Chapter V

Establishment of Adventitious Root Cultures of *Achyranthes aspera* Linn. and Enhancing the Production of 20-hydroxyecdysone by Elicitor and Precursor Feeding

**ABSTRACT**

Adventitious roots were initiated from the root and leaf explants obtained from two weeks old *in vitro* raised seedlings of *A. aspera* cultured on Murashige and Skoog (MS) medium. Different types of nutrient media *i.e.* MS, B5 and Whites media supplemented with various concentrations of IBA were tested for their effect on biomass production. Among different media tested MS medium supplemented with 1.5 mg L\(^{-1}\) of IBA showed highest biomass accumulation so this media was adopted for further studies. After three weeks of culture on MS medium, the biomass accumulated was 3.94±0.04 g FW/flask and contained 20-hydroxyecdysone of 0.32 mg g\(^{-1}\) DW. HPLC and LC-Q-TOF MS/MS analysis confirmed the ability of adventitious roots to produce phytoecdysteroid 20-hydroxyecdysone. To enhance the accumulation of 20-hydroxyecdysone, adventitious root cultures of *A. aspera* were treated with methyl jasmonate, cholesterol and 7-dehydrocholesterol. When 15-day-old cultures were treated with 0.6 mM methyl jasmonate for 3 days produced 20-hydroxyecdysone of about 0.47 mg g\(^{-1}\) DW, which was greater than that of the untreated cells. Similarly, precursor feeding (cholesterol and 7-dehydrocholesterol) also improved the production of 20-hydroxyecdysone to 0.403 mg g\(^{-1}\) DW and 0.37 mg g\(^{-1}\) DW respectively.

**Key words:** Adventitious root cultures, 20-hydroxyecdysone, IBA, Methyl jasmonate, Cholesterol, 7-Dehydrocholesterol.
5.1 INTRODUCTION

Ability of plant cell, tissue, and organ cultures to produce valuable chemical compounds has been documented since the inception of *in vitro* technology. However, when it was detected that production of secondary metabolites is usually higher in differentiated plant tissues, efforts were done to cultivate whole plant organs, *i.e.* shoots or roots in *in vitro* conditions with the objective to produce medicinally important compounds (Biondi et al. 2002). The organ cultures are relatively stable in producing secondary metabolites compared to cultures of undifferentiated cells, such as cells in callus or suspension culture (Rao and Ravishankar, 2002). Recently, the organ cultures, mainly adventitious root culture, have been established from numerous plant species. Due to its rapid growth rate, they are considered as substitutes for production of secondary metabolites of pharmaceutical and nutraceutical interest (Murthy et al. 2008b).

Root system is the descendent portion of higher plants, which is responsible for creating a dynamic habitat below the ground (Rost and Bloom, 2006). Despite its major functions like anchorage, absorption and conduction of water and minerals, plant root system can synthesize, store and secrete a vast array of compounds and secondary metabolites (Dubrovsky et al. 2006). These make root cultures, treasured sources of medicinal compounds (Li et al. 2002) and they can be established with the objective of secondary metabolite production. Adventitious roots were induced from leaves, roots and stems of many plants and their formation is controlled by a range of exogenous and endogenous factors such as auxins, polyamines, ethylene, nitric oxide, calcium ions, sugars, etc. These factors might function via auxin signalling during the formation of adventitious roots (Li et al. 2009). Adventitious roots cultured under aseptic conditions on a suitable medium containing plant growth regulators show high stability, growth rate and
synthesize abundant amount of secondary metabolites in intercellular spaces, which can be extracted and roots can be grown in a medium with low inoculums (Sivakumar et al. 2006). Adventitious root cultures are considered as the experimental system to investigate the coordination among primary and secondary metabolism and also for the improvement of plant-based pharmaceutical compounds.

