Materials and Methods
3. MATERIALS AND METHODS

3.1 Plant material

The seeds of *Withania somnifera* were collected from Centre for medicinal and aromatic plants, Division of Horticulture, University of Agricultural Sciences, Dharwad. Mature seeds of *Gymnema sylvestre* were collected from plants grown at Karnataka University campus, Dharwad.

*Withania somnifera* L. Dunal (Solanaceae) is an erect, grayish, tomentose, undershrub with 30-75 cm high (Figure 5A), long tuberous roots and is distributed throughout the drier sub-tropical regions of India. Leaves are alternate or sub-opposite, broadly ovate, sub-acute, entire, 5-10 x 2.5-7 cm. Flowers are small, greenish, axillary, solitary or in few flowered cymes and bisexual. Calyx is gamosepalous with 5 lobes, accrescent and inflated in a fruit. Corolla is campanulate, greenish-yellow with 5 lobes. 5 included stamens are present. Ovary is ovoid/globose, glabrous, many ovuled. Style is filiform and stigma is 2-lobed. Fruit is a globose berry, orange-red when ripe and enclosed in the enlarged calyx (Figure 5B). Seeds are many, discoid, yellow and reniform (Figure 5C).

*Gymnema sylvestre* R. Br. (Asclepiadaceae) is a large woody climber (Figure 14A), rooting at nodes, leaves elliptic, acuminate, base acute to acuminate, glabrous above sparsely or densely tomentose beneath; flowers small, in axillary and lateral umbel like cymes, pedicels long; calyx-lobes long, ovate, obtuse, pubescent; corolla pale yellow campanulate, valvate, corona single with 5 fleshly scales. Scales adnate to throat of corolla tube between lobes; anther connective produced into a membranous tip, pollinia 2, erect, carpels 2, unilocular; locules many ovule; follicle long, fusiform; fruit follicle (Figure 14B).
3.2 Chemicals and Glassware

Analytical grade mineral salts, vitamins (nicotinic acid, pyridoxine HCl, thiamine HCl), sucrose, glucose, fructose, maltose, agar, laboline, sodium hypochlorite, mercuric chloride, growth regulators like Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), 2,4-Dichlorophenoxyacetic acid (2,4-D), α-naphthalene acetic acid (NAA), N6-benzyladenine (BAP) and Kinetin (KN) were procured from Hi-Media Laboratories, Mumbai, India. Solvents like methanol and acetonitrile (AR and HPLC grade) were procured from Ranbaxy fine chemicals ltd, Mumbai, India. Micro filters were obtained from Sigma Chemicals, U.S.A. Glass wares were procured from Borosil, Mumbai, India and they were autoclaved before use.

3.3 Media Preparation

The medium was prepared using standard compositions of Chu (N6, 1978), Gamborg (B5, 1968), and Nitsch and Nitsch (NN, 1969), Murashige and Skoog (MS, 1962) media as mentioned in Table 11. Media was solidified with 0.8% agar and pH was adjusted to 5.8 using 0.1 N NaOH and HCl. The hot medium (15 ml) was dispensed into Borosil rimless culture tubes (15 x 2.5 cm) or Borosil flasks (100 ml) which were plugged with non-absorbent cotton wrapped in cheese cloth or caps and sterilized by autoclaving at 121 °C and 1.06 Kg/cm² pressure. Culture tubes, flasks, Petri plates, filter unit, autoclaved media, sterile distilled water and sterilized instruments were kept in laminar airflow chamber under UV light for 45-60 min before inoculation. Forceps and scalpels were dipped in 95% alcohol, flamed over spirit lamp and cooled before use at regular intervals.

3.4 Surface sterilization

The seeds of Withania somnifera were first washed under running tap water for 10 mins and they were rinsed with 70% alcohol for 1 min. The final step of
sterilization was carried out in a horizontal laminar air flow chamber by rinsing the seeds twice in sterile distilled water, followed by 0.1% mercuric chloride solution for 3 min. Finally the seeds were rinsed several times (x5) in sterile distilled water. The seeds were inoculated onto the MS basal medium (Murashige and Skoog, 1962) for germination.

