Summary and Conclusion
6. SUMMARY AND CONCLUSIONS

Secondary metabolites are the natural chemical compounds synthesized by the plants as the byproduct during the primary metabolism. These secondary metabolites are low molecular weight compounds with unique complex structures. The numerous plant secondary metabolites such as alkaloids, anthocyanins, flavonoids, quinines, lignans, steroids and terpenoids have found commercial application as pharmaceuticals, agronomical, flavours, fragrance, colours, biopesticides and food additives.

Plant secondary metabolites are currently isolated from naturally growing medicinal plants or by cultivation. However accumulation of plant secondary metabolites is often restricted to a species or genus and might be activated only during a particular growth or developmental stage, or under specific seasonal, stress or nutrient availability conditions. The cell, tissue and organ culture technologies would help in producing the active compounds in vitro with better productivities without cutting down the natural resources. Recombinant DNA technologies would also supplement plant cell technologies.

Withania somnifera (L.) Dunal also known as ashwagandha, Indian ginseng and winter cherry belongs to the family Solanaceae. The roots of the plant contain biologically active chemical constituents (alkaloids, steroidal compounds) including ergostane type steroidal lactone, withaferin A, withanolides A-Z, withasominiferin A etc. The withanolides like withaferin A and withanolide-A of the plant have demonstrated to possess therapeutic action in carcinogenesis, Parkinson’s disease and Alzheimer’s disease.

Gymnema sylvestre R. Br. is known as ‘Gur-mur’ belongs to family Asclepiadaceae. The leaves of the species contain triterpene saponins belonging to the oleanane and dammarenene classes known as gymnemic acid, gymnemasaponins and gymnemasides. These compounds are used against diabetes.
Attempts have been made for the production of in vitro production of secondary metabolites from *Withania somnifera* and *Gymnema sylvestre*, but there are no reports on the optimization of the physical and chemical parameters for the production of metabolites. In view of this, the present work deals with the establishment of cell, adventitious roots and transformed root suspension cultures and subsequent optimization of the physical and chemical parameters for the production of withanolide A from *Withania somnifera*. With respect to *Gymnema sylvestre*, experiments were carried out to establish cell suspension culture and subsequent optimization of the physical and chemical parameters for the production of gymnemic acid.

### 6.1.1 Effect of auxins in combination with cytokinins.

Leaf explants of *Withania somnifera* were cultured on MS medium supplemented with auxins such as 2,4-D, NAA, IBA and IAA, and cytokinins such as BAP and KN individually at concentrations of 0.1, 0.5, 1.0, 2.0 and 5.0 mg l\(^{-1}\) and 3% sucrose. The highest percentage of 91.66% of callus formation was observed in the explants cultured on 2.0 mg l\(^{-1}\) 2,4-D. Highest callus production of 3.45 g in fresh weight (FW) and 0.330 g of dry weight (DW) was observed in the explants cultured on 2.0 mg l\(^{-1}\) 2,4-D followed by 2.0 mg l\(^{-1}\) NAA which yielded 3.23 g FW of callus and 0.310 g of DW (Table 12).

The highest percentage of 83.33% of callus induction was observed on medium containing 2.0 mg l\(^{-1}\) KN followed by 2.0 mg l\(^{-1}\) BAP, 1.0 mg l\(^{-1}\) KN and 5.0 mg l\(^{-1}\) KN (80.55%) (Table 13). The highest amount of callus was observed with the explants cultured on medium supplemented with 1.0 mg l\(^{-1}\) KN which weighed 1.88 g of FW and 0.178 g of DW, followed by 1.0 mg l\(^{-1}\) BAP, which yielded 1.75 g of FW and 0.162 g of DW.
6.1.2 Effect of auxins in combination with cytokinins.

The leaf explants were cultured on MS medium supplemented with 2,4-D/NAA (1.0 and 2.0 mg \text{l}^{-1}) in combination with cytokinins such as BAP and KN (0.1, 0.5, 1.0 and 2.0 mg \text{l}^{-1}). The percentage of responding leaf explants differed with varied auxin and cytokinin concentration. The optimum amount of callus was obtained in the culture medium supplemented with 2.0 mg \text{l}^{-1} 2,4-D + 0.5 mg \text{l}^{-1} KN, which yielded 4.45 g of FW and 0.428 g of DW, followed by 1.0 mg \text{l}^{-1} 2,4-D + 0.1 mg \text{l}^{-1} KN, 2.0 mg \text{l}^{-1} 2,4-D + 0.1 mg \text{l}^{-1} KN and 1.0 mg \text{l}^{-1} 2,4-D + 0.5 mg \text{l}^{-1} KN which yielded 3.95 g of FW and 0.38 g of DW, 3.98 g of FW and 0.375 g DW and 4.08 g FW and 0.398 g DW respectively.

The effect of NAA (1.0 and 2.0 mg \text{l}^{-1}) in combination with cytokinins (BAP and KN at concentrations of 0.1, 0.5, 1.0 and 2.0 mg \text{l}^{-1}) on callus formation was studied. The highest number of explants (83.33\%) developed callus on the medium supplemented with 2.0 mg \text{l}^{-1} NAA alone and in combination of 2.0 mg \text{l}^{-1} NAA + 1.0 mg \text{l}^{-1} KN (83.33). The highest amount of callus was observed in the medium supplemented with 2.0 mg \text{l}^{-1} NAA + 1.0 mg \text{l}^{-1} KN, which yielded 3.75 g of FW and 0.360 g of DW, followed by 2.0 mg \text{l}^{-1} NAA + 1.0 mg \text{l}^{-1} BAP which produced about 3.55 g of FW and 0.340 g of DW.

6.1.3 Effect of 2,4-D in combination with NAA

The effect of 2,4-D (1.0 and 2.0 mg \text{l}^{-1}) in combination with NAA (0.1, 0.5, 1.0 and 2.0 mg \text{l}^{-1}) for callus induction was studied. The highest responding explants was observed on medium supplemented with 2.0 mg \text{l}^{-1} 2,4-D (91.66\%) alone followed by 1.0 mg \text{l}^{-1} 2,4-D and 2.0 mg \text{l}^{-1} 2,4-D + 1.0 mg \text{l}^{-1} NAA (83.33\%). The combination of 2.0 mg \text{l}^{-1} 2,4-D + 1.0 mg \text{l}^{-1} NAA induced the highest production of callus in terms of FW (3.90 g) and DW (0.375 g) followed by 1.0 mg \text{l}^{-1} 2,4-D + 2.0 mg \text{l}^{-1} NAA, which produced 3.78 g of FW and 0.362 g of DW.
6.1.4 Effect of NAA in combination with 2,4-D

The effect of NAA (1.0 and 2.0 mg l\(^{-1}\)) in combination with 2,4-D (0.1, 0.5, 1.0 and 2.0 mg l\(^{-1}\)) on callus formation was studied. The highest responding explants was observed on 2.0 mg l\(^{-1}\) NAA and 1.0 mg l\(^{-1}\) NAA + 2.0 mg l\(^{-1}\) 2,4-D supplemented medium (83.33%) followed by 1.0 mg l\(^{-1}\) NAA containing medium (81.66%). The combination of 1.0 mg l\(^{-1}\) NAA + 2.0 mg l\(^{-1}\) 2,4-D resulted in the highest production of callus with 3.90 g of FW and 0.375 g of DW.

