemented with different concentrations of plant growth regulators such as BAP, KIN and IAA coded as MSLM_0 to MSLM_32 shown in Table 3.6 for their proliferation as a multiplication media. The effects of additives viz. adenine sulphate, casein hydrolysate, citric acid and ascorbic acid of various concentrations (10, 20, 30, 40 and 50 mg l^-1), with a constant concentration of 0.25 mg l^-1 BAP and 0.25 mg l^-1 KIN coded as MSLM_33 to MSLM_47 as shown in Table 3.7 on the multiplication of nodal segments from in vitro raised cultures were studied. Cultures were maintained by regular subculturing after every 15 days of incubation. The entire experiment was conducted in three replicates.

The following observations were made and data was recorded:

- Percent explants showing direct bud initiation
- Number of shoots per explant
- Shoot length per explant
### Materials and Methods

Table 3.5 MS medium supplemented with different concentrations of plant growth regulators employed in present investigation for establishment of *Lawsonia inermis*

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Medium code</th>
<th>Plant Growth Regulators (mg l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BAP</td>
</tr>
<tr>
<td>1</td>
<td>MSLE₀</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>MSLE₁</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>MSLE₂</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>MSLE₃</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
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<td>1.5</td>
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<td>MSLE₅</td>
<td>2.0</td>
</tr>
<tr>
<td>7</td>
<td>MSLE₆</td>
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</tr>
<tr>
<td>8</td>
<td>MSLE₇</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>MSLE₈</td>
<td>-</td>
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<tr>
<td>10</td>
<td>MSLE₉</td>
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<td>-</td>
</tr>
<tr>
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<td>13</td>
<td>MSLE₁₂</td>
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<td>14</td>
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<tr>
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</tr>
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<td>24</td>
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<td>26</td>
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<td>27</td>
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<td>-</td>
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<tr>
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<td>MSLE₂₈</td>
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<tr>
<td>30</td>
<td>MSLE₂₉</td>
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</tr>
<tr>
<td>31</td>
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<tr>
<td>32</td>
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<tr>
<td>33</td>
<td>MSLE₃₂</td>
<td>-</td>
</tr>
</tbody>
</table>

MSLE denotes MS medium supplemented with various concentration of Plant Growth Regulators for *Lawsonia inermis* establishment.

MSLE₀⁻ MSLE₃₂ denotes various treatments of MS medium supplemented with different concentration of Plant Growth Regulators for establishment of *Lawsonia inermis*.

---

Micropropagation of *Lawsonia inermis* L. and *in vitro* Manipulations 53
Table 3.6 MS medium supplemented with different concentrations of plant growth regulators either alone or in combination employed in present investigation for multiplication of *Lawsonia inermis*

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Medium code</th>
<th>Plant Growth Regulators (mg l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BAP</td>
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<td>MSLM₁</td>
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</tr>
<tr>
<td>3</td>
<td>MSLM₂</td>
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</tr>
<tr>
<td>4</td>
<td>MSLM₃</td>
<td>1.0</td>
</tr>
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<td>6</td>
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<td>MSLM₆</td>
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<td>MSLM₉</td>
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</tr>
<tr>
<td>33</td>
<td>MSLM₃₂</td>
<td>-</td>
</tr>
</tbody>
</table>

MSLM denotes MS medium supplemented with various concentrations of Plant Growth Regulators for *Lawsonia inermis* multiplication.

MSLM₀⁻ MSLM₃₂ denotes various treatments of MS medium supplemented with different concentrations of Plant Growth Regulators for multiplication of *Lawsonia inermis*.
Materials and Methods

Table 3.7 MS medium supplemented with different concentrations of plant growth regulators either alone or with additives employed in present investigation for multiplication of nodal segments of *Lawsonia inermis*