The seeds of *Gymnema sylvestre* were first washed thoroughly under running tap water for 30 mins followed by several rinses in distilled water (x5). They were surface sterilized in sodium hypochlorite solution (2%, v/v) for 20 mins. After draining off the sodium hypochlorite solution, the final step of sterilization was carried out in a horizontal laminar air flow chamber by rinsing the seeds in sterile distilled water (x2), followed by 0.5% mercuric chloride solution for 5 min. Finally the seeds were rinsed several times (x5) in sterile distilled water. The seeds (Figure 14C) were inoculated onto the MS basal medium (Murashige and Skoog, 1962) for germination.
Table 11. Composition of MS (Murashige and Skoog, 1962), B5 (Gamborg et al., 1968), N6 (Chu, 1978) and NN (Nitsch and Nitsch, 1969) basal media.

<table>
<thead>
<tr>
<th>Component</th>
<th>MS</th>
<th>B5</th>
<th>N6</th>
<th>NN</th>
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<tr>
<td><strong>Major salts</strong></td>
<td>mg/l</td>
<td>mg/l</td>
<td>mg/l</td>
<td>mg/l</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1650</td>
<td>-</td>
<td>-</td>
<td>720</td>
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<tr>
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<td>2500</td>
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<tr>
<td>MgSO₄.7H₂O</td>
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<td>KH₂PO₄</td>
<td>170</td>
<td>-</td>
<td>400</td>
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<td>(NH₄)₂SO₄</td>
<td>-</td>
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<td>-</td>
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<tr>
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<td>-</td>
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<tr>
<td>ZnSO₄.7H₂O</td>
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<td>0.025</td>
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<td>mg/l</td>
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<td>Myo-inositol</td>
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<tr>
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<td>-</td>
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<td>6.4</td>
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<td>-</td>
<td>40</td>
<td>-</td>
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<td>5.8</td>
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3.5 Induction of callus from leaf explants of *Withania somnifera*.

To induce the callus from leaf explants of *Withania somnifera*, the following experiments were carried out

**Experiment 1**

Leaf explants obtained from 4 weeks old germinated seedling (Figure 5D) were inoculated on MS medium containing 3% sucrose alone and medium supplemented with auxins and cytokinins such as IAA, IBA, NAA, 2,4-D, BAP and KN at concentrations of 0.1, 0.5, 1.0, 2.0 and 5.0 mg l\(^{-1}\) individually for induction of callus.

**Experiment 2**

To evaluate the effect of auxins and cytokinins in combinations at various concentrations on callus induction, the leaf explants were cultured on MS medium containing 3% sucrose and supplemented with following combinations of auxins and cytokinins.

a. 2, 4-D (1.0 and 2.0 mg l\(^{-1}\)) + KN/ BAP (0.1, 0.5, 1.0 and 2.0 mg l\(^{-1}\)).

b. NAA (1.0 and 2.0 mg l\(^{-1}\)) + KN/ BAP (0.1, 0.5, 1.0 and 2.0 mg l\(^{-1}\)).

c. 2,4-D (1.0 and 2.0 mg l\(^{-1}\)) + NAA (0.1, 0.5, 1.0 and 2.0 mg l\(^{-1}\)).

d. NAA (1.0 and 2.0 mg l\(^{-1}\)) + 2,4-D (0.1, 0.5, 1.0 and 2.0 mg l\(^{-1}\)).

3.6 Establishment of cell suspension culture for the accumulation of biomass and withanolide-A production

To produce the withanolide-A from cell suspension cultures of *Withania somnifera*, the following experiments were carried out.
Experiment 3

To evaluate the effect of auxins on the accumulation of biomass in suspension cultures and the production of withanolide-A, the callus was cultured in MS liquid medium containing 3% sucrose and supplemented with various concentrations (0.1, 0.5, 1.0, 2.0 and 5.0 mg l\(^{-1}\)) of auxins (2,4-D, NAA and IBA).

Experiment 4

To evaluate the effect of cytokinins for the accumulation of biomass in suspension cultures and the production of withanolide-A, the callus was cultured in MS liquid medium supplemented with 2 mg l\(^{-1}\) 2,4-D and 3% sucrose and with various concentrations (0.1, 0.5, 1.0 and 2.0 mg l\(^{-1}\)) of cytokinins (BAP and KN).

Experiment 5

To study the kinetics of growth of cell suspension cultures and production of withanolide A, 0.5 g of cells were cultured in MS medium supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.5 mg l\(^{-1}\) KN and 3% sucrose.

Experiment 6

To study the effect of inoculum density on biomass accumulation and withanolide-A production, 2.5, 5.0, 10.0 and 20.0 g l\(^{-1}\) cells were cultured in MS liquid medium supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.5 mg l\(^{-1}\) KN and 3% sucrose for 4 weeks.