6.2 Production of withanolide-A from cell suspension cultures of *Withania somnifera*

6.2.1 Effect of auxins on biomass accumulation and withanolide-A production in cell suspension culture.

The effect of different concentrations of auxins (0.1, 0.5, 1.0, 2.0 and 5.0 mg l\(^{-1}\)) on the biomass accumulation and withanolide-A production was studied. The highest accumulation of biomass (92.63 g l\(^{-1}\) FW and 8.11 g l\(^{-1}\) DW) with a growth ratio of 7.37 and the highest production of withanolide-A (1.27 mg g\(^{-1}\) DW) was observed in the medium supplemented with 2.0 mg l\(^{-1}\) 2,4-D, followed by 5.0 mg l\(^{-1}\) 2,4-D which accumulated 91.15 g l\(^{-1}\) of FW and 7.96 g l\(^{-1}\) DW with a growth ratio of 7.24 and withanolide-A production was 1.19 mg g\(^{-1}\) DW.

6.2.2 Effect of cytokinins in combination with 2.0 mg l\(^{-1}\) 2,4-D on biomass accumulation and withanolide-A production in cell suspension culture.

The effect of 2,4-D (2.0 mg l\(^{-1}\)) in combination with cytokinins (BAP and KN at concentrations of 0.1, 0.5, 1.0 and 2.0 mg l\(^{-1}\)) on biomass accumulation and withanolide-A production was studied. The maximum accumulation of biomass (118.40 g l\(^{-1}\) FW and 10.79 g l\(^{-1}\) DW) with a growth ratio of 9.48 was observed in the medium supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.5 mg l\(^{-1}\) KN with a withanolide-A production of 2.26 mg g\(^{-1}\) DW.
6.2.3 Growth kinetics of *Withania somnifera* cell suspension cultures.

The growth kinetics and withanolide-A production from cell suspension cultures was studied. The media was supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.5 mg l\(^{-1}\) KN and 3% sucrose. The maximum accumulation of biomass (124.17 g l\(^{-1}\) FW and 11.02 g l\(^{-1}\) DW) with a growth ratio of 10.02 was recorded after four weeks of culture initiation. The production of withanolide-A was maximum (2.03 mg g\(^{-1}\) DW) at the end of four weeks and declined thereafter and reached (1.13 mg g\(^{-1}\) DW) at the end of five weeks.

6.2.4 Effect of inoculum density on biomass accumulation and withanolide-A production

The effect of inoculum density (2.5, 5.0, 10.0 and 20.0 g l\(^{-1}\)) on biomass accumulation and withanolide-A production from cell suspension cultures was studied. The media was supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.5 mg l\(^{-1}\) KN and 3% sucrose. 10 g l\(^{-1}\) inoculum was found suitable and in this medium maximum biomass was recorded (119.08 g l\(^{-1}\) of FW and 10.88 g l\(^{-1}\) DW) at a growth ratio of 9.89. The highest production of withanolide-A (2.42 mg g\(^{-1}\) DW) was recorded at 10.0 g l\(^{-1}\) of inoculum density.

6.2.5 Effect of different media on biomass accumulation and withanolide-A production

Various media such as MS, B5, NN and N6 were tested on the biomass accumulation and withanolide-A production from cell suspension cultures of *Withania somnifera*. All the media were supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.5 mg l\(^{-1}\) KN and 3% sucrose. Highest accumulation of biomass with respect to FW (126.80 g l\(^{-1}\)) and DW (11.79 g l\(^{-1}\)) was observed in the MS medium with a growth ratio of 10.72. The highest production of 2.39 mg g\(^{-1}\) DW of withanolide-A was recorded in the MS medium.
6.2.6 Effect of medium strength on biomass accumulation and withanolide-A production

The effect of medium strength (0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 MS medium) on the biomass accumulation and withanolide-A production from cell suspension cultures was studied. The media was supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.5 mg l\(^{-1}\) KN and 3% sucrose. The highest accumulation of biomass (138.52 g l\(^{-1}\) of FW and 12.13 g l\(^{-1}\) DW) was observed in the full strength medium. Full strength MS medium favoured the production of withanolide-A which recorded 2.35 mg g\(^{-1}\) DW.

6.2.7 Effect of different carbohydrate sources on biomass accumulation and withanolide-A production

The effect of different carbohydrate sources (sucrose, glucose, fructose, maltose, glucose + fructose, fructose + sucrose and sucrose + glucose at 3%) on the biomass accumulation and withanolide-A content from cell suspension cultures was studied. The media was supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.5 mg l\(^{-1}\) KN. Sucrose was found to be the ideal carbohydrate source for the biomass accumulation, which yielded the biomass of 115.63 g l\(^{-1}\) FW and 10.47 g l\(^{-1}\) DW. The highest production of withanolide-A content (2.95 mg g\(^{-1}\) DW) was recorded in the medium supplemented with sucrose.

6.2.8 Effect of sucrose concentration on biomass accumulation and withanolide-A production

The effect of different sucrose concentration (1, 2, 3, 4, 6 and 8%) on the biomass accumulation and withanolide-A production from cell suspension cultures was studied. The media was supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.5 mg l\(^{-1}\) KN. The optimum of 4% sucrose favoured the biomass accumulation of 134.94 g l\(^{-1}\) FW and
Summary and Conclusions

10.75 g l\(^{-1}\) DW. The highest production of withanolide-A (2.95 mg g\(^{-1}\) DW) was recorded in the medium supplemented with 3% sucrose.