<table>
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<tr>
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<th>Plant Growth Regulators (mg l⁻¹)</th>
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<td>3</td>
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<td>0.25</td>
</tr>
<tr>
<td>4</td>
<td>MSLM₃₆</td>
<td>0.25</td>
</tr>
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<td>5</td>
<td>MSLM₃₇</td>
<td>0.25</td>
</tr>
<tr>
<td>6</td>
<td>MSLM₃₈</td>
<td>0.25</td>
</tr>
<tr>
<td>7</td>
<td>MSLM₃₉</td>
<td>0.25</td>
</tr>
<tr>
<td>8</td>
<td>MSLM₄₀</td>
<td>0.25</td>
</tr>
<tr>
<td>9</td>
<td>MSLM₄₁</td>
<td>0.25</td>
</tr>
<tr>
<td>10</td>
<td>MSLM₄₂</td>
<td>0.25</td>
</tr>
<tr>
<td>11</td>
<td>MSLM₄₃</td>
<td>0.25</td>
</tr>
<tr>
<td>12</td>
<td>MSLM₄₄</td>
<td>0.25</td>
</tr>
<tr>
<td>13</td>
<td>MSLM₄₅</td>
<td>0.25</td>
</tr>
<tr>
<td>14</td>
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</tr>
<tr>
<td>15</td>
<td>MSLM₄₇</td>
<td>0.25</td>
</tr>
</tbody>
</table>

MSLM₃₃ - MSLM₃₇ denotes MS medium supplemented with 0.25 mg l⁻¹ BAP and 0.25 mg l⁻¹ KIN along with different concentrations of additive AdSO₄ (10-50 mg l⁻¹)

MSLM₃₈ - MSLM₄₂ MS medium supplemented with 0.25 mg l⁻¹ BAP and 0.25 mg l⁻¹ KIN along with different concentrations of additive CH (10-50 mg l⁻¹)

MSLM₄₃ - MSLM₄₇ MS medium supplemented with 0.25 mg l⁻¹ BAP and 0.25 mg l⁻¹ KIN along with different concentrations of additive each of CA+AA (10-50 mg l⁻¹)

3.5 Root Induction

Regenerated shoots of *Lawsonia inermis* were excised aseptically along with some of the surrounding basal portion and were cultured on half strength MS media coded as MSLR₀ to MSLR₂₁ given in Table 3.8, for induction of rooting. The following observations were made and data was recorded:

- Number of days taken for visible root formation
- Percentage rooting of plantlets
- Average root length per plantlet
- Number of roots per plantlet
Materials and Methods

The following methods have been used for calculating the percentage of (%) direct plant regeneration and rooting:

\[
\text{i) Percentage Direct Plant} = \frac{\text{Number of explants showing direct shoot bud induction}}{\text{Total number of explants cultured}} \times 100
\]

\[
\text{ii) Percentage Rooting} = \frac{\text{Number of shoots responding to rooting}}{\text{Total number of shoots cultured on rooting medium}} \times 100
\]

Table 3.8 MS Media (half strength) supplemented with different concentrations of plant growth regulators employed in present investigation for in vitro root induction of \textit{Lawsonia inermis}

<table>
<thead>
<tr>
<th>S. No</th>
<th>Media code</th>
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<th>IBA</th>
<th>NAA</th>
<th>Medium strength</th>
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<tr>
<td>2</td>
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<td>-</td>
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<td>3</td>
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<td>-</td>
<td>-</td>
<td>Half strength</td>
</tr>
<tr>
<td>4</td>
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<td>-</td>
<td>-</td>
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</tr>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>6</td>
<td>MSLR\textsubscript{5}</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>Half strength</td>
</tr>
<tr>
<td>7</td>
<td>MSLR\textsubscript{6}</td>
<td>4.0</td>
<td>-</td>
<td>-</td>
<td>Half strength</td>
</tr>
<tr>
<td>8</td>
<td>MSLR\textsubscript{7}</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
<td>Half strength</td>
</tr>
<tr>
<td>9</td>
<td>MSLR\textsubscript{8}</td>
<td>-</td>
<td>0.25</td>
<td>-</td>
<td>Half strength</td>
</tr>
<tr>
<td>10</td>
<td>MSLR\textsubscript{9}</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
<td>Half strength</td>
</tr>
<tr>
<td>11</td>
<td>MSLR\textsubscript{10}</td>
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<td>1.0</td>
<td>-</td>
<td>Half strength</td>
</tr>
<tr>
<td>12</td>
<td>MSLR\textsubscript{11}</td>
<td>-</td>
<td>2.0</td>
<td>-</td>
<td>Half strength</td>
</tr>
<tr>
<td>13</td>
<td>MSLR\textsubscript{12}</td>
<td>-</td>
<td>3.0</td>
<td>-</td>
<td>Half strength</td>
</tr>
<tr>
<td>14</td>
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</tr>
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<td>16</td>
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<td>0.25</td>
<td>Half strength</td>
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<td>17</td>
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</tr>
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<td>-</td>
<td>1.0</td>
<td>Half strength</td>
</tr>
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<td>19</td>
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<td>-</td>
<td>2.0</td>
<td>Half strength</td>
</tr>
<tr>
<td>20</td>
<td>MSLR\textsubscript{19}</td>
<td>-</td>
<td>-</td>
<td>3.0</td>
<td>Half strength</td>
</tr>
<tr>
<td>21</td>
<td>MSLR\textsubscript{20}</td>
<td>-</td>
<td>-</td>
<td>4.0</td>
<td>Half strength</td>
</tr>
<tr>
<td>22</td>
<td>MSLR\textsubscript{21}</td>
<td>-</td>
<td>-</td>
<td>5.0</td>
<td>Half strength</td>
</tr>
</tbody>
</table>