Elicitation has been proved to be an effective method for inducing or enhancing the synthesis of secondary metabolites in plants to ensure their survival, persistence and competitiveness (Namdeo, 2007). Synthesis of numerous valuable secondary metabolites by various elicitors are reported (Lee and Shuler, 2000; Dong and Zhong, 2001). To increase the production of secondary metabolites in plant cell and tissue culture, precursor feeding is an effective method. Precursors are compounds, which are present upstream in a biosynthetic pathway of target compounds and most of the intermediates can be used as precursors.

Roots of *A.aspera* are used as astringents to wounds, in abdominal tumour, stomach pain and it has application in infantile diarrhoea and cold (Borthakur and Goswami, 1995). Banerji et al. (1971) isolated ecdysterone (20-Hydroxyecdysone) from the methanolic extract of roots of *A. aspera*. In spite of these medicinal uses, there have been no reports on adventitious root culture of *A. aspera* till date. Consequently, the objective of the current study was to establish an efficient *in vitro* method for adventitious root cultures of *A. aspera* for the production of valuable secondary metabolite 20-hydroxyecdysone. To our knowledge, this is the first report on 20-hydroxyecdysone production from normal root culture of *A. aspera* and no elicitation studies with methyl jasmonate and precursor feeding studies with cholesterol and 7-dehydrocholesterol has been reported in *A. aspera*.
5.2 SPECIFIC OBJECTIVES

The objectives of the present study are

1. To develop a protocol for initiating and establishing adventitious root cultures of *A.aspera*.
2. To study the effect of different media on adventitious root growth.
3. To study the effect of different IBA concentrations on growth of adventitious root culture.
4. To extract, identify and quantify 20-hydroxyecdysone from adventitious root cultures of *A.aspera*.
5. To study the effect of methyl jasmonate as elicitor on the production of 20-hydroxyecdysone from adventitious root cultures of *A.aspera*.
6. To study the effect of cholesterol and 7-dehydrocholesterol as precursors in the production of 20-hydroxyecdysone from adventitious root cultures of *A.aspera*.

5.3 REVIEW OF LITERATURE

Roots of numerous plant families are the site for various metabolic pathways, which include biosynthesis or accumulation of specific metabolites; although in plants these phytochemicals might be stored in some other organs (Dewick, 2002). Their genetic and biosynthetic stability enables their *in vitro* culture for the production of valuable secondary metabolites. For exploiting the potential of roots, adventitious roots are induced from various plants and are applied from small-scale to large scale, intended for the production of secondary metabolites, which have their uses as pharmaceuticals, agrochemicals, flavours, fragrances, colours or food additives (Murthy et al. 2008b).
Auxins are one of the most important endogenous hormones involved in the development of adventitious rooting (Wiesman et al. 1988). Besides, the physiological stages of rooting are associated with variations in endogenous auxin concentrations (Heloir et al. 1996). Elevation in endogenous auxin concentration is generally associated with increased rooting rate at the beginning of the rooting process (Blazkova et al. 1997). Auxins are reported to be effective inducers of adventitious roots in several woody species (Diaz-Sala et al. 1996; DeKlerk et al. 1999). It is believed that the adventitious root formation is usually inhibited by cytokinins (Bollmark and Eliasson, 1986). Conversely, adventitious root formation is promoted by low concentrations of cytokinins in sunflower (Fabijan et al. 1981) and pea (Bollmark and Eliasson, 1986). On the other hand, higher concentrations of cytokinins combined with auxins promote adventitious root formation in some plants such as Kampheria glanga (Vincent et al. 1992) and Aristolochia bractiolata (Remeshree et al. 1994).

*In vitro* adventitious root induction can be achieved from various explants, such as leaves, roots, stems and petioles. Adventitious roots are induced from the root explants of Panax ginseng through the initial callus stage on Murashige and Skoog medium containing 4.53 µM of 2,4-Dichlorophenoxy acetic acid (2,4-D) and 0.46 µM of kinetin and 3 % (w/v) of sucrose. When callus was subcultured on MS medium augmented with 14.70 µM of IBA and 3 % (w/v) of sucrose, adventitious roots grow successfully. Adventitious root was induced from *in vitro* leaf explants of Chicorium intybus (Nandagopal and Kumari, 2007); Plumbago zeylanica (Sivanesan and Jeong, 2009); and Withania somnifera (Sivanandhan et al. 2012).