6.2.9 Effect of hydrogen ion concentration (pH) on biomass accumulation and withanolide-A production

The effect of hydrogen ion concentration (pH) (pH; 4.0, 4.5, 5.0, 5.5, 5.8, 6.0 and 6.5) on the biomass accumulation and withanolide-A production from cell suspension culture were studied. The media was supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.5 mg l\(^{-1}\) KN and 3% sucrose. The highest accumulation of biomass in terms of FW and DW was recorded in the medium set at the pH of 5.8 and 6.0 and cells grown in this medium accumulated 119.34 g l\(^{-1}\) FW and 10.27 g l\(^{-1}\) DW at pH 5.8 and 115.98 g l\(^{-1}\) FW and 10.15 g l\(^{-1}\) DW at pH 6.0. The highest production of withanolide-A (2.51 mg g\(^{-1}\) DW) content was recorded at the pH level of 6.0.

6.2.10 Effect of NH\(_4^+\)/NO\(_3^-\) ratios on biomass accumulation and withanolide-A production

The effect of different NH\(_4^+\)/NO\(_3^-\) concentrations (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) on the biomass accumulation and withanolide-A production from cell suspension cultures were studies. The media was supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.5 mg l\(^{-1}\) KN and 3% sucrose. The lower concentration (7.19 mM) of NH\(_4^+\) with moderate concentration (18.80 mM) of NO\(_3^-\) favoured the highest accumulation of biomass yielding 110.45 g l\(^{-1}\) FW and 9.29 g l\(^{-1}\) DW, followed by NH\(_4^+\) free medium with moderate concentration (18.80 mM) of NO\(_3^-\), which yielded 100.12 g l\(^{-1}\) FW and 8.47 g l\(^{-1}\) DW of biomass. The highest production of withanolide-A content was recorded at highest concentration of NO\(_3^-\) (37.60 mM), which produced 3.96 mg g\(^{-1}\) DW.
6.2.11 Effect of different concentrations of macro elements on biomass accumulation and withanolide-A production

The effects of different concentrations of macro elements (NH$_4$NO$_3$, KNO$_3$, CaCl$_2$, MgSO$_4$ and KH$_2$PO$_4$ at 0.0, 0.5, 1.0, 1.5 and 2.0 X) on the accumulation of biomass and withanolide-A production of cell suspension cultures were tested. All the media was supplemented with 2.0 mg l$^{-1}$ 2,4-D + 0.5 mg l$^{-1}$ KN and 3% sucrose. The highest accumulation of biomass (147.81 g l$^{-1}$ FW and 14.02 g l$^{-1}$ DW) was recorded in the medium containing 0.5 X NH$_4$NO$_3$ and in the 0.5 X KNO$_3$ which yielded the biomass of 143.75 g l$^{-1}$ FW and 14.33 g l$^{-1}$ DW. The highest production of withanolide-A was recorded in the medium with 2.0 X KNO$_3$ which produced 4.36 mg g$^{-1}$ DW, followed by 0.0 X NH$_4$NO$_3$ which produced 3.96 mg g$^{-1}$ DW of withanolide-A content.

6.3 Induction of adventitious roots from leaf explants of Withania somnifera.

6.3.1 Effect of individual auxins on adventitious root induction from leaf explants.

The effect of different concentrations of auxins on the induction of adventitious roots from leaf explants of Withania somnifera was studied. Leaf explants were cultured on full and half strength MS medium supplemented with auxins such as 2,4-D, NAA, IBA and IAA at concentration of 0.1, 0.5, 1.0, 2.0 and 5.0 mg l$^{-1}$ at 3% sucrose. The explants cultured on full strength MS medium failed to induce adventitious roots in all the tested auxin concentrations. They produced callus with the full strength MS medium. The leaf explants cultured on half strength MS medium induced adventitious roots. The auxins 2,4-D and NAA failed to induce adventitious roots, where they formed the callus. The highest response of 100% explants forming adventitious roots was observed in the medium supplemented with 0.5 mg l$^{-1}$ IBA, 0.1 mg l$^{-1}$ IAA and 0.5 mg l$^{-1}$ IAA. The highest mean of adventitious
roots of 17.50 was recorded in the medium supplemented with 0.5 mg l\(^{-1}\) IBA followed by 1.0 mg l\(^{-1}\) IBA which induced about 13.75 roots. The lowest induction of mean adventitious roots was observed in the medium supplemented with 5.0 mg l\(^{-1}\) IAA which produced 3.16 roots.

6.4 Production of withanolide-A from adventitious root suspension of *Withania somnifera*

6.4.1 Growth kinetics of *Withania somnifera* adventitious root suspension cultures.

The growth kinetics and withanolide-A production from adventitious root suspension cultures was studied. The adventitious roots were cultured in half strength MS medium supplemented with 0.5 mg l\(^{-1}\) IBA and 3% sucrose. The highest accumulation of biomass (108.48 g l\(^{-1}\) FW and 10.76 g l\(^{-1}\) DW) with a growth ratio of 8.28 was recorded after 4 weeks. The production of withanolide-A was maximum (8.80 mg g\(^{-1}\) DW) at the end of 4 weeks and decreased thereafter (8.00 mg g\(^{-1}\) DW).

6.4.2 Effect of inoculum density on biomass accumulation and withanolide-A production

The effect of inoculum density (2.5, 5.0, 10.0 and 20.0 g l\(^{-1}\)) on biomass accumulation and withanolide-A production from adventitious root suspension cultures was studied. The adventitious roots were cultured in half strength MS medium supplemented with 0.5 mg l\(^{-1}\) IBA and 3% sucrose. There was increase in the accumulation of biomass with increase in the inoculum density and reached maximum (109.08 g l\(^{-1}\) of FW and 10.88 g l\(^{-1}\) DW) at a growth ratio of 8.37 with 10.0 g l\(^{-1}\) of inoculum density and thereafter there was decrease in the biomass with increase in the inoculum density. The highest production of withanolide-A (8.92 mg g\(^{-1}\) DW) was recorded at 10.0 g l\(^{-1}\) of inoculum density.
6.4.3 Effect of different media on biomass accumulation and withanolide-A production

The effect of different media such as MS, B5, NN and N6 on the biomass accumulation and withanolide-A production from adventitious root suspension cultures were studied. Highest accumulation of biomass (107.48 g l⁻¹ FW and 10.53 g l⁻¹ DW) was recorded in the MS medium with a growth ratio of 8.10. The highest production of 8.27 mg g⁻¹ DW of withanolide-A was also recorded in the MS medium.

6.4.4 Effect of medium strength on biomass accumulation and withanolide-A production

The effect of medium strength (MS medium of 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 strength) on the biomass accumulation and withanolide-A production from adventitious root suspension cultures were studied. The adventitious roots were cultured in MS medium supplemented with 0.5 mg l⁻¹ IBA and 3% sucrose. The highest accumulation of biomass (107.48 g l⁻¹ of FW and 10.53g l⁻¹ DW) was observed in the half strength medium. Half strength MS medium favoured the production of withanolide-A which recoded 8.65 mg g⁻¹ DW.