MSLR denotes MS (half strength) medium supplemented with various concentrations of auxins for \textit{Lawsonia inermis} rooting

MSLR\textsubscript{0} - MSLR\textsubscript{21} denotes various treatments of MS (half strength) medium supplemented with various concentrations of auxins: IAA, IBA and NAA for rooting of \textit{Lawsonia inermis}.

3.5.1 Acclimatization and hardening

Rooted plants (5-6 cm) were carefully removed from the Jam bottles, washed with sterile water to remove agar media. They were transferred to transparent polythene bags.
Materials and Methods

to prevent desiccation and maintain high humidity. These plants were maintained in the culture room at 24 ± 2 °C and 16 h photoperiod with a light intensity of 50 μmol m⁻² s⁻¹ PPFD. The relative humidity was gradually reduced after 4 weeks the plants were removed from the polybags and transferred to pots filled with 1:1:1 mixture of soil, vermi compost and farmyard manure (FYM) (w/w). Pots were kept in the greenhouse for 4-6 weeks till they attained a height of approximately 15cm. The acclimatized plants were successfully transferred and established in the field.

PM₁ = Sand,
PM₂ = Sand + FYM (1:1)
PM₃ = Sand + Soil + FYM (1:1:1)
PM₄ = Sand + Soil + Vermi compost (1:1:1)

Data was recorded for:
• Percent survival of rooted plantlets in different potting mixtures.

3.6 Protocol for Micropropagation of *Lawsonia inermis*

Micropropagation is a versatile technique in which a variety of plant species can be propagated by cell, tissue and organ culture. Rapid multiplication of *Lawsonia inermis* plants was achieved using clonal propagation method. For better understanding flow chart for micropropagation of *Lawsonia inermis* given below.

<table>
<thead>
<tr>
<th>Explants (Shoot tips/Nodal segment) of <em>Lawsonia inermis</em></th>
<th>↓</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washed with mild detergent (teepol) followed by washing</td>
<td>↓</td>
</tr>
<tr>
<td>Antibacterial and antifungal treatment followed by washing with distilled water</td>
<td>↓</td>
</tr>
<tr>
<td>HgCl₂ (0.1%) treatment followed by washing with double distilled sterilized water</td>
<td>↓</td>
</tr>
<tr>
<td>Establishment</td>
<td>↓</td>
</tr>
<tr>
<td>Multiplication</td>
<td>↓</td>
</tr>
<tr>
<td>Elongation</td>
<td>↓</td>
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3.7 Genomic DNA Extraction

Genomic DNA from young leaves from field grown mother plant (MP) and in vitro grown plant in Green House (Li₁ to Li₉) of Lawsonia inermis were isolated using modified CTAB method of Saghai Maroof et al. (1984) with some modifications. Approximately 3-5 g of plant material such as leaf samples were ground into fine powder using liquid nitrogen in a sterilized pestle and mortar. Powder was transferred immediately into 50 ml polypropylene tube (Tarsons Products Pvt. Limited; Kolkata, India) taking care that thawing does not take place. Added 15 ml of preheated (65 °C) CTAB buffer (Appendix 2) to the powder mixture. The samples were thoroughly mixed by gently inverting the tubes several times. The tubes were then incubated at 65 °C for 90 min in a water bath (Pharmacia Biotech, USA) with regular mixing of samples. After incubation, samples were cooled to room temperature and 15 ml of Phenol: Chloroform: Isoamylalcohol (25:24:1) mixture was added to each tube. Contents of the tubes were mixed gently for 10-15 min. Tubes were then centrifuged at 10,000 rpm for 20 min at 25 °C (4K15; Sigma Laboratory Centrifuge, Germany). After centrifugation, aqueous layer was transferred to clean centrifuge tubes and again treated with Phenol: Chloroform: Isoamylalcohol followed by centrifugation. This step enhanced purity of DNA. DNA was precipitated by adding equal volume of ice cold isopropanol followed by centrifugation at 10,000 rpm for 20 min. Washed DNA pellet with 70% ethanol and dissolved genomic DNA in TE buffer.