The optimization of cultural parameters are essential, since biomass accumulation and secondary metabolite production can be greatly affected
by factors such as, inoculum density, media components, oxygen transfer, mixing, and other physico-chemical factors (Kim et al. 2002; Jeong et al. 2009a; Jeong et al. 2009b). Influence of inoculum density on adventitious roots and secondary metabolite production are reported in a number of plant systems (Jeong et al. 2009a; Praveen and Murthy 2010; Lee et al. 2011a; Lee et al. 2011b). Praveen and Murthy (2010, 2014) while studying the production of withanolide-A from *Withania sominifera* adventitious root cultures at various inoculum densities of 2.5, 5, 10 and 20 g L\(^{-1}\), reported the highest biomass production and withanolide-A accumulation at 10 g L\(^{-1}\). In *Eleutherococcus koreanum* adventitious root culture, inoculum density of 15 g L\(^{-1}\) has been found suitable for production of biomass, whereas inoculum density 5 g L\(^{-1}\) was appropriate for secondary metabolite production (Lee et al. 2011a; Lee et al. 2011b). In *Raphanus sativus* (cv. Peking Koushin), adventitious roots were induced from root segments on half strength MS medium containing 0.5 mg L\(^{-1}\) of IBA and the cultured roots produced anthocyanin in dark conditions (Betsui et al. 2004). The adventitious root cultures of *E. purpurea*, biomass accumulation and production of caffeic acid derivatives was ideal under incubation temperature of 20 °C among different incubation temperatures tested (10, 15, 20, 25 and 30 °C). Biomass production of adventitious roots was highest in cultures developed under dark conditions whereas synthesis and accumulation of caffeic acid derivatives was optimum in the cultures grown under 3/12 h light and dark cultural regimes (Wu et al. 2007b).

Elicitation approach was adopted to enhance the ginsenoside production in the adventitious root cultures of ginseng (Yu et al. 2002; Kim et al. 2004). Elicitors may be abiotic or biotic, besides it act individually or in combination and turn on the synthesis of molecules that might only be produced in lesser amounts or it might produce new compounds (Weathers
et al. 2010). One efficient method to improve the production of secondary metabolites in the plant is treatment with chemical elicitors such as methyl jasmonate. Jasmonates are endogenous plant phytohormones. Jasmonic acid and its methyl ester (methyl jasmonate) are stated to play a significant part in a signal transduction process which regulates defence genes in plants (Farmer and Ryan, 1990). Addition of methyl jasmonate was reported to induce the production of secondary metabolites by switching on the transcription of numerous genes involved in their biosynthesis (Van der fits et al. 2000). Furthermore, the application of exogenous methyl jasmonate is reported to stimulate the production of alkaloids in plants, such as *Eschscholtzia californica* (Cho et al. 2008) and *Atropa baetica* (Jaber et al. 2008).

Limited number of studies has been conducted for the production of adventitious roots in large scale at the industrial level. The first attempt to scale-up this process was carried out by Choi et al. (2000), who fruitfully attained 150-fold growth increase in ginseng adventitious roots when they were cultured in 500 L balloon type bubble bioreactors for 7 weeks. Another successful instance of the scale-up process was achieved by Wu et al. (2007a) by culturing adventitious roots of *Echinacea purpurea* in 1000 L air lift bioreactors. They attained 5.1 kg dry biomass of adventitious roots containing higher amounts of chichoric acid (22 mg g$^{-1}$ dry mass), clorogenic acid (5 mg g$^{-1}$ dry mass) and caftaric acid (4 mg g$^{-1}$ dry mass).

### 5.4 MATERIALS AND METHODS

#### 5.4.1 Initiation of adventitious root from different explants

Adventitious roots were induced from leaves and roots of *in vitro* grown plants. Leaves excised from *in vitro* grown plantlets were cut into small segments (0.5 cm × 0.5 cm) and placed on MS medium supplemented with 0.5 mg L$^{-1}$ IBA for induction of adventitious roots.
Chapter V

Root tip explants from two week old seedling were aseptically removed and were also used as explants for the root culture.