6.4.5 Effect of different carbohydrate sources on biomass accumulation and withanolide-A production

The effect of different carbohydrate source [sucrose, glucose, fructose, maltose, glucose + fructose (1:1), fructose + sucrose (1:1) and sucrose + glucose (1:1) at 3%] on the biomass accumulation and withanolide-A content from adventitious root suspension cultures were studied. The adventitious roots were cultured in half strength MS medium supplemented with 0.5 mg l⁻¹ IBA. Sucrose was found to be the ideal carbohydrate source for the biomass accumulation, which yielded the biomass of 105.63 g l⁻¹ FW and 10.47 g l⁻¹ DW. The highest production of withanolide-A content
(8.73 mg g\(^{-1}\) DW) was recorded in the medium supplemented with sucrose followed by sucrose + glucose which produced 8.38 mg g\(^{-1}\) DW of withanolide-A content.

### 6.4.6 Effect of sucrose concentration on biomass accumulation and withanolide-A production

The effect of different sucrose concentration (1, 2, 3, 4, 6 and 8\%) on the biomass accumulation and withanolide-A production from adventitious root suspension cultures were studied. The adventitious roots were cultured in half strength MS medium supplemented with 0.5 mg l\(^{-1}\) IBA. The optimum of 2\% sucrose favoured the biomass accumulation of 113.58 g l\(^{-1}\) FW and 10.64 g l\(^{-1}\) DW. The highest production of withanolide-A (8.73 mg g\(^{-1}\) DW) was recorded in the medium supplemented with 2\% sucrose.

### 6.4.7 Effect of hydrogen ion concentration (pH) on biomass accumulation and withanolide-A production

The effect of hydrogen ion concentration (pH; 4.0, 4.5, 5.0, 5.5, 5.8, 6.0 and 6.5) on the biomass accumulation and withanolide-A production from adventitious root suspension culture was studied. The adventitious roots were cultured in half strength MS medium supplemented with 0.5 mg l\(^{-1}\) IBA and 3\% sucrose. Medium set at pH 6.0 favoured highest accumulation of biomass of 113.26 g l\(^{-1}\) FW and 11.33 g l\(^{-1}\) DW and 109.08 g l\(^{-1}\) FW and 10.88 g l\(^{-1}\) DW at pH 5.8. The highest production of withanolide-A (9.09 mg g\(^{-1}\) DW) content was recorded at the pH level of 5.5, followed by pH 5.8 which produced 8.92 mg g\(^{-1}\) DW of withanolide-A content.

### 6.4.8 Effect of NH\(_4\)/NO\(_3\) ratios on biomass accumulation and withanolide-A production

The effect of different NH\(_4\)/NO\(_3\) concentrations (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) on the biomass accumulation and withanolide-A
Summary and Conclusions

Production from adventitious root suspension cultures were studied. The adventitious roots were cultured in half strength MS medium supplemented with 0.5 mg l\(^{-1}\) IBA and 3% sucrose. The higher concentration (37.60 mM) of NO\(_3^-\) with moderate (14.38 mM) concentration of NH\(_4^+\) favoured the highest accumulation of biomass yielding 145.84 g l\(^{-1}\) FW and 14.49 g l\(^{-1}\) DW, followed by lower concentration (7.19 mM) of NH\(_4^+\) with moderate (18.80 mM) concentration of NO\(_3^-\), which yielded 130.45 g l\(^{-1}\) FW and 12.29 g l\(^{-1}\) DW of biomass. The highest production of withanolide-A content was recorded at lowest concentration of NH\(_4^+\) (0.00 mM), which produced 11.76 mg g\(^{-1}\) DW.

6.4.9 Effect of different concentrations of macro elements on biomass accumulation and withanolide-A production

The effect of different concentrations of macro elements (NH\(_4\)NO\(_3\), KNO\(_3\), CaCl\(_2\), MgSO\(_4\) and KH\(_2\)PO\(_4\) at 0.0, 0.5, 1.0, 1.5 and 2.0 X) on the accumulation of biomass and withanolide-A production from adventitious root suspension cultures was studied. The adventitious roots were cultured in half strength MS medium supplemented with 0.5 mg l\(^{-1}\) IBA and 3% sucrose. The highest accumulation of biomass (127.52 g l\(^{-1}\) FW and 12.45 g l\(^{-1}\) DW) was recorded in the 0.5 X concentration of NH\(_4\)NO\(_3\) followed by 1.5 X MgSO\(_4\), 2.0 X KNO\(_3\) which yielded the biomass of 126.92 g l\(^{-1}\) FW and 12.48 g l\(^{-1}\) DW and 126.40 g l\(^{-1}\) FW and 12.51 g l\(^{-1}\) DW respectively. The highest production of withanolide-A was recorded in the medium with 2.0 X KNO\(_3\) which produced 14.00 mg g\(^{-1}\) DW, followed by 2.0 X KH\(_2\)PO\(_4\) which produced 13.59 mg g\(^{-1}\) DW of withanolide-A content.

6.5 Induction of hairy roots from leaf explants of *Withania somnifera*.

The explants from seedlings roots, stems, hypocotyls, cotyledons, cotyledonary nodes and leaf segments were inoculated with Agrobacterium
Summary and Conclusions

rhizogenes strain R1601. However only cotyledon and leaf explants responded to inoculation by A. rhizogenes by developing transformed roots, representing 3 and 40% of responses respectively. Initial root protuberances developed within 10-15 days of inoculation at the wounded edges of leaf explants with hairy roots developing within one month of inoculation. In contrast, root, stem, hypocotyls and cotyledonary nodal explants failed to respond to Agrobacterium and became necrotic within one month of inoculation. Four weeks post-inoculation, roots, 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 16/8 h light condition. Roots were grown for 4 weeks, before 500 mg 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 4 weeks. 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.

The integration of the A. rhizogenes Ri plasmid T-DNA into the W. somnifera genome was confirmed by polymerase chain reaction (PCR) for the nptII and rolB genes. The primers for rolB were used according to Zhao et al., (2004). Primers amplified the expected 261 base pair (bp) fragment of the nptII gene and a 863 bp fragment of rolB. Similar PCR products were absent from roots excised from non-transformed plants. PCR amplification of the nptII and rolB in A. rhizogenes-induced roots were confirmed by the stable and typical hairy root phenotype of the cultured roots.
Summary and Conclusions

The transformed nature of roots incited by *A. rhizogenes* strain R1601 was confirmed by southern analysis for the presence of the *nptII* and *rolB* genes in extracted DNA. All *A. rhizogenes*-induced roots exhibited strong hybridization bands of 10 kb for the *nptII* gene, and 2.0 kb for the *rolB* gene. Southern hybridization was negative for DNA from roots of non-transformed plants.