Grind 3-5 g of young leaf plant material in liquid N₂
i) Add CTAB (15ml)
ii) Incubate for 90 min at 65 °C
iii) Add Phenol: Chloroform: Isoamylalcohol (25:24:1)
Centrifugation at 10,000 rpm for 20 min
Aqueous phase Supernatant (Pellet discarded)
Add Phenol: Chloroform: Isoamylalcohol (25:24:1)
Add equal volume of ice cold isopropanol & precipitate genomic DNA by centrifugation at 10,000 rpm for 20 min
Add 70% ethanol for Washing
Dissolved genomic DNA in TE buffer
Genomic DNA

Micropropagation of Lawsonia inermis L. and in vitro Manipulations 58
allowed to air dry. DNA pellet was re-suspended in 1X TE buffer (Appendix 2). For
better understanding flow chart of isolation of genomic DNA from leaf samples of
Lawsonia inermis is given.

Flowchart for isolation of Genomic DNA

3.7.1 RNase treatment

For removal of RNA contamination, 2 µl of RNase A (10 mg ml⁻¹) was added to each
tube. Samples were mixed gently and then incubated at 37 °C for 1 h. DNA was
extracted by adding equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1)
mixture. Samples were mixed well and centrifuged at 10,000 rpm for 15 min at 4 °C.
Supernatant (aqueous phase) was transferred to fresh eppendorf tubes (Tarsons
Products Pvt. Limited; Kolkata, India) and 1/10 volume of 3 M sodium acetate
(Appendix II) and 2 volumes of chilled absolute ethanol were added. Placed tube at -20
°C for 40 min to overnight. Centrifuged at 10,000 rpm for 15 min at 4 °C to pellet down
the DNA. The Pellet was washed with 70% alcohol; air dried and finally dissolved in
appropriate volume 1X TE buffer. Samples were stored at -20 °C until further use. For
better understanding flowchart for purification of genomic DNA after RNase treatment
is given

Flow Chart for purification of Genomic DNA after RNase treatment
3.8 DNA Quantification and Purity Check

Isolated genomic DNA was quantified using spectrophotometer and the quality was checked by agarose gel electrophoresis.

3.8.1 Spectrophotometric analysis

The approximate purity of double standard DNA was estimated by determination of the ratio of absorbance at 260 and 280 nm (A$_{260}$/A$_{280}$). This ratio is 1.8 for pure double standard DNA. A$_{260}$/A$_{280}$ ratio greater than 1.8 suggests RNA contamination, whereas, one less than 1.8 suggests protein in the sample. The sample was quantified by equation given below. After quantifying the DNA each sample was diluted with TE buffer so as to get similar working concentration of DNA (~ 25 ng µl$^{-1}$) for use in PCR.

The concentration of DNA was estimated from the following formula:–

$$\text{Concentration of DNA (µg ml}^{-1}) = A_{260} \times 50 \times \text{dilution factor}$$

The quality of DNA was checked by following formula:–

$$A_{260}/A_{280} = 1.8 \text{ (Pure DNA)}$$

3.8.2 Agarose gel electrophoresis

For accurate estimation of quantity of isolated genomic DNA, 5µl of aliquot was mixed with 1 µl of 6X gel loading dye and subjected to agarose gel electrophoresis. Weighed 0.32 g of agarose (Hi-Media, Mumbai, India) and melted in 40 ml TBE buffer using microwave oven (LG Electronics, MG-605AP) at 65 °C for 2-3 min. The molten agar was cooled to 37 °C and 5 µl ethidium bromide was added from a stock of 10 mg l$^{-1}$ and mixed by swiring and poured into gel caster (Sub Cell GT Bio-Rad Laboratories, CA, USA). DNA samples were then loaded in the wells and electrophoresis was carried out at constant voltage (3v/cm of gel) till the dye migrated to the other end of gel. Gel visualized under UV light and photograph was taken using gel documentation system (Gene Genius, Syngene, UK).
3.9 Genetic Fidelity of Regenerated Plants

Genetic fidelity of tissue culture raised plants was done by employing ISSR primer MP1 to MP12 and compared to field grown mother plant (MP) by PCR amplification.