5.4.2 Effect of different media on adventitious root culture

Induced adventitious roots of about 2 cm (inoculum size, 0.5 g), were cultured in 250 mL Erlenmeyer flask containing 100 mL MS basal liquid medium, B5 medium and White root culture medium supplemented with various concentrations of IBA (0.5, 1.0, 1.5, 2.0 mg L^{-1}). All cultures were incubated under total darkness at 25±2°C with continuous agitation at 100 rpm on a rotary shaker. At the end of every 20 days, the roots were aseptically separated from the medium and cut into 1.0- to 1.5-cm long segments which were sub cultured into 250 mL Erlenmeyer flask containing medium with the above mentioned concentrations of IBA under complete darkness at 100 rpm.

5.4.3 Measurement of root biomass

Fresh weight (FW) and dry weight (DW) of roots were estimated by separating roots from the liquid media. The fresh weight was measured after blotting away the surface water with filter paper (Himedia, India) to remove excess water and finally weighed. The dry weight was recorded after drying the fresh roots at 60°C for 5-6 hours. Fresh and dry biomasses of adventitious roots are recorded as gram/flask.

5.4.4 Preparation and addition of Methyl jasmonate as elicitor

Methyl jasmonate was prepared by diluting in dimethylsulfoxide to obtain 1M stock solution and then filter-sterilized through a sterile microfilter of 0.22 μm. Elicitor was aseptically added to the 15day old adventitious root culture medium in the following final concentration of 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mM. The roots were maintained on a rotary shaker at 100 rpm in complete darkness. The roots were harvested after, 3, 6 and
9th days after addition of methyl jasmonate and dried. Respectively, control cultures were also treated with DMSO.

5.4.5 Addition of cholesterol and 7-dehydrocholesterol as precursors

Two different precursors such as cholesterol and 7-dehydrocholesterol were used at different concentrations (5, 10, 50, 100 mg L⁻¹) by adding aseptically to the 15 day old adventitious root culture medium. The cultures were maintained on a rotary shaker at 100 rpm in complete darkness. The roots were harvested after, 3, 6, 9 and 12 days after the addition of precursor. An appropriate control culture was maintained for each experiment.

5.4.6 Preparation of root extract for extraction and analysis of 20-hydroxyecdysone

Dried powdered roots (0.3g) were extracted with 20 mL methanol. The extract was then evaporated to dryness and dissolved in 1 mL methanol and filtered through a nylon syringe filter of pore size 0.22 µm to remove any cellular debris and 20 µL was injected for analysis by HPLC.

The aqueous culture medium was extracted with ethyl acetate. The ethyl acetate layer was concentrated and filtered through a nylon syringe filter of pore size 0.22 µm and was analyzed by HPLC.

5.4.7 HPLC analysis

HPLC analysis was done using the procedure given in the materials and methods of section 2.4.3.

5.4.8 LC-Q-TOF analysis

The 20-hydroxyecdysone was confirmed using LC-Q-TOF analysis as explained in the materials and methods of section 2.4.4.
5.4.9 Quantification of 20-hydroxyecdysone from adventitious root cultures

The quantitative analysis of 20-hydroxyecdysone present in the adventitious root cultures were conducted using the method described in the section 2.4.5.

5.4.10 Statistical Analysis

All experiments were performed in triplicates. The data shown represent the mean ± SE for three independent experiments. One way ANOVA analysis followed by Duncan’s Test was used to compare the means. All statistical analyses were performed using SPSS Ver.18 (SPSS Inc. Chicago, IL, USA) statistical software package.

5.5 RESULTS

5.5.1 Root induction from different explants

In this study, adventitious root cultures were established from in vitro grown leaf and root segments of *A. aspera* and their proliferation capacity, as well as growth kinetics were examined. After seven days of culture adventitious roots started emerging from the cut ends of the leaves. The fully developed roots were observed from leaf explants after 4 weeks of culture (Fig.5.1a).