6.6 Production of withanolide-A from hairy root suspension culture of *Withania somnifera*

6.6.1 Growth kinetics of *Withania somnifera* hairy root suspension cultures.

The growth kinetics and withanolide-A production from hairy root suspension cultures were studied. The hairy roots were cultured in MS basal medium with 3% sucrose. The highest accumulation of biomass (121.42 g l⁻¹ FW and 11.99 g l⁻¹ DW) with a growth ratio of 9.52 was recorded after 4 weeks of culture. The production of withanolide-A was maximum (11.60 mg g⁻¹ DW) at the end of 4 weeks and decreased thereafter and it decreased (9.59 mg g⁻¹ DW).

6.6.2 Effect of inoculum density on biomass accumulation and withanolide-A production

The effect of inoculum density (2.5, 5.0, 10.0 and 20.0 g l⁻¹) on biomass accumulation and withanolide-A production from hairy root suspension cultures was studied. The hairy roots were cultured in MS basal medium supplemented with 3% sucrose. There was increase in the accumulation of biomass with increase in the inoculum density and reached maximum (120.42 g l⁻¹ of FW and 11.98 g l⁻¹ DW) with a growth ratio of 9.51 when inoculum density was 10.0 g l⁻¹ and thereafter there was decrease in the biomass with increase in the inoculum density. The highest production of withanolide-A (11.96 mg g⁻¹ DW) was recorded at 10.0 g l⁻¹ of inoculum density.
6.6.3 Effect of different media on biomass accumulation and withanolide-A production

The effect of different media such as MS, B5, NN and N6 on the biomass accumulation and withanolide-A production from hairy root suspension cultures of Withania somnifera were studied. The hairy roots were cultured in basal medium supplemented with 3% sucrose. Highest accumulation of biomass (121.15 g l\(^{-1}\) FW and 11.96 g l\(^{-1}\) DW) was recorded in the MS medium with a growth ratio of 9.49. The highest production of 11.50 mg g\(^{-1}\) DW of withanolide-A was recorded in the MS medium.

6.6.4 Effect of medium strength on biomass accumulation and withanolide-A production

The effect of medium strength (MS medium of 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 strength) on the biomass accumulation and withanolide-A production from hairy root suspension cultures were studied. The hairy roots were cultured in MS basal medium with 3% sucrose. The highest accumulation of biomass (122.82 g l\(^{-1}\) of FW and 12.10 g l\(^{-1}\) DW) was observed in the full strength MS medium. Full strength MS medium favoured the production of withanolide-A which recorded 14.70 mg g\(^{-1}\) DW.

6.6.5 Effect of different carbohydrate sources on biomass accumulation and withanolide-A production

The effect of different carbohydrate sources (sucrose, glucose, fructose, maltose, glucose + fructose (1:1), sucrose + glucose (1:1) and fructose + sucrose (1:1) at 3%) on the biomass accumulation and withanolide-A content from hairy root suspension cultures were studied. The hairy roots were cultured in MS basal medium. Sucrose was found to be the ideal carbohydrate source for the biomass accumulation, which yielded the biomass of 121.05 g l\(^{-1}\) FW and 11.92 g l\(^{-1}\) DW, followed by
combination of sugars i.e. sucrose + glucose which accumulated the biomass of 108.40 g l⁻¹ FW and 10.79 g l⁻¹ DW. The highest production of withanolide-A content (12.55 mg g⁻¹ DW) was recorded in the medium supplemented with sucrose + glucose followed by sucrose which produced 11.96 mg g⁻¹ DW of withanolide-A content.

6.6.6 Effect of sucrose concentration on biomass accumulation and withanolide-A production

The effect of different sucrose concentration (1, 2, 3, 4, 6 and 8%) on the biomass accumulation and withanolide-A production from hairy root suspension cultures were studied. The hairy roots were cultured in MS basal medium. The optimum of 3% sucrose favoured the biomass accumulation of 121.05 g l⁻¹ FW and 11.92 g l⁻¹ DW. The highest production of withanolide-A (13.28 mg g⁻¹ DW) was recorded in the medium supplemented with 4% sucrose.

6.6.7 Effect of hydrogen ion concentration (pH) on biomass accumulation and withanolide-A production

The effect of hydrogen ion concentration (pH; 4.0, 4.5, 5.0, 5.5, 5.8, 6.0 and 6.5) on the biomass accumulation and withanolide-A production from hairy root suspension culture were studied. The hairy roots were cultured in MS basal medium with 3% sucrose. The medium set at pH 5.8 accumulated the highest biomass of 122.82 g l⁻¹ FW and 12.10 g l⁻¹ DW. The highest production of withanolide-A (13.84 mg g⁻¹ DW) content was recorded at the pH level of 6.0, followed by pH 5.8 which produced 11.60 mg g⁻¹ DW.

6.6.8 Effect of NH₄⁺/NO₃⁻ ratios on biomass accumulation and withanolide-A production

The effect of different NH₄⁺/NO₃⁻ concentrations (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)
Summary and Conclusions

14.38/28.20 and 14.38/37.60 mM) on the biomass accumulation and withanolide-A production from hairy root suspension cultures were studied. The hairy roots were cultured in MS basal medium with 3% sucrose. The higher concentration (37.60 mM) of NO$_3^-$ with moderate (14.38 mM) concentration of NH$_4^+$ favoured the highest accumulation of biomass yielding 148.17 g l$^{-1}$ FW and 14.79 g l$^{-1}$ DW, followed by lower concentration (7.19) of NH$_4^+$ medium with moderate (18.80 mM) concentration of NO$_3^-$, which yielded 140.45 g l$^{-1}$ FW and 13.29 g l$^{-1}$ DW of biomass. The highest production of withanolide-A content was recorded at NH$_4^+$ (0.00 mM) free medium, which produced 14.68 mg g$^{-1}$ DW. Increase in the concentration of NH$_4^+$ source lead to decrease in the production of withanolide-A.