3.9.1 Polymerase chain reaction (PCR) amplification

DNA amplification of *Lawsonia inermis* mother plant (MP) and nine tissues cultured raised lines Li1-Li9 were employed for analysis of genetic fidelity using a set of twelve Inter Simple Sequence Repeat (ISSR) primers designated as MP1-MP12 (Table 3.9). PCR amplification was carried out in MY-CYCLER (Programmable thermal cycler from Bio-Rad, Research Laboratories, CA, USA). PCR amplification conditions viz. concentration of template DNA, primers, MgCl2, Taq DNA polymerase and annealing temperature were optimized initially.

For ISSR primers, varying concentrations of template DNA (12.5, 25 and 50 ng), primer (0.2, 0.4, 0.8 and 1.0 µM), MgCl2 (1.0, 1.5 and 2.0 mM), and Taq DNA polymerase (0.8, 1.0 and 1.5 U) were used. Different annealing temperatures (45 to 55°C) were used during PCR amplification. Final PCR reaction was conducted in 20µl of reaction mix containing lX PCR buffer, 200 µM dNTPs mix, 0.4 µM primers, 1.0 mM MgCl2, 1Unit Taq DNA polymerase and 25 ng of template genomic DNA.

The following protocols was used for PCR amplification

**PCR Amplification conditions**

1. Initial denaturation/Preheating 94°C for 3 min
2. Denaturation 94°C for 45 sec
3. Annealing 45- 55°C for 1 min 45cycles
4. Extension 72°C for 1min
5. Final Extension 72°C for 10 min

Amplified products were stored at -20 °C till profiling on 1.5% agarose gel electrophoresis. The amplified DNA products in the gel were viewed under UV light with the help of transilluminator and gel was photographed on gel documentation system (Gene Genius, Syngene, UK). For better understanding flowchart is given:-
Flow Chart for PCR amplification

1. 25 ng of Genomic DNA
   - 1X PCR buffer
   - dNTPs mix (200 µM)
   - Primer (0.4 µM)
   - Taq DNA polymerase (1 U)
   - MgCl₂ (1.0 mM)

2. Preheating (94 °C, 3 min)

3. Denaturation (94 °C, 45 sec)

4. Anneling (45-55 °C, 1 min)
   - 45 Cycles

5. Extension (72 °C, 1 min)

6. Final Extension (72 °C, 10 min)

7. Soak (4 °C)
3.10 ISSR Primers

Inter simple sequence repeat (ISSR) are detected by the use of repeat anchored primer that amplify between simple sequence repeats (SSRs). ISSR primers mostly preferred in assessing the genetic integrity among clonally propagated plants as ISSR technique is very simple, fast, cost-effective, highly discriminative and reliable.

3.10.1 ISSR primers used for studying genetic fidelity in *Lawsonia inermis*

Total 12 ISSR Primers designated as MP1 to MP2 were employed in present investigation, synthesized from University of British Columbia (UBC, Vancouver, Canada) and IDT (Integrated DNA Technologies, Inc.CA, USA). Table 3.9 represents primer 5’-3’ sequences and $T_m$ values.

**Table 3.9 List of ISSR primers used for testing genetic fidelity of *in vitro* raised plants of *Lawsonia inermis***

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<th>Primers</th>
<th>Sequence (5’-3’)</th>
<th>$T_m$ (°C)</th>
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3.10.2 Data analysis for genetic fidelity

- ISSR banding pattern of mother plant (MP) was compared to tissue culture raised nine lines Li1-Li9 using 1 kb DNA ladder.
3.11 Induction of Callus for Lawsone Production

3.11.1 Effect of plant growth regulators on induction of callus

Leaf explants were cultured on MS basal with various concentrations of auxins (2, 4-D, NAA) and cytokinins (BAP) either alone or in combinations coded as MSLC0 to MSLC60 shown in Table 3.10. Visual observations like number of days required for induction of callus, per cent explant callus induction, colour, shape and texture of callus were recorded periodically and used to select best callus induction medium. The entire experiment was conducted in three replicates.