Experiment 7

To study the effect of different media on biomass accumulation and withanolide-A production, 0.5 g of cells were cultured in MS, B5, NN and N6 liquid medium supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.5 mg l\(^{-1}\) KN and 3% sucrose for 4 weeks.
Experiment 8

To study the effect of different medium strength on biomass accumulation and
withanolide-A production, 0.5 g of cells were cultured in MS liquid medium of
strength 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 and supplemented with 2.0 mg l⁻¹ 2,4-D + 0.5
mg l⁻¹ KN and 3% sucrose for 4 weeks.

Experiment 9

To study the effect of different carbohydrate sources on biomass accumulation
and withanolide-A production, 0.5 g of cells were cultured in MS liquid medium
supplemented with 2.0 mg l⁻¹ 2,4-D + 0.5 mg l⁻¹ KN and 3% sucrose, glucose,
fructose, maltose, glucose + fructose (1:1), fructose + sucrose (1:1) and sucrose +
glucose (1:1) individually for 4 weeks.

Experiment 10

To evaluate the effect of sucrose concentration on biomass accumulation and
withanolide-A production, 0.5 g of cells were cultured in MS liquid medium
supplemented with 2.0 mg l⁻¹ 2,4-D + 0.5 mg l⁻¹ KN and 1.0, 2.0, 3.0, 4.0, 6.0 and
8.0% sucrose individually for 4 weeks.

Experiment 11

To study the effect of different pH on biomass accumulation and withanolide-
A production, 0.5 g of cells were cultured in MS liquid medium supplemented with
2.0 mg l⁻¹ 2,4-D + 0.5 mg l⁻¹ KN and 3% sucrose at medium pH of 4.0, 4.5, 5.0, 5.5,
5.8, 6.0 and 6.5 individually for 4 weeks.

Experiment 12

To study the effect of different NH₄⁺/NO₃⁻ ratios on biomass accumulation and
withanolide-A production, 0.5 g of cells were cultured in MS liquid medium
containing NH₄⁺/NO₃⁻ ratios of 0.00/18.80, 7.19/18.80, 14.38/18.80, 21.57/18.80,
28.75/18.80, 14.38/0.00, 14.38/9.40, 14.38/18.80, 14.38/28.20 and 14.38/37.60 mM individually and supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.5 mg l\(^{-1}\) KN and 3% sucrose for 4 weeks.

**Experiment 13**

To study the effect of different concentrations of macro elements on biomass accumulation and withanolide-A production, 0.5 g of cells were cultured in MS liquid medium with 0.0, 0.5, 1.0, 1.5 and 2.0 x of NH\(_4\)NO\(_3\), KNO\(_3\), CaCl\(_2\), MgSO\(_4\) and KH\(_2\)PO\(_4\) supplemented individually with 2.0 mg l\(^{-1}\) 2,4-D + 0.5 mg l\(^{-1}\) KN and 3% sucrose for 4 weeks.

**3.7 Induction of adventitious roots from leaf explants of *Withania somnifera***

To induce adventitious root (untransformed root) from leaf explants of *Withania somnifera*, the following experiments were carried out.

**Experiment 14**

Leaf explants were inoculated on full strength and half strength MS medium containing 3% sucrose and medium supplemented with auxins such as IAA, IBA, NAA and 2,4-D, at concentrations of 0.1, 0.5, 1.0, 2.0 and 5.0 mg l\(^{-1}\) individually for induction of adventitious roots.

**3.8 Establishment of adventitious root suspension culture for the accumulation of biomass and withanolide-A production**

To produce the withanolide-A from adventitious root (untransformed root) suspension cultures of *Withania somnifera*, the following experiments were carried out.

**Experiment 15**

To study the kinetics of growth of adventitious root suspension cultures and production of withanolide-A, 0.5 g of adventitious roots were cultured in MS liquid medium supplemented with 0.5 mg l\(^{-1}\) IBA and 3% sucrose.
Experiment 16

To study the effect of inoculum density on biomass accumulation and withanolide-A production, 2.5, 5.0, 10.0 and 20.0 g l\(^{-1}\) adventitious roots were cultured in MS liquid medium supplemented with 0.5 mg l\(^{-1}\) IBA and 3% sucrose for 4 weeks.

Experiment 17

To study the effect of different media on biomass accumulation and withanolide-A production, 0.5 g of adventitious roots were cultured in MS, B5, NN and N6 liquid medium supplemented with 0.5 mg l\(^{-1}\) IBA and 3% sucrose for 4 weeks.