6.6.9 Effect of different concentrations of macro elements on biomass accumulation and withanolide-A production

The effects of different concentrations of macro elements (NH$_4$NO$_3$, KNO$_3$, CaCl$_2$, MgSO$_4$ and KH$_2$PO$_4$ at 0.0, 0.5, 1.0, 1.5 and 2.0 X) on the accumulation of biomass and withanolide-A production from hairy root suspension cultures were studied. The hairy roots were cultured in MS basal medium with 3% sucrose. The highest accumulation of biomass (139.42 g l$^{-1}$ FW and 13.11 g l$^{-1}$ DW) was recorded in the 2.0 X concentration of KH$_2$PO$_4$ followed by 2.0 X KNO$_3$ which yielded the biomass of 137.87 g l$^{-1}$ FW and 13.69 g l$^{-1}$ DW. The highest production of withanolide-A was recorded in the medium with 2.0 X KNO$_3$ which produced 15.27 mg g$^{-1}$ DW, followed by 2.0 X KH$_2$PO$_4$ which produced 14.68 mg g$^{-1}$ DW of withanolide-A content.

6.7 Induction of callus from leaf explants of Gymnema sylvestre

6.7.1 Effect of individual auxins and cytokinins on callus induction from leaf explants.

Leaf explants of Gymnema sylvestre were cultured on MS medium supplemented with auxins such as 2,4-D, NAA, IBA and IAA, and cytokinins such as...
Summary and Conclusions

BAP and KN individually at concentrations of 0.1, 0.5, 1.0, 2.0 and 5.0 mg l\(^{-1}\) and 3\% sucrose. The highest percentage of 97.22\% callus formation was observed in the explants cultured on 2.0 mg l\(^{-1}\) 2,4-D. Highest callus production of 3.50 g in fresh weight and 0.338 g of dry weight was observed in the explants cultured on 2.0 mg l\(^{-1}\) 2,4-D.

The percentage of explants forming callus varied with the cytokinins and its concentrations. The highest percentage of 80.55\% of callus induction was observed in 1.0 mg l\(^{-1}\) KN. The highest amount of callus was observed in the explants cultured on medium supplemented with 1.0 mg l\(^{-1}\) KN which weighed 1.31 g of fresh weight and 0.121 g of dry weight.

6.7.2 Effect of auxins in combination with cytokinins.

The leaf explants were cultured on MS medium supplemented with 2,4-D (1.0 and 2.0 mg l\(^{-1}\)) and NAA (2.0 and 5.0 mg l\(^{-1}\)) in combination with cytokinins such as BAP and KN (0.1, 0.5, 1.0 and 2.0 mg l\(^{-1}\)). Highest response was observed with 2.0 mg l\(^{-1}\) 2,4-D alone (97.22\%). The highest amount of callus was obtained in the culture medium supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.1 mg l\(^{-1}\) KN, which yielded 4.12 g of fresh weight and 0.412 g of dry weight.

The effect of NAA (2.0 and 5.0 mg l\(^{-1}\)) in combination with cytokinins (BAP and KN at concentrations of 0.1, 0.5, 1.0 and 2.0 mg l\(^{-1}\)) on callus formation was studied. The highest number of explants responding was observed in the medium supplemented with 2.0 mg l\(^{-1}\) NAA alone (88.88\%). The highest amount of callus was observed in the medium supplemented with 5.0 mg l\(^{-1}\) NAA + 1.0 mg l\(^{-1}\) KN, which yielded 3.58 g of fresh weight and 0.340 g of dry weight.
6.7.3 Effect of 2,4-D in combination with NAA

The explants cultured on 2,4-D (1.0 and 2.0 mg l\(^{-1}\)) in combination with NAA (0.1, 0.5, 1.0 and 2.0 mg l\(^{-1}\)) for callus induction was studied. The highest responding explants was observed in 2.0 mg l\(^{-1}\) 2,4-D (97.22%) alone. The combination of 2.0 mg l\(^{-1}\) 2,4-D + 1.0 mg l\(^{-1}\) NAA resulted in the highest production of callus in terms of fresh weight (3.85 g) and dry weight (0.370 g).

6.7.4 Effect of NAA in combination with 2,4-D

The effect of NAA (2.0 and 5.0 mg l\(^{-1}\)) in combination with 2,4-D (0.1, 0.5, 1.0 and 2.0 mg l\(^{-1}\)) on callus formation was studied. The highest responding explants was observed in 2.0 mg l\(^{-1}\) NAA (86.88%). The combination of 5.0 mg l\(^{-1}\) NAA + 1.0 mg l\(^{-1}\) 2,4-D resulted in the highest production of callus with 3.75 g of fresh weight and 0.362 g of dry weight.

6.8 Production of gymnemic acid from cell suspension cultures of \textit{Gymnema sylvestre}

6.8.1 Effect of auxins on biomass accumulation and gymnemic acid production in cell suspension culture.

The effect of different concentrations of auxins on the biomass accumulation and gymnemic acid production was studied. The biomass accumulation and gymnemic acid content was differing with varied concentrations of auxins. The highest accumulation of biomass (98.16 g l\(^{-1}\) FW and 8.68 g l\(^{-1}\) DW) with a growth ratio of 7.89 and the highest production of gymnemic acid (6.70 mg g\(^{-1}\) DW) was observed in the medium supplemented with 2.0 mg l\(^{-1}\) 2,4-D followed by 5.0 mg l\(^{-1}\) 2,4-D which accumulated 90.67 g l\(^{-1}\) of FW and 7.96 g l\(^{-1}\) DW with a growth ratio of 7.24.
6.8.2 Effect of cytokinins in combination with 2.0 mg l\(^{-1}\) 2,4-D on biomass accumulation and gymnemic acid production in cell suspension culture.

The effect of 2,4-D (2.0 mg l\(^{-1}\)) in combination with cytokinins (BAP and KN at concentrations of 0.1, 0.5, 1.0 and 2.0 mg l\(^{-1}\)) on biomass accumulation and gymnemic acid production was studied. The maximum accumulation of biomass (117.76 g l\(^{-1}\) FW and 10.77 g l\(^{-1}\) DW) with a growth ratio of 9.47 was observed in the medium supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.1 mg l\(^{-1}\) KN with a gymnemic acid production of 9.53 mg g\(^{-1}\) DW.

6.8.3 Growth kinetics of *Gymnema sylvestre* cell suspension cultures.

The growth kinetics and gymnemic acid production from cell suspension cultures were studied. The media was supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.1 mg l\(^{-1}\) KN and 3% sucrose. The maximum accumulation of biomass (125.67 g l\(^{-1}\) FW and 11.56 g l\(^{-1}\) DW) with a growth ratio of 10.51 was recorded after 4 weeks of culture initiation and later biomass accumulation decreased. The production of gymnemic acid was maximum (9.95 mg g\(^{-1}\) DW) at the end of 4 weeks and decreased thereafter.