Initiation of roots was also observed from the 1cm long root tip explants after 7 days of culture. The fully developed roots were removed from the medium, washed in sterile distilled water and cut into 1.5- 2.0 cm long segments and placed in fresh liquid medium for sub culturing.

5.5.2 Initiation of root culture and effect of different culture media and IBA on root growth

Root explants obtained from *in vitro* leaves and roots were used to initiate adventitious root culture. Adventitious roots (0.5g) were cultured on MS, B5 and White root culture medium supplemented with various
concentrations of IBA for 4 weeks. The greatest biomass accumulation was observed on the MS medium containing 1.5 mgL\(^{-1}\) of IBA (3.94±0.04g fresh weight and 0.993±0.008 g dry weight) followed by MS with 1 mgL\(^{-1}\) of IBA. Table: 5.1 show the effect of different media types and different concentrations of IBA on adventitious root growth. In the middle of 7 to 20 days, there was an active growth phase, where proliferating thin and white roots (Fig.5. 1b and Fig.5. 1c) were produced. Besides a linear increase in biomass was also recorded. Enhanced biomass accumulation was observed till 20 days, proceeding to a stationary phase (Fig.5.1d), where there was no subsequent increase in the biomass.

The auxin IBA was found to be effective in proliferation of roots and improves the biomass accumulation. Increase in the concentration of IBA beyond 1.5 mgL\(^{-1}\) lead to the development of shorter and thicker lateral roots. In the absence of IBA roots proliferation and elongation stayed less than that observed in medium containing IBA. In control MS media devoid of IBA, only limited numbers of lateral roots were formed and growth was also delayed. Additionally, largest root biomass was observed after 25 days of culture. Supplementation of exogenous auxin IBA was necessary for production and growth of adventitious roots in liquid media, since the endogenous auxin levels of the explants were not sufficient to induce lateral roots in media devoid of auxin IBA.

In B5 and White root culture medium the largest biomass was recorded after 21 and 30 days of growth as 3.42±0.02 g FW and 1.79±0.05 g FW respectively. Root growth in the B5 and White root culture medium devoid of IBA was 1.47±0.05 g FW and 0.84±0.01 g FW respectively. MS medium showed superior growth to B5 and White root culture medium so it was used for the maintenance, growth and further studies regarding the production of 20-hydroxyecdysone from adventitious root culture.
### Table 5.1 Influence of Different Type of Media and Various Concentrations of IBA on Adventitious Root Growth of *A. aspera*.