Experiment 18

To study the effect of different medium strength on biomass accumulation and withanolide-A production, 0.5 g of adventitious roots were cultured in MS liquid medium of strength 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 supplemented with 0.5 mg l\(^{-1}\) IBA and 3% sucrose for 4 weeks.

Experiment 19

To study the effect of different carbohydrate sources on biomass accumulation and withanolide-A production, 0.5 g of adventitious roots were cultured in MS liquid medium supplemented with 0.5 mg l\(^{-1}\) IBA and 3% of sucrose, glucose, fructose, maltose glucose + fructose (1:1), fructose + sucrose (1:1) and sucrose + glucose (1:1) individually for 4 weeks.

Experiment 20

To evaluate the effect of sucrose concentration on biomass accumulation and withanolide-A production, 0.5 g of adventitious roots were cultured in MS liquid
medium supplemented with 0.5 mg l⁻¹ IBA and 1.0, 2.0, 3.0, 4.0, 6.0 and 8.0% of sucrose individually for 4 weeks.

**Experiment 21**

To study the effect of different pH on biomass accumulation and withanolide-A production, 0.5 g of adventitious roots were cultured in MS liquid medium supplemented with 0.5 mg l⁻¹ IBA and 3% sucrose with medium pH of 4.0, 4.5, 5.0, 5.5, 5.8, 6.0 and 6.5 individually for 4 weeks.

**Experiment 22**

To study the effect of different NH₄⁺/NO₃⁻ ratios on biomass accumulation and withanolide-A production, 0.5 g of adventitious roots were cultured in MS liquid medium with NH₄⁺/NO₃⁻ ratios of 0.00/18.80, 7.19/18.80, 14.38/18.80, 21.57/18.80, 28.75/18.80, 14.38/0.00, 14.38/9.40, 14.38/18.80, 14.38/28.20 and 14.38/37.60 mM and supplemented with 0.5 mg l⁻¹ IBA and 3% sucrose for 4 weeks.

**Experiment 23**

To study the effect of different concentrations of macro elements on biomass accumulation and withanolide-A production, 0.5 g of adventitious roots were cultured in MS liquid medium with 0.0, 0.5, 1.0, 1.5 and 2.0 x of NH₄NO₃, KNO₃, CaCl₂, MgSO₄ and KH₂PO₄ individually and medium supplemented with 0.5 mg l⁻¹ IBA and 3% sucrose for 4 weeks.

**3.9 Induction of hairy roots from seedling explants of Withania somnifera**

**Experiment 24**

**3.9.1 Plant material**

*In vivo* leaf explants and *in vitro* seedlings (4 weeks old plantlet) were used for the induction of hairy roots from *Agrobacterium rhizogenes*. 

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3.9.2 Bacterial cultures

*Agrobacterium rhizogenes* strain R1601, carrying pRiA4b with a chimaeric *nptII* gene co-integrated into *HindIII* fragment 21 of the TL-DNA, with pTVK291 in trans conferring a supervirulent phenotype (Pythoud et al. 1987), was used in the present experiments. Bacteria were cultured on semi-solid Luria Bertani (LB) medium (Sambrook et al. 1989), supplemented with kanamycin sulfate (100 mg l\(^{-1}\)) and ampicillin (100 mg l\(^{-1}\)).

3.9.3 Transformation and establishment of transformed root cultures

Cotyledons and leaves (5 × 5 mm), roots, stems, hypocotyls and cotyledonary nodal segments (each 5 mm in length) were immersed in an overnight grown bacterial suspension (OD 600nm =1.0) of *A. rhizogenes* strain R1601 for 30 min. Explants were blotted dry on sterile filter papers (Whatman No. 1) and placed on the surface of 20-ml aliquots of MS-based semi-solid medium (0.8% (w/v) agar; Sigma-Aldrich) contained in 9-cm diameter Petri dishes. Inoculated explants were maintained at 25±2°C with a 16 h photoperiod. Two days after co-cultivation, explants were transferred to MS-basal medium supplemented with 400 mg l\(^{-1}\) cefotaxime (Claforan; Roussel Laboratories, Uxbridge, UK). Four weeks post-inoculation, roots (each 1.5–2.0 cm in length), which developed on the explants were excised aseptically from the explants and transferred onto the surface of 20-ml aliquots of MS-basal medium supplemented with 400 mg l\(^{-1}\) cefotaxime and 100 mg l\(^{-1}\) kanamycin sulfate, contained in 9 cm diameter Petri dishes. Cultures were incubated under the same conditions as described above. Roots were grown for 28 d, before 500 mg (fresh weight (FW)) of roots were transferred into 50-ml aliquots of liquid MS-basal medium lacking growth regulators in 250-ml Erlenmeyer flasks. Cultures were placed on a horizontal shaker (100 rpm), and incubated under the same conditions as before.
Roots were subcultured every 28 d. The concentrations of cefotaxime and kanamycin sulfate were reduced progressively. At the third passage, roots were transferred to 50-ml aliquots of MS-based liquid medium lacking cefotaxime and kanamycin sulfate. Roots excised from in vitro germinated seedlings were cultured in growth regulator-free MS-based liquid medium as controls.