6.8.4 Effect of inoculum density on biomass accumulation and gymnemic acid production

The effect of inoculum density (2.5, 5.0, 10.0 and 20.0 g l\(^{-1}\)) on biomass accumulation and gymnemic acid production from cell suspension cultures were studied. The media was supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.1 mg l\(^{-1}\) KN and 3% sucrose. There was increase in the accumulation of biomass with increase in the inoculum density and reached maximum (122.63 g l\(^{-1}\) of FW and 11.25 g l\(^{-1}\) DW) at a growth ratio of 10.23 with 10.0 g l\(^{-1}\) of inoculum density. The highest production of gymnemic acid (9.95 mg g\(^{-1}\) DW) was recorded at 10.0 g l\(^{-1}\) of inoculum density.
6.8.5 Effect of different media on biomass accumulation and gymnemic acid production

The effect of different media such as MS, B5, NN and N6 on the biomass accumulation and gymnemic acid production from cell suspension cultures was studied. All the media were supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.1 mg l\(^{-1}\) KN and 3% sucrose. Highest accumulation of biomass with respect to fresh wt (120.38 g l\(^{-1}\)) and dry wt (10.99 g l\(^{-1}\)) was observed in the MS medium with a growth ratio of 9.99. The highest production of 9.55 mg g\(^{-1}\) DW of gymnemic acid was recorded in the MS medium.

6.8.6 Effect of medium strength on biomass accumulation and gymnemic acid production

The effect of medium strength (MS medium of 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 strength) on the biomass accumulation and gymnemic acid production from cell suspension cultures were studied. The media was supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.1 mg l\(^{-1}\) KN and 3% sucrose. The highest accumulation of biomass (125.91 g l\(^{-1}\) of FW and 11.54 g l\(^{-1}\) DW) was recorded in the full strength MS medium. Full strength MS medium favoured the production of gymnemic acid which recoded 8.93 mg g\(^{-1}\) DW.

6.8.9 Effect of different carbohydrate sources on biomass accumulation and gymnemic acid production

The results of the different carbohydrate sources [sucrose, glucose, fructose, maltose, glucose + fructose (1:1), sucrose + glucose (1:1) and fructose + sucrose (1:1) at 3%] on the biomass accumulation and gymnemic acid content from cell suspension cultures were studied. The media was supplemented with 2.0 mg l\(^{-1}\) 2,4-D + 0.1 mg l\(^{-1}\) KN. Sucrose was found to be the ideal carbohydrate source for the biomass accumulation, which yielded the biomass of 120.38 g l\(^{-1}\) FW and 10.99 g l\(^{-1}\) DW,
Summary and Conclusions
followed by glucose and combination of sugars i.e. sucrose + glucose which accumulated the biomass of 118.00 g l⁻¹ FW and 10.66 g l⁻¹ DW and 117.22 g l⁻¹ FW and 10.66 g l⁻¹ DW respectively. The highest production of gymnemic acid content (9.95 mg g⁻¹ DW) was recorded in the medium supplemented with sucrose followed by fructose + sucrose which produced 9.26 mg g⁻¹ DW of gymnemic acid content.

6.8.10 Effect of sucrose concentration on biomass accumulation and gymnemic acid production

The effect of different sucrose concentration (1, 2, 3, 4, 6 and 8%) on the biomass accumulation and gymnemic acid production from cell suspension cultures were studied. The media was supplemented with 2.0 mg l⁻¹ 2,4-D + 0.1 mg l⁻¹ KN. The optimum of 3% sucrose favoured the biomass accumulation of 124.21 g l⁻¹ FW and 11.36 g l⁻¹ DW. The biomass decreased with increase or decrease in the concentration of sucrose. The highest production of gymnemic acid (10.01 mg g⁻¹ DW) was recorded in the medium supplemented with 4% sucrose.

6.1.11 Effect of hydrogen ion concentration (pH) on biomass accumulation and gymnemic acid production.

The effect of hydrogen ion concentration (pH; 4.0, 4.5, 5.0, 5.5, 5.8, 6.0 and 6.5) on the biomass accumulation and gymnemic acid production from cell suspension culture were studied. The media was supplemented with 2.0 mg l⁻¹ 2,4-D + 0.1 mg l⁻¹ KN and 3% sucrose. The highest accumulation of biomass in terms of FW and DW was recorded at the pH of 5.8 (120.38 g l⁻¹ FW and 10.99 g l⁻¹ DW). The highest production of gymnemic acid (9.95 mg g⁻¹ DW) content was recorded at the pH level of 5.8, followed by pH 6.0 which produced 9.39 mg g⁻¹ DW.

6.8.12 Effect of NH₄⁺/NO₃⁻ ratios on biomass accumulation and gymnemic acid production

The effect of different NH₄⁺/NO₃⁻ concentrations (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) on the biomass accumulation and gymnemic acid production from cell suspension cultures were studied. The media was supplemented with 2.0 mg l⁻¹ 2,4-D + 0.1 mg l⁻¹ KN and 3% sucrose. The lower concentration (7.19 mM) of NH₄⁺ with moderate concentration (18.80 mM) of NO₃⁻ favoured the highest accumulation of biomass yielding 159.72 g l⁻¹ FW and 14.95 g l⁻¹ DW, followed by NH₄⁺ free medium (0.00mM) with moderate concentration (18.80mM) of NO₃⁻, which yielded 137.07 g l⁻¹ FW and 12.69 g l⁻¹ DW of biomass. The highest production of gymnemic acid content was recorded at moderate concentration (7.19 mM) of NH₄⁺, which produced 11.35 mg g⁻¹ DW.

6.8.13 Effect of different concentrations of macro elements on biomass accumulation and gymnemic acid production

The effects of different concentrations of macro elements (NH₄NO₃, KNO₃, CaCl₂, MgSO₄ and KH₂PO₄ at 0.0, 0.5, 1.0, 1.5 and 2.0 X) on the accumulation of biomass and gymnemic acid production of cell suspension cultures were studied. The media was supplemented with 2.0 mg l⁻¹ 2,4-D + 0.1 mg l⁻¹ KN and 3% sucrose. The highest accumulation of biomass (165.00 g l⁻¹ FW and 15.42 g l⁻¹ DW) was recorded in the 0.5 X concentration of NH₄NO₃ and in the 2.0 X KNO₃ which yielded the biomass of 163.10 g l⁻¹ FW and 15.30 g l⁻¹ DW. The highest production of gymnemic acid was recorded in the medium with 2.0 X KH₂PO₄ which produced 11.32 mg g⁻¹ DW, followed by 2.0 X KNO₃ which produced 10.58 mg g⁻¹ DW of gymnemic acid content.