<table>
<thead>
<tr>
<th>Medium</th>
<th>IBA</th>
<th>7 days (Fresh weight)</th>
<th>7 days (Dry weight)</th>
<th>14 days (Fresh weight)</th>
<th>14 days (Dry weight)</th>
<th>21 days (Fresh weight)</th>
<th>21 days (Dry weight)</th>
<th>30 days (Fresh weight)</th>
<th>30 days (Dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.71±0.03</td>
<td>0.07±0.005</td>
<td>0.93±0.02</td>
<td>0.08±0.003</td>
<td>1.32±0.02</td>
<td>0.45±0.05</td>
<td>1.52±0.02</td>
<td>0.45±0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.88±0.04</td>
<td>0.09±0.004</td>
<td>1.59±0.02</td>
<td>0.46±0.005</td>
<td>2.60±0.01</td>
<td>0.68±0.008</td>
<td>2.55±0.02</td>
<td>0.65±0.02</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>1.70±0.04</td>
<td>0.40±0.01</td>
<td>2.87±0.04</td>
<td>0.61±0.01</td>
<td>3.94±0.04</td>
<td>0.99±0.008</td>
<td>3.86±0.05</td>
<td>0.92±0.01</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1.90±0.17</td>
<td>0.41±0.02</td>
<td>2.90±0.03</td>
<td>0.65±0.008</td>
<td>3.62±0.02</td>
<td>0.81±0.005</td>
<td>3.57±0.02</td>
<td>0.81±0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.74±0.02</td>
<td>0.07±0.003</td>
<td>0.97±0.02</td>
<td>0.08±0.005</td>
<td>1.22±0.01</td>
<td>0.45±0.07</td>
<td>1.47±0.05</td>
<td>0.43±0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.73±0.02</td>
<td>0.82±0.006</td>
<td>1.80±0.02</td>
<td>0.55±0.01</td>
<td>2.31±0.01</td>
<td>0.66±0.01</td>
<td>2.32±0.01</td>
<td>0.66±0.03</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>1.23±0.03</td>
<td>0.40±0.02</td>
<td>2.46±0.04</td>
<td>0.69±0.01</td>
<td>3.14±0.02</td>
<td>0.91±0.01</td>
<td>3.06±0.08</td>
<td>0.90±0.02</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.48±0.01</td>
<td>0.40±0.02</td>
<td>2.71±0.04</td>
<td>0.63±0.01</td>
<td>3.30±0.03</td>
<td>0.81±0.03</td>
<td>3.20±0.14</td>
<td>0.81±0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.54±0.02</td>
<td>0.40±0.005</td>
<td>0.65±0.01</td>
<td>0.05±0.003</td>
<td>0.80±0.17</td>
<td>0.08±0.006</td>
<td>0.84±0.01</td>
<td>0.07±0.003</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.56±0.02</td>
<td>0.04±0.003</td>
<td>0.99±0.01</td>
<td>0.08±0.008</td>
<td>1.12±0.06</td>
<td>0.39±0.003</td>
<td>1.31±0.01</td>
<td>0.39±0.01</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>0.60±0.01</td>
<td>0.06±0.005</td>
<td>1.10±0.02</td>
<td>0.45±0.03</td>
<td>1.47±0.02</td>
<td>0.49±0.05</td>
<td>1.63±0.02</td>
<td>0.51±0.06</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.61±0.01</td>
<td>0.04±0.003</td>
<td>1.39±0.01</td>
<td>0.43±0.03</td>
<td>1.53±0.03</td>
<td>0.54±0.02</td>
<td>1.61±0.02</td>
<td>0.57±0.03</td>
</tr>
</tbody>
</table>
Fig: 5.1  *A. aspera* adventitious root induction and culture (a) adventitious root induction from leaf explant on MS medium supplemented with 0.5 mg L$^{-1}$ IBA. (b) The growth of adventitious roots on MS medium after 7 (c) 14 days (d) adventitious root mass after 30 days.

### 5.5.3 Identification of 20-hydroxyecdysone using HPLC and LC-Q-TOF analysis

HPLC analysis of 20-hydroxyecdysone and root culture extract is presented in Fig.5. 2. Peaks of 20-hydroxyecdysone assigned in the HPLC chromatograms of the root extract was compared by individual peak retention time with that of the authentic reference standard. The identification of the compound was confirmed by HPLC retention behaviour, ultraviolet absorbance and the molecular structural information provided by LC-Q-TOF MS/MS (Fig.5.3 and Fig.5.4) data in comparison with reference standard
20-hydroxyecdysone. The mass spectrum of the standard solution (20-hydroxyecdysone) is shown in the Fig.2.4a and Fig.2.5a of chapter two.

**Fig.5.2** HPLC analysis of 20-hydroxyecdysone from adventitious root cultures of *A. aspera*. (a) 20-hydroxyecdysone reference standard (b) adventitious root extract of *A. aspera*. 
Establishment of Adventitious Root Cultures of Achyranthes aspera Linn.…

**Fig. 5.3** LC-Q-TOF analysis of 20-hydroxyecdysone from adventitious root cultures of *A. aspera* (*m/z* 479 [M-H]⁻ and *m/z* 525 [M+HCOO]⁻).