3.9.4 Detection of transgenes in *Agrobacterium*-induced roots

3.9.4.1 Polymerase chain reaction analysis: plasmid RiA4b was used as a positive control. Plasmid DNA was isolated from 12 h grown cultures of *A. rhizogenes* (OD600nm =0.8) using a Wizard plus Miniprep Kit (Promega, Southampton, UK). Genomic DNAs of the nontransformed roots and transformed roots were isolated by using a GenElute Plant Genomic DNA Kit (Sigma-Aldrich). PCR was carried out to screen for the presence of *nptII* and *rolB* genes in the T-DNA of pRiA4b and the genomic DNAs of transformed and non-transformed roots. Primers for amplification of the 261 bp *nptII* gene fragment were 5'-AGACAATCGGCTGCTCTGAT- 3' and 5'- TACTTTTCTCGGCAGGAGCA-3'. The primers for amplification of an 863 bp *rolB* gene fragment were 5'-TACTGCAGCAGGCTTCATGCA-3' and 5'-GCTTTCCGACCAGAGACTG-3'. PCR was carried out using RED Taq Ready Mix (Sigma-Aldrich) according to the manufacturer’s instructions. Amplification was carried out in a DNA Thermal Cycler 480 (Perkin Elmer Applied Biosystems Division, Warrington, UK), with initial denaturing (one cycle, 94°C, 3 min), denaturing (35 cycles, 94°C, 1 min), primer annealing (35 cycles; 57°C (*nptII*) or 55°C (*rolB*), 1 min), primer extension (35 cycles, 72°C, 90 s), final extension (one cycle, 72°C, 10 min) and holding at 4°C (5 min to∞). Amplified PCR products were examined by electrophoresis in 1.2% (w/v) agarose gels.
3.9.4.2 Southern hybridization

Genomic DNA was isolated using a GenElute Plant Genomic DNA Kit (Sigma-Aldrich). The integration of foreign genes into the host genome was determined by Southern blot analysis (Holtke et al. 1995). A 10-μg aliquot of pRiA4b and 15 μg of plant DNA were digested overnight at 37°C with HindIII, and separated on 0.8% (w/v) agarose gels. DNA was transferred to nylon membranes (Roche Diagnostics Ltd., Lewes, UK) and later prehybridized in DIG-Easy-Hyb buffer (Roche, Mannheim, Germany) at 68°C for 1 h. For hybridization, 10 ml of DIG-Easy-Hyb, to which had been added DIG-labeled DNA probes (100 ng ml⁻¹; Roche), were used per 100 cm² of membrane. Hybridization was at 68°C for 16 h. Membranes were washed for 2 × 5 min in 2X standard saline citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) (Roche) and for 2 × 15 min in 0.1 × SSC/0.1% SDS at 68°C, preincubated for 45 min in blocking solution (Roche) and incubated for 30 min with anti-DIG-AP conjugate (diluted 1:10 000, v:v). Two 15 min washing steps preceded equilibration in detection buffer for 5 min. Detection involved CDPStar (Roche) with a 20 min exposure to X-ray film (Kodak).

3.10 Establishment of hairy root suspension cultures for the production of withanolide-A

To produce the withanolide-A from hairy root (transformed root) suspension cultures of *Withania somnifera*, the following experiments were carried out.

*Experiment 25*

To study the kinetics of growth of hairy root suspension cultures and production of withanolide-A, 0.5 g of hairy roots were cultured in MS liquid medium supplemented with 3% sucrose.
Experiment 26

To study the effect of inoculum density on biomass accumulation and withanolide-A production, 2.5, 5.0, 10.0 and 20.0 g l\(^{-1}\) of hairy roots were cultured in MS liquid medium supplemented with 3% sucrose for 4 weeks.

Experiment 27

To study the effect of different media on biomass accumulation and withanolide-A production, 0.5 g of hairy roots were cultured in MS, B5, NN and N6 liquid medium supplemented with 3% sucrose for 4 weeks.