6.9 Induction of adventitious roots from leaf explants of Gymnema sylvestre.

6.9.1 Effect of individual auxins on adventitious root induction from leaf explants.

The effect of different concentrations of auxins on the induction of adventitious roots from leaf explants of Gymnema sylvestre was studied. Leaf
explants were cultured on full and half strength MS medium supplemented with auxins such as 2,4-D, NAA, IBA and IAA at concentration of 0.1, 0.5, 1.0, 2.0 and 5.0 mg l\(^{-1}\) at 3% sucrose. The explants cultured on full strength MS medium failed to induce adventitious roots, where they formed callus. The leaf explants cultured on half strength MS medium induced adventitious roots. The highest response of 67% explants forming adventitious roots was observed in the medium supplemented with 2.0 mg l\(^{-1}\) NAA. The highest mean number of adventitious roots of 1.41 was recorded in the medium supplemented with 2.0 mg l\(^{-1}\) NAA followed by 5.0 mg l\(^{-1}\) IBA which induced about 1.16 roots.

6.9.2 Effect of NAA in combination IBA for the induction of adventitious roots from leaf explants

The effect of the different concentrations of NAA (0.5, 1.0, 2.0 and 5.0 mg l\(^{-1}\)) in combination with IBA at concentrations of 0.1, 0.5, 1.0 and 2.0 mg l\(^{-1}\) for the induction of adventitious roots was studied. The highest number of adventitious roots was recorded in the medium supplemented with 2.0 mg l\(^{-1}\) NAA + 1.0 mg l\(^{-1}\) IBA, which produced 1.58 roots with callus, followed by 1.14 roots in the medium with 2.0 mg l\(^{-1}\) NAA. The higher concentration of NAA with the combination of IBA induced roots with callus.

6.10 Induction of hairy roots from leaf explants of Gymnema sylvestre

The induction of hairy roots from Gymnema sylvestre was carried out. The in vivo and in vitro leaf explants failed to induce the hairy roots with the bacterial strain 15834 with the OD of 0.6-1.2 at 600 nm wavelength and infected for different duration of time (5, 10, 15, 30 and 60 mins) and co cultured for 1 and 2 days with light and dark incubations at 25°C. Irrespective of various treatments, induction of hairy roots was not possible in this system.
Summary and Conclusions

Based on the above results the following conclusions were drawn

*Withania somnifera* (L.)Dunal

1. Induction of callus and establishment of cell suspension cultures
   1.1 MS medium supplemented with 2.0 mg l⁻¹ 2,4-D + 0.5 mg l⁻¹ KN was found suitable for induction of callus from leaf explants of *Withania somnifera*, which yielded 4.45 g of FW and 0.428 g of DW callus after 4 weeks of culture.
   1.2 Cell suspension cultures could be established by using MS liquid medium. MS medium supplemented with 2.0 mg l⁻¹ 2,4-D and 0.5 mg l⁻¹ KN found suitable for highest biomass accumulation and withanolide-A production.
   1.3 Among various parameters tested on biomass and withanolide-A accumulation, 10 g l⁻¹ of inoculum, 3% sucrose, medium pH set at 5.8, 7.19/18.80 mM NH₄⁺/NO₃⁻ ratio were found suitable.

2. Induction of adventitious roots and establishment of adventitious root suspension cultures.
   2.1 MS medium supplemented with 0.5 mg l⁻¹ IBA was found suitable for adventitious root induction.
   2.2 MS medium supplemented with 0.5 mg l⁻¹ IBA and 3% sucrose was found suitable for biomass (108.48 g l⁻¹ FW and 10.76 g l⁻¹ DW) and withanolide-A (8.80 mg g⁻¹ DW) after 4 weeks of culture.
   2.3 Among various parameters tested, half strength MS medium, 10 g l⁻¹ inoculum, 2% sucrose, medium pH set at 5.5 favour biomass accumulation and withanolide-A production. The NH₄⁺/NO₃⁻ ratio of 14.38/37.60 mM favoured the accumulation of biomass whereas 0.00/18.80 mM NH₄⁺/NO₃⁻ concentration favoured the production of withanolide-A.

3. Induction of hairy roots and establishment of hairy root suspension culture
   3.1 Hairy roots were successfully induced by co cultivation of leaf explants with *Agrobacterium rhizogenes* strain R1601. Hairy roots were selected on antibiotic containing medium and presence of trans genes (*nptII* and *rolB*) through polymerase chain reaction and southern hybridization.
3.2 Hairy roots cultured in MS basal medium with 3% sucrose produced highest accumulation of biomass (121.42 g l$^{-1}$ FW and 11.99 g l$^{-1}$ DW) and withanolide-A (11.60 mg g$^{-1}$ DW) after 4 weeks of culture.

3.3 Among various parameters tested on biomass and withanolide-A accumulation, 10 g l$^{-1}$ inoculum, full strength MS medium, 3% sucrose, medium pH set at 5.8 were found suitable. The NH$_4^+$/NO$_3^-$ ratio of 14.38/37.60 mM favoured the accumulation of biomass whereas 0.00/18.80 mM NH$_4^+$/NO$_3^-$ ratio favoured the production of withanolide-A.

Gymnema sylvestre R. Br.

1. Induction of callus and establishment of cell suspension culture

1.1 MS medium supplemented 2.0 mg l$^{-1}$ 2,4-D and 0.1 mg l$^{-1}$ KN was found suitable for callus induction from leaf explants of Gymnema sylvestre, which yielded 4.12 g FW and 0.412 g DW callus after 4 weeks of culture.

1.2 MS medium supplemented with 2.0 mg l$^{-1}$ 2,4-D and 0.1 mg l$^{-1}$ KN was found suitable for biomass (118.76 g l$^{-1}$ FW and 10.77 g l$^{-1}$ DW) and gymnemic acid (9.53 mg g$^{-1}$ DW) accumulation.

1.3 Among various parameters tested for biomass and gymnemic acid accumulation, 10 g l$^{-1}$ inoculum density, full strength MS medium, medium pH set at 5.8 were found suitable, medium supplemented with 3% sucrose favoured the accumulation of biomass and 4% sucrose favoured the withanolide-A production. The NH$_4^+$/NO$_3^-$ ratio of 14.38/37.60 mM favoured the accumulation of biomass whereas 0.00/18.80 mM NH$_4^+$/NO$_3^-$ ratio were favoured production of gymnemic acid.

2. Induction of adventitious root was successful on MS medium supplemented with 2.0 mg l$^{-1}$ NAA or 2.0 mg l$^{-1}$ NAA + 1.0 mg l$^{-1}$ IBA. However establishment of suspension cultures was not successful.