**Fig. 5.4** LC-Q-TOF MS/MS (Negative mode) of the mass *m/z* 479(M-H)⁻, and its fragment.
5.5.4 Quantification of 20-hydroxyecdysone from adventitious root cultures of *A. aspera*

The accumulation of 20-hydroxyecdysone increased linearly with time and reached its highest value of 0.32 mg g\(^{-1}\) DW at the end of third week (20 days) (Fig. 5.5). Analysis of the liquid culture medium did not reveal the presence of 20-hydroxyecdysone.

![Graph showing production of 20-hydroxyecdysone from *A. aspera* in vitro adventitious root cultures.](image)

**Fig. 5.5** Production of 20-hydroxyecdysone from *in vitro* adventitious root cultures of *A. aspera*. Data represent mean ± SE of three replicates and the means with the same letter are not significantly different according to the Duncan’s multiple range test (\(p \leq 0.05\)).
5.5.5 Impact of elicitor (methyl jasmonate) addition on 20-hydroxyecdysone production in adventitious root cultures of *A. aspera*

Addition of methyl jasmonate improved the synthesis of 20-hydroxyecdysone in adventitious root cultures of *A. aspera*. Methyl jasmonate at 0.6 mM produced highest concentration of 20-hydroxyecdysone (0.47mg g$^{-1}$ DW) after 3 day elicitation, which was higher as compared to the control cells. Fig. 5.6 shows the effect of different concentration of methyl jasmonate on 20-hydroxyecdysone production in adventitious root cultures of *A. aspera*. After elicitation with methyl jasmonate the fresh weight of treated roots was lower than that of the control and darkening of the roots were also observed in the elicited cultures.

**Fig. 5.6** Effect of different concentrations of methyl jasmonate on 20-hydroxyecdysone production in adventitious root cultures of *A. aspera*. Methyl jasmonate incubation period 3, 6 and 9 days are treated as different groups and data represents mean ± SE. The means with the same letter for the same parameter do not differ significantly according to the Duncan’s multiple range test ($p \leq 0.05$).
5.5.6 Impact of cholesterol treatments on 20-hydroxyecdysone production in adventitious root cultures of *A. aspera*

Fig 5.7 shows the effect of cholesterol on 20-hydroxyecdysone production in adventitious root cultures of *A. aspera*. The addition of cholesterol enhanced the production of 20-hydroxyecdysone. The maximum amount of 20-hydroxyecdysone was found to be 0.403 mg g\(^{-1}\) DW after 6 days of treatment using 10 mg L\(^{-1}\) of cholesterol. Results showed that there was no significant variation observed between the growths of roots under different treatments of precursor when compared with that of the control.

![Graph showing the effect of different concentrations of cholesterol on 20-hydroxyecdysone production in adventitious root cultures of *A. aspera*](image)

**Fig. 5.7** Effect of different concentrations of cholesterol on 20-hydroxyecdysone production in adventitious root cultures of *A. aspera*. Cholesterol incubation period 3, 6, 9 and 12 days are treated as different groups and data represents mean ± SE. The means with the same letter for the same parameter do not differ significantly according to the Duncan’s multiple range test ($p\leq0.05$).
5.5.7 Impact of 7-dehydrocholesterol treatments on 20-hydroxyecdysone production in adventitious root cultures of *A. aspera* 

Effect of 7-dehydrocholesterol on 20-hydroxyecdysone production in adventitious root cultures of *A. aspera* was also studied. The maximum amount of 20-hydroxyecdysone was found to be 0.37 mg g\(^{-1}\) DW after 6 days of treatment using 10 mg L\(^{-1}\) of 7-dehydrocholesterol (Fig.5.8). The supplementation of 7-dehydrocholesterol does not affect the cell growth when compared with that of the control.