Experiment 28

To study the effect of different medium strength on biomass accumulation and withanolide-A production, 0.5 g of hairy roots were cultured in MS liquid medium of strength 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 supplemented with 3% sucrose for 4 weeks.

Experiment 29

To study the effect of different carbohydrate sources on biomass accumulation and withanolide-A production, 0.5 g of hairy roots were cultured in MS liquid medium supplemented with 3% of sucrose, glucose, fructose, maltose glucose + fructose (1:1), fructose + sucrose (1:1) and sucrose + glucose (1:1) individually for 4 weeks.

Experiment 30

To evaluate the effect of sucrose concentration on biomass accumulation and withanolide-A production, 0.5 g of hairy roots were cultured in MS liquid medium supplemented with 1.0, 2.0, 3.0, 4.0, 6.0 and 8.0% of sucrose individually for 4 weeks.
Experiment 31

To study the effect of different pH on biomass accumulation and withanolide-A production, 0.5 g of hairy roots were cultured in MS liquid medium supplemented with 3% sucrose with medium pH of 4.0, 4.5, 5.0, 5.5, 5.8, 6.0 and 6.5 individually for 4 weeks.

Experiment 32

To study the effect of different NH$_4^+$/NO$_3^-$ ratios on biomass accumulation and withanolide-A production, 0.5 g of hairy roots were cultured in MS liquid medium with NH$_4^+$/NO$_3^-$ ratios of 0.00/18.80, 7.19/18.80, 14.38/18.80, 21.57/18.80, 28.75/18.80, 14.38/0.00, 14.38/9.40, 14.38/18.80, 14.38/28.20 and 14.38/37.60 mM and supplemented with 3% sucrose for 4 weeks.

Experiment 33

To study the effect of different concentrations of macro elements on biomass accumulation and withanolide-A production, 0.5 g of hairy roots were cultured in MS liquid medium with 0.0, 0.5, 1.0, 1.5 and 2.0 x of NH$_4$NO$_3$, KNO$_3$, CaCl$_2$, MgSO$_4$ and KH$_2$PO$_4$ individually and medium supplemented with 3% sucrose for 4 weeks.

3.11 Induction of callus from leaf explants of Gymnema sylvestre

To induce the callus from leaf explants of Gymnema sylvestre, the following experiments were carried out

Experiment 34

Leaf explants obtained from 4 weeks old germinated seedling (Figure 14D) were inoculated on MS medium containing 3% sucrose and medium supplemented with auxins and cytokinins such as IAA, IBA, NAA, 2,4- D, BAP and
KN at concentrations of 0.1, 0.5, 1.0, 2.0 and 5.0 mg l\(^{-1}\) individually for induction of callus.

**Experiment 35**

To evaluate the effect of auxins and cytokinins in combinations at various concentrations, the leaf explants were cultured on MS medium containing 3% sucrose and supplemented with following combinations of auxins and cytokinins.

a. 2, 4-D (1.0 and 2.0 mg l\(^{-1}\)) + KN/ BAP (0.1, 0.5, 1.0 and 2.0 mg l\(^{-1}\)).

b. NAA (2.0 and 5.0 mg l\(^{-1}\)) + KN/ BAP (0.1, 0.5, 1.0 and 2.0 mg l\(^{-1}\)).

c. 2,4-D (1.0 and 2.0 mg l\(^{-1}\)) + NAA (0.1, 0.5, 1.0 and 2.0 mg l\(^{-1}\)).

d. NAA (2.0 and 5.0 mg l\(^{-1}\)) + 2,4-D (0.1, 0.5, 1.0 and 2.0 mg l\(^{-1}\)).

**3.12 Establishment of cell suspension culture for the accumulation of biomass and gymnemic acid production**

To produce the gymnemic acid from cell suspension cultures of *Gymnema sylvestre*, the following experiments were carried out.

**Experiment 36**

To evaluate the effect of auxins for the accumulation of biomass and gymnemic acid production in suspension cultures, the callus was cultured in MS liquid medium containing 3% sucrose and supplemented with various concentrations (0.1, 0.5, 1.0, 2.0 and 5.0 mg l\(^{-1}\)) of auxins (2,4-D, NAA and IBA).

**Experiment 37**

To evaluate the effect of cytokinins for the accumulation of biomass and gymnemic acid production in suspension cultures, the callus was cultured in MS liquid medium supplemented with 2 mg l\(^{-1}\) 2,4-D and 3% sucrose and with various concentrations (0.1, 0.5, 1.0 and 2.0 mg l\(^{-1}\)) of cytokinins (BAP and KN).
**Experiment 38**

To study the kinetics of growth of cell suspension cultures and production of gymnemic acid, 0.5 g of cells were cultured in MS medium supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.1 mg l\(^{-1}\) KN and 3% sucrose.

