CHAPTER V
5.1 Introduction

The production of mass amounts of plant tissue by submerged culture (Tulecke & Nickell, 1959) and its potential use for the study of biosynthesis of secondary compounds such as alkaloids and steroids have been suggested (Nickell & Tulecke, 1960).

Cell suspension cultures offer the possibility to establish nearly homogeneous culture conditions for all cells. Nevertheless, individual cells often differ considerably in their secondary metabolite content. Suspension cultures are initiated by transfer of the most friable sectors of an established callus tissue into an agitated liquid medium. Cells are freed from the parent callus and dispersed in the liquid medium where they divide to form small cell aggregates. They are maintained by serial subculture under the right conditions of agitation. After several passages, the friability of the culture increases. A suspension culture consists of cells and cell aggregates dispersed in liquid medium and actively growing under agitation and aeration. In this chapter, the initiation of suspension culture of *S. rhombifolia* Linn and the growth and production pattern were studied.
5.2 Materials and methods

5.2.1 Source of plant material

The source of plant material was as described under section 3.2.2.

5.2.2 Callus induction and maintenance

The callus culture of *S.rhombifolia* was initiated and maintained as described under section 3.2.3.

5.2.3 Initiation and maintenance of suspension culture

Callus tissue (approx. 4g) was transferred to 40 ml of liquid MS medium (2.5 mg l\(^{-1}\) of 2,4-D + 1 mg l\(^{-1}\) of BAP) in 150 ml Erlenmeyer flasks. They were incubated for 2 weeks on a rotary shaker (Orbitek, India) at 90 rpm under light intensity of 1000 lux for 16 h photoperiod. The cell suspensions were filtered through sieves (850 \(\mu\)m) to obtain single cells and few celled aggregates for use as inoculum for subsequent subculturing. Fine cell suspension culture was obtained by repeated subculturing of callus and removing small clumps at every stage. The cell suspension culture was maintained by subculturing at 2 weeks interval.

5.2.4 Detection and identification of the contaminants

The contaminant was detected by sterility test as described by Cassels (1986). Sap from the surface sterilized leaf explants of *S.rhombifolia* was plated on two different solidified sterility test media (Nutrient agar & Potato Dextrose Agar, HI media, India) and incubated at 30°C for 48h. and assessed for the growth of contaminants.
Isolates obtained as contaminants were identified based on their morphological, physiological and biochemical properties (Claus & Berkeley, 1986).

5.2.5 Antibiotic sensitivity test

Antibiotic sensitivity tests for the contaminants were carried out employing disk diffusion test using antimicrobial octodiscs (OD 002 GII plus and OD 005 GI Minus from HI media, India). Nutrient agar plates were inoculated with the log phase broth culture employing spread plate technique and the antibiotic octodiscs were placed gently over it using a sterile forceps and pressed gently. The petriplates were then incubated at 30°C for 18-24h. Antibiotic sensitivity was assessed by measuring the diameter of the clear zone around the colonies developed on the plates.

5.2.6 Dilution susceptibility test

Based on the results of antibiotic sensitivity test, the antibiotics which expressed maximum sensitivity were selected, filter sterilized using millipore filter sterilizer assembly and tested for Minimal Inhibition Concentration (MIC). The two most sensitive antibiotics viz., gentamycin and ciprofloxacin were incorporated at a concentration, varying from 2 μg ml\(^{-1}\) to 20 μg ml\(^{-1}\), in 10 ml of nutrient broth, inoculated with the contaminant bacteria (1% v/v) at a conc. of 0.1 OD and determined the turbidity at 660 nm after 20h of incubation at 30°C. The minimum concentration at which growth of the contaminant bacteria was completely inhibited was selected as the MIC of each selected antibiotics. The
MLC (Minimal Lethal Concentration) was ascertained by subculturing the tubes showing no growth into fresh medium lacking antibiotics. The lowest antibiotic concentration from which the microorganisms do not recover and grow when transferred to fresh medium was selected as the MLC.

5.2.7 Control of the contaminant bacteria

The selected antibiotics were filter sterilized using millipore filter sterilizer assembly. The shake flask cultures of *S. rhombifolia* were added with different concentrations of selected antibiotics and incubated at 25±2°C in an orbital shaker (90 rpm) for two weeks. The plant cell density and bacterial count (number/ml) were observed using a haemocytometer under Phase Contrast Microscope and the plant cell viability was determined by dye exclusion procedure for viable cells using Evan's blue (Gaff & Okong'O-Ogola, 1971).