![Graph showing the effect of different concentrations of 7-dehydrocholesterol on 20-hydroxyecdysone production in adventitious root cultures of *A. aspera*.](image)

**Fig. 5.8** Effect of different concentrations of 7-dehydrocholesterol on 20-hydroxyecdysone production in adventitious root cultures of *A. aspera*. 7-dehydrocholesterol incubation period 3, 6, 9 and 12 days are treated as different groups and data represents mean ± SE. The means with the same letter for the same parameter do not differ significantly according to the Duncan’s multiple range test (\(p\leq0.05\)).
5.6 DISCUSSION

Different types of culture media MS, B5 and Whites media were tested for their ability to establish adventitious root cultures of *A. aspera*. Among these media MS medium showed increased biomass accumulation compared to other media. This report coincided with the previous studies of Khalafalla et al. (2009) in *in vitro* root culture of *Vernonia amygdalina*. In the same way, *Bupleurum falcatum* adventitious root cultures, MS medium was appropriate for root development as well as saikosaponin production (Kusakari et al. 2000). Therefore MS medium was adopted for studying the production of 20-hydroxyecdysone from adventitious root cultures of *A. aspera*.

Growth and multiplication of adventitious roots are influenced by the growth regulators augmented in the culture medium. All the tested IBA concentrations were capable for adventitious root induction. In current study, 1.5 mgL$^{-1}$ of IBA was found to be optimal for adventitious roots formation and biomass accumulation. IBA is used commercially to induce roots in many plant species (Hartmann et al. 1990). In *Vigna radiata* adventitious root induction was observed after IBA application but not after IAA application (Riov and Yang, 1989). Treatment containing IBA was more efficient in promoting biomass production from root cultures of *Panax ginseng* (Kim et al. 2003).

This work demonstrates for the first time establishment of adventitious root cultures in *A. aspera*. An increase in the fresh weight of the roots was observed till 21 days and entered a decline phase where a gradual decrease in the biomass was observed. The obtained data point out that the production of 20-hydroxyecdysone is related to the growth of roots. Highest biomass accumulation and 20-hydroxyecdysone production (0.32 mg g$^{-1}$ DW)
was observed in MS medium supplemented with IBA 1.5 mgL\(^{-1}\) after three weeks of culture (20 day). It coincides with the previous studies in *Ajuga reptans* var. *atropurpurea* which suggest that the production of 20-hydroxyecdysone is related to the growth of hairy roots (Matsumoto and Tanaka, 1991).

Application of methyl jasmonate as elicitor enhanced the production of 20-hydroxyecdysone in adventitious root cultures of *A.aspera*. The adventitious root cultures elicited with 0.6 mM methyl jasmonate for 3-days produced 0.47 mg g\(^{-1}\) DW of 20-hydroxyecdysone. Previous experiments specified the methyl jasmonate signalling and tissue damage induced accumulation of 20-hydroxyecdysone and other phytoecdysterols in spinach (Schmelz et al. 1999). Schmelz et al (1999) also reported that wound signal analogue methyl jasmonate can be used for the manipulation of root 20-hydroxyecdysone levels in growing plants. When adventitious root cultures of ginseng elicited with 50–150 µM of methyl jasmonate enhanced the accumulation of ginsenosides compared to control cultures, but growth inhibition of adventitious roots were observed in methyl jasmonate treatments (Yu et al. 2002; Kim et al. 2004). In the present study methyl jasmonate treatments led to the darkening of the roots followed by a decline in the biomass.

Precursor feeding at suitable concentrations enhances the production of secondary metabolites in plant cell cultures (Ouyang et al. 2005). Phytoecdysteroid biosynthesis takes place through terpene synthesis and mevalonic acid pathway, which uses acetyl-CoA, mevalonic acid and cholesterol as direct precursors (Adler and Grebenok, 1999; Bathori and Pongracz, 2005). Addition of precursor cholesterol and 7-dehydrocholesterol (10 mg L\(^{-1}\)) to the *A.aspera* adventitious root cultures improved the
accumulation of 20-hydroxyecdysone, compared to that of the control cultures.

In this study, adventitious root cultures were established from *A. aspera* and demonstrated enhanced production of 20-hydroxyecdysone using elicitor and precursor addition. Further studies have to be conducted for improving the production of 20-hydroxyecdysone accumulation in adventitious root cultures of *A. aspera*. 