**Experiment 39**

To study the effect of inoculum density on biomass accumulation and gymnemic acid production, 2.5, 5.0, 10.0 and 20.0 g l\(^{-1}\) cells were cultured in MS liquid medium supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.1 mg l\(^{-1}\) KN and 3% sucrose for 4 weeks.

**Experiment 40**

To study the effect of different media on biomass accumulation and gymnemic acid production, 0.5 g of cells were cultured in MS, B5, NN and N6 liquid medium supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.1 mg l\(^{-1}\) KN and 3% sucrose for 4 weeks.

**Experiment 41**

To study the effect of different medium strength on biomass accumulation and gymnemic acid production, 0.5 g of cells were cultured in MS liquid medium of strength 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 and supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.1 mg l\(^{-1}\) KN and 3% sucrose for 4 weeks.

**Experiment 41**

To study the effect of different carbohydrate sources on biomass accumulation and gymnemic acid production, 0.5 g of cells were cultured in MS liquid medium supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.1 mg l\(^{-1}\) KN and 3% sucrose, glucose, fructose, maltose, glucose + fructose (1:1), fructose + sucrose (1:1) and sucrose + glucose (1:1) individually for 4 weeks.
**Experiment 42**

To evaluate the effect of sucrose concentration on biomass accumulation and gymnemic acid production, 0.5 g of cells were cultured in MS liquid medium supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.1 mg l\(^{-1}\) KN and 1.0, 2.0, 3.0, 4.0, 6.0 and 8.0\% sucrose individually for 4 weeks.

**Experiment 43**

To study the effect of different pH on biomass accumulation and gymnemic acid production, 0.5 g of cells were cultured in MS liquid medium supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.1 mg l\(^{-1}\) KN and 3\% sucrose at medium pH of 4.0, 4.5, 5.0, 5.5, 5.8, 6.0 and 6.5 individually for 4 weeks.

**Experiment 44**

To study the effect of different NH\(_4^+\)/NO\(_3^-\) ratios on biomass accumulation and gymnemic acid production, 0.5 g of cells were cultured in MS liquid medium containing NH\(_4^+\)/NO\(_3^-\) ratios of 0.00/18.80, 7.19/18.80, 14.38/18.80, 21.57/18.80, 28.75/18.80, 14.38/0.00, 14.38/9.40, 14.38/18.80, 14.38/28.20 and 14.38/37.60 mM individually and supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.1 mg l\(^{-1}\) KN and 3\% sucrose for 4 weeks.

**Experiment 45**

To study the effect of different concentrations of macro elements on biomass accumulation and gymnemic acid production, 0.5 g of cells were cultured in MS liquid medium with 0.0, 0.5, 1.0, 1.5 and 2.0 x of NH\(_4\)NO\(_3\), KNO\(_3\), CaCl\(_2\), MgSO\(_4\) and KH\(_2\)PO\(_4\) supplemented individually with 2.0 mg l\(^{-1}\) 2,4-D + 0.1 mg l\(^{-1}\) KN and 3\% sucrose for 4 weeks.
3.13 Induction of adventitious roots from leaf explants of *Gymnema sylvestre*

To induce adventitious root (untransformed root) from leaf explants of *Gymnema sylvestre*, the following experiments were carried out.

**Experiment 46**

Leaf explants were inoculated on full strength and half strength MS medium containing 3% sucrose alone and medium supplemented with auxins such as IAA, IBA, NAA and 2,4-D, at concentrations of 0.1, 0.5, 1.0, 2.0 and 5.0 mg l⁻¹ individually for induction of adventitious roots.

**Experiment 47**

Leaf explants were inoculated on half strength MS medium containing 3% sucrose and medium supplemented with NAA (0.5, 1.0, 2.0 and 5.0) in combination with IBA at concentrations of 0.1, 0.5, 1.0 and 2.0 mg l⁻¹ for induction of adventitious roots.