5.2.8 Growth measurements

The growth of suspension cultures was measured in terms of wet weight and dry weight of cells. To determine the fresh cell weight, the cell suspension was filtered through a preweighed whatman No.1 (wet condition) filter paper on a buchner funnel under slight vacuum. The cells were washed with distilled water and drained fully under vacuum, reweighed the cells with filter paper and expressed the weight of cell biomass as g wet cell weight l⁻¹.

To determine the dry cell weight, the cell suspension was filtered through a preweighed whatman No.1 filter paper on a buchner funnel under slight vacuum.
The cells were washed with distilled water and drained fully under vacuum. Dried the cells with filter paper in a hot air oven at 60°C to a constant weight (approx. 12-24h), reweighed the cells with filter and expressed the weight of cell biomass as g dry cell weight l\(^{-1}\).

### 5.2.9 Extraction procedure

a) From the cells

The cells were separated from the suspension by centrifugation (Kubota, Japan), at 10000 rpm for 10 minutes and washed with distilled water thrice and drained under vacuum. The alkaloid was extracted from the cells as described under section 3.2.4.

b) From the medium

Ephedrine from the culture medium was extracted into (3:1) ether: chloroform mixture (3x10 ml), rendered strongly alkaline with ammonia, and the organic fractions were pooled and used for assay.

### 5.2.10 Analysis of ephedrine content

The ephedrine contents of both the cells and medium were analysed as detailed under section 3.2.6.

### 5.2.11 Influence of pH

The impact of pH on biomass production and ephedrine yield of S.rhombifolia Linn. suspension culture was determined by subjecting the cultures
to various pH levels (4.5-6.0) adjusted in the medium using 1N HCl/1N NaOH. After incubation for 20 days at 25±2°C, the cell biomass and ephedrine content were analysed as described under sections 5.2.8 and 5.2.10, respectively.

5.2.12 Influence of inoculum concentration

The effect of inoculum concentration on growth was determined with various levels of inoculum (1-7% w/v). Ten day old actively growing cell suspension cultures were used as inoculum. Cells were harvested by centrifugation (Kubota 6700, Japan) at 10,000 rpm at 4°C for 10 minutes under aseptic conditions. The harvested cells were added to preweighed flasks containing 20 ml sterile media, using sterile spatula and the required inoculum concentration was adjusted based on the final weight. Further, 1 ml of cell free supernatant of the actively growing inoculum was also added to each 20 ml shake flask cultures as a conditioner. After 20 days of incubation in an orbital shaker (90 rpm) at 25±2°C under 1000 lux light intensity, the cell biomass was determined as described under section 5.2.8.

5.2.13 Growth curve and production profile

Growth curve studies were carried out in MSSR 4 medium. The inoculum selected after optimisation was added aseptically to the media and incubated at 25 ±2°C in an orbital shaker (Orbitek, India) at 90 rpm, under 1000 lux light intensity (16h photoperiod). Samples were analysed for growth and alkaloid production at regular intervals of 2 days as detailed under sections 5.2.8 and 5.2.10 respectively.
The specific growth rate ($\mu$) and the doubling time ($td$) were estimated as described earlier under section 4.2.7.

### 5.2.14 Extraction and isolation of ephedrine

The cells were separated by centrifugation (10,000 rpm for 10 minutes), washed with distilled water and drained fully under vacuum. These cells were used for extraction and isolation of alkaloid.

The extraction of the alkaloid was performed following the method suggested by Yamasaki et al. (1973) as detailed below. The cells were moistened with 0.1N HCl and homogenized in a tissue homogenizer (Remi motors, India) and extracted with a mixture of ether: chloroform (3:1). The mixture was made strongly alkaline with potassium carbonate and almost saturated with sodium chloride and kept overnight in an orbital shaker (150 rpm). The extract was then centrifuged (10,000 rpm for 10 minutes) and the residue was repeatedly (3 times) extracted with ether: chloroform mixture. The extracts were pooled and lyophilized in a lyophilizer (Yamato, Japan). The residue after lyophilization was dissolved in small (2 ml) aliquots of chloroform and used for separation by preparative TLC.