Table 3.10 MS media supplemented with different concentrations of plant growth regulators employed in present investigation for in vitro callus induction in Lawsonia inermis

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<th>Medium Code</th>
<th>Plant Growth Regulators (mg l⁻¹)</th>
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**Materials and Methods**

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MSLC denotes MS medium supplemented with various combinations of plant growth regulators for \textit{Lawsonia inermis} callus induction from leaf.

MSLC<sub>24</sub> - MSLC<sub>60</sub> denotes various treatments MS medium supplemented with various combinations of plant growth regulators: BAP, 2,4-D and NAA for \textit{Lawsonia inermis} callus induction from leaf.
3.11.2 Effect of additives on induction of lawsone

For monitoring the effect of additives on lawsone induction calli were induced on MS medium supplemented with or without 0.5 mg l⁻¹ 2,4-D along with 10-40 mg l⁻¹ Casein hydrolysate (CH), 10-40 mg l⁻¹ Adenine Sulphate (AdSO₄) and 10-40 mg l⁻¹ Yeast Extract (YE) coded as MSLL₀ to MSLL₁₃ shown in Table 3.11.

**Table 3.11 MS Media supplemented with or without 0.5 mg l⁻¹ 2, 4-D along with additives employed in present investigation for in vitro induction of lawsone production in calli derived from leaf explants of Lawsonia inermis**

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<th>Adenine sulphate</th>
<th>Yeast Extract</th>
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<td>-</td>
<td>-</td>
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<td>40</td>
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</table>

MSLL denotes MS medium supplemented with 0.5 mg l⁻¹ 2,4-D with various concentrations of additives CH, AdSO₄ and YE for lawsone induction in calli derived from leaf explants of Lawsonia inermis;
MSLL₁ - MSLL₅ denotes various treatment of MS medium supplemented with 0.5 mg l⁻¹ 2,4-D along for induction of lawsone production in callus culture derived from leaf explants of Lawsonia inermis;
MSLL₀ - MSLL₁₃ denotes various treatment of MS medium supplemented with 0.5 mg l⁻¹ 2,4-D along with various concentrations of CH (10-40 mg l⁻¹) for induction of lawsone production in callus culture derived from leaf explants of Lawsonia inermis;
MSCLL₁₄ - MSLL₁₉ denotes various treatment of MS medium supplemented with 0.5 mg l⁻¹ 2,4-D along with various concentrations of AdSO₄ (10-40 mg l⁻¹) for induction of lawsone production in callus culture derived from leaf explants of Lawsonia inermis;
MSCLL₂₀ - MSLL₂₅ denotes various treatment of MS medium supplemented with 0.5 mg l⁻¹ 2,4-D along with various concentrations of YE (10-40 mg l⁻¹) for induction of lawsone production in callus culture derived from leaf explants of Lawsonia inermis.

**Materials and Methods**

Micropropagation of Lawsonia inermis L. and in vitro Manipulations
3.12 Analysis of Lawsone Content Using HPLC

For HPLC analysis leaf samples were taken from field grown mother plant (MP) and in vitro raised *Lawsonia inermis* plants (TCL). Callus samples were also collected after 6-8 weeks of culture for HPLC analysis of lawsone content. All HPLC sample were analyzed by Oniosome Healthcare Pvt. Ltd., Mohali, Punjab. For better understanding flowchart for extraction of lawsone from field grown, *in vitro* grown leaf samples and callus is given.

**Flowchart for extraction of Lawsone from Leaf Samples of *Lawsonia inermis***

- **Collection Leaf samples from field/*in vitro* of *Lawsonia inermis***
- dried in oven at 50 °C

- Samples powdered in grinder without increasing temperature

- Powdered sample extracted with methanol (100 ml) in soxhlet apparatus at 40 °C – 60 °C for 8-10 h, continuously till extract became colourless

- Concentrated under vaccum using rotary evaporator to near dryness

- Concentrated sticky samples were reconstituted quantitatively in methanol in volumetric flask & filtered through 0.45μm filter paper

- Concentrated extract subjected to HPLC analysis
Flowchart for extraction of Lawsone from callus samples derived from leaf of *Lawsonia inermis*