3.14 Induction of hairy roots from leaf explants of *Gymnema sylvestre*

Leaf (5 x 5 mm) explants were immersed in an overnight grown bacterial suspension (OD 600nm =0.6-1.2) of *A. rhizogenes* strain ATCC 15834 for 5, 10, 15, 30 and 60 min. Explants were blotted dry on sterile filter papers (Whatman No. 1) and placed on the surface of 20-ml aliquots of MS-based semi-solid medium (0.8% (w/v) agar; Sigma-Aldrich) contained in 9-cm diameter Petri dishes. Inoculated explants were maintained at 25±2°C with a 16 h photoperiod. One to two days for cocultivation, explants were transferred to MS-basal medium supplemented with 400 mg l⁻¹ cefotaxime (Claforan; Roussel Laboratories, Uxbridge, UK). The explants were cultured in light and dark conditions at 25±2°C.
3.15 Culture conditions

The cultures were incubated at 25±2°C temperature and 16 h photoperiod of 40 μmol m⁻² s⁻¹ light provided by cool white fluorescent tubes (Philips, India) for callus induction and adventitious root induction. The suspension cultures were placed in an orbital shaker (Scigenics) at 100 rpm and the temperature was maintained at 25±2°C and 16 h photoperiod of 40 μmol m⁻² s⁻¹ light provided by cool white fluorescent tubes.

3.16 Extraction of the withanolide-A

The extraction of withanolide was done following the method of Ganzera et al., (2003) with some modifications. Cell suspension and root cultures were dried overnight in an oven at 60°C. The dried, powdered material (200 mg) was extracted with 2 ml methanol by sonication for 30 mins at room temperature. Methanolic extracts were evaporated to dryness in a vacuum oven. For analysis, the remainder was redissolved in 1 ml of HPLC grade methanol and transferred to a polypropylene microcentrifuge tube, vortexed for 10s and centrifuged for 5 min at 3000 X g. After centrifugation, the clear supernatant was filtered through 0.45 μm nylon membrane filter (Sigma, USA) and was used for the HPLC analysis.

3.17 HPLC analysis of withanolide-A

The HPLC analysis of withanolide was done following the method of Ganzera et al., (2003) with some modifications. The analytical HPLC experiments were performed with a Waters High Performance Liquid Chromatography (HPLC) equipped with a variable dual wavelength detector operating at 230 nm (W 2487). Separations was carried out with C18 (5 μm) column with reagent alcohol : water (80 : 20) as an eluent at a flow rate of 1 ml min⁻¹ and the column temperature was
maintained at 27°C. Withanolide A standard was obtained from Chromadex (Laguna Hills, CA, USA). Validation of quantitative method was performed with samples for five injections of 20 µl each.

### 3.18 Extraction of gymnemic acid

The extraction of gymnemic acid was done following the standard protocol of Chromadex Inc. (Laguna Hills, CA, USA). 500 mg of sample was weighed into a 500 ml round bottom flask and 50 ml of extraction solvent (1:1 volume of methanol : water) and 10 ml of 11% potassium hydroxide solution was added. The mixture was refluxed for an hour; 9 ml of conc. HCl was added and refluxed again for one hour. The mixture was cooled to room temperature, the extract was filtered through 0.45 µm nylon filter (Sigma, USA) and the volume was made up to 100 ml with extraction solvent and the clean supernatant was used for HPLC analysis.

### 3.19 HPLC analysis of gymnemic acid

The HPLC analysis of gymnemic acid was done following the standard protocol of Chromadex Inc. (Laguna Hills, CA, USA) with some modifications. The analytical HPLC experiments were performed with a Waters High Performance Liquid Chromatography (HPLC) equipped with a variable dual wavelength detector operating at 210 nm (W 2487). Separations was carried out with C18 (5 µm) column with acetonitrile : water (80 : 20) as an eluent at a flow rate of 1 ml min⁻¹ and the column temperature was maintained at 27°C. Gymnemic acid standard was obtained from Chromadex (Laguna Hills, CA, USA). Validation of quantitative method was performed with samples for five injections of 20 µl each. The conversion of Gymnemagenin to Gymnemic acid was done using the formula,

\[
\text{Molecular weight conversion of Gymnemagenin to Gymnemic acid (809.0/506.7)} = \text{conversion.}
\]
3.20 Data collection and analysis

For callus and adventitious root induction, 12 explants (12 replicated test tubes containing one explant each) were cultured in each treatment and each experiment was repeated twice. The cell, adventitious and hairy root suspension cultures were maintained in triplicates and each experiment was repeated twice. The cultures were observed periodically and morphological changes were recorded at weekly intervals. The number of explants producing callus or adventitious roots was expressed as percentage of responding explants. The HPLC analysis was performed in triplicates. All the data was statistically analyzed by variance (ANOVA) using SPSS software and mean values were separated according to Duncan's multiple range test at $P < 0.05$ level.