1. Collection callus samples of *Lawsonia inermis* dried in oven at 50 °C
2. Samples powdered in grinder without increasing temperature
3. Powdered sample extracted with methanol (100 ml) in soxhlet apparatus at 40 °C – 60 °C for 8-10 h, continuously till extract became colourless
4. Concentrated under vacumm using rotary evaporator to near dryness
5. Concentrated sticky samples were reconstituted quantitatively in methanol in volumetric flask and filtered through 0.45 µ filter paper
6. Concentrated extract subjected to HPLC analysis

3.12.1 Preparation of lawsone stock

Lawsone standard (H46805) was procured from Sigma Chemical, Co. St. Louis, MO, U.S.A. Accurately weighed (10.1 mg) lawsone standard was transferred to 10 ml volumetric flask and volume was made using methanol 10 ml. Standard solution was further diluted to obtain working stock solution of different concentrations ranging from 10 to 30 µg ml⁻¹ and stored at 4 °C till further use.

3.12.2 HPLC instrumentation and chromatographic conditions

Extracts were analyzed using RP-HPLC system (Shimadzu Corporation, LC2010C HT, Kyoto, Japan) coupled with auto sampler, ultra violet - photo diode array detector (UV-
PDA). Chromatographic separation was performed at 40°C using C18 phenomenex column (250 x 4.6 mm, 5µm).

The mobile phase was a binary gradient system consisting a mixture of eluents 0.1M l⁻¹ acetic acid: methanol in ratio 35: 65. Mobile phase filtered and degassed (for 20 min) in ultrasonic bath prior to use. A volume of 20µl was injected automatically for each assay at a constant flow rate (0.5 ml min⁻¹). The spectral data was collected at 244 nm wavelength with UV detector and data acquisition was performed by LC-Solution software version 1.25.

The optimized conditions for quantification of lawsone through HPLC are summarized in Table 3.12

<table>
<thead>
<tr>
<th>S. No.</th>
<th>HPLC Instrumentation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Detector</td>
<td>RP-HPLC+ UV-PDA</td>
</tr>
<tr>
<td>2</td>
<td>Column</td>
<td>C18 phenomenex column (250 x 4.6 mm, 5µm)</td>
</tr>
<tr>
<td>3</td>
<td>Pump</td>
<td>LC2010 pump</td>
</tr>
<tr>
<td>4</td>
<td>Mobile phase</td>
<td>Acetic acid + Methanol (35:65) 0.1 Ml⁻¹</td>
</tr>
<tr>
<td>5</td>
<td>Flow rate</td>
<td>0.5 ml min⁻¹</td>
</tr>
<tr>
<td>6</td>
<td>Column Temperature</td>
<td>40 °C</td>
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<tr>
<td>7</td>
<td>Injection Volume</td>
<td>20 µl</td>
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<tr>
<td>8</td>
<td>Wavelength (λ)</td>
<td>244 nm</td>
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<tr>
<td>9</td>
<td>Retention time</td>
<td>7.2 min</td>
</tr>
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</table>

3.12.3 Identification and quantification

Peaks of lawsone in the samples were identified by comparing their retention time with standard lawsone under similar conditions.

3.12.4 Calculation of lawsone content

The concentration of lawsone in each sample was determined with reference to a calibration curve made from the following lawsone concentrations in MeOH with the corresponding peak areas: 10, 20 and 30 µg ml⁻¹. The resultant diluted solutions of lawsone were analyzed by HPLC. The relationship of lawsone concentration and integrated peak area was found to be linear as shown in standard curve:-
Materials and Methods

Micropropagation of *Lawsonia inermis* L. and *in vitro* Manipulations

Where:-

\[ Y = 14756 \times X + 3075 \]

\[ Y = \text{Peak Area of lawsone} \]

\[ X = \text{Concentration of lawsone} \]
3.13 Statistical Analysis

All the experiments were carried out under completely randomized design (CRD). The data of all the experiments, recorded during the present investigation was subjected to statistical analysis in the following way:-

\[
\bar{x} = \frac{\sum x_i}{n} = \frac{\text{Sum of } i^{th} \text{ treatment}}{\text{Number of observation}}
\]

\[
\sigma = \sqrt{\frac{\sum x^2 - \left(\frac{\sum x}{n}\right)^2}{n}}
\]

\[
(\text{SEm}) = \frac{\sigma (\text{SD})}{\sqrt{n}}
\]

Statistical package for agricultural workers OPSTAT 1 Software of CCS HAU, Hisar was used for data analysis.