The solvent system and the spray reagent for preparative TLC were the same as described under section 3.2.5 previously. About 1 cm width of silica gel corresponds to $R_f$ value 0.56 was scraped off and extracted in methanol. The methanol extract was lyophilized to get pure ephedrine hydrochloride.
5.2.15 NMR spectrum

The $^1$H NMR spectrum (300 MHz) of the isolated alkaloid from the cells was taken and compared with the spectrum of pure ephedrine (Aldrich Chemicals, USA).

5.3 Results

5.3.1 Presence of latent contamination

In solid culture, no symptoms of contamination was observed even after ten generations of subculturing. The callus was healthy and friable at the time of initiation of suspension culture. On the third day of incubation of suspension culture, turbidity was observed in all the shake flask cultures. On analysis, the rapid increase in turbidity revealed the presence of microbial contamination. The presence of contaminants in all the shake flask cultures and in repeated experiments with fresh callus revealed that it was not accidental contamination, but latent in nature.

5.3.2 Detection and identification of the contaminant

The colonies developed on the plates, for the surface sterilized samples of sterility tests were similar in morphology to those obtained by plating the contaminated suspension culture. All the isolates were picked and identified to their generic level. Interestingly it was observed that all the isolates belonged to *Bacillus* sp (Claus & Berkley, 1986).
5.3.3 Antibiotic sensitivity test

From the results presented in fig.5.1 it is evident that among the ten antibiotics tested, ciprofloxacin and gentamycin showed maximal sensitivity at a concentration of 10 μg each, compared to others on nutrient agar plate (Plate 5).

5.3.4 Minimal inhibition concentration (MIC)

The minimal inhibition concentration of gentamycin and ciprofloxacin were observed as 10 μg ml⁻¹ and 12 μg ml⁻¹ respectively (Fig.5.2).

5.3.5 Minimal lethal concentration (MLC)

The minimal lethal concentration of gentamycin and ciprofloxacin were observed as 14 μg ml⁻¹ and 18 μg ml⁻¹ respectively (Fig.5.3).

5.3.6 Control of contaminant bacteria

Administration of filter sterilized gentamycin and ciprofloxacin at concentrations of 14 μg ml⁻¹ and 18 μg ml⁻¹ respectively were proved to be very effective to control the contaminants which occurred as latent contaminant in S.rhombifolia and was safe for the plant cells which retained more than 80% viability (Fig.5.4a&b).

5.3.7 Influence of pH

Ephedrine production by S.rhombifolia was influenced by the pH of the medium (Fig.5.5). Both the biomass and alkaloid yield were on par over a pH
Comparative efficacy of sensitive antibiotics against the contaminant

*Bacillus sp.*

G-gentamycin (10 μg), Cf-ciprofloxacin (10 μg), T-tetracyclin (30 μg), S-streptomycin (10 μg), Co-co-trimoxazol (25 μg), Cm-co-trimazine (25 μg), Nx-norfloxacin (10 μg), Cl-colistin (10 μg). The data are the mean of 5 replicates. The cultures were incubated at 30°C for 24 h.
Plate 5  Antibiogram of the contaminant Bacillus sp.

Antibiotic octodisc OD – 002

Antibiotic octodisc OD – 005
Minimal inhibition concentration (MIC) of gentamycin and ciprofloxacin against the contaminant *Bacillus* sp.

The data are the mean of 5 replicates. The cultures were incubated at 30°C in an orbital shaker (150 rpm) for 24h.
Minimal latent concentration (MLC) of gentamycin and ciprofloxacin against the contaminant *Bacillus* sp.

The data are the mean of 5 replicates. The cultures were incubated at 30°C in an orbital shaker (150 rpm) for 24h.
Influence of gentamycin on control of the contaminant *Bacillus* sp in *S.rhombifolia* suspension culture

Data represent average of 5 replicates. Cultures were incubated for 10 days on a rotary shaker (90 rpm) at 25±2°C under 1000 lux light intensity (16h photoperiod).
Influence of ciprofloxacin on control of the contaminant *Bacillus* sp in *S. rhombifolia* suspension culture

![Graph showing the influence of ciprofloxacin on bacterial counts and plant cell viability.](image)

Data represent average of 5 replicates. Cultures were incubated for 10 days on a rotary shaker (90 rpm) at 25±2°C under 1000 lux light intensity (16h photoperiod).
Influence of pH on growth and ephedrine production by *S. rhombifolia* under suspension culture

Data represent average of 5 replicates. The cultures were incubated for 20 days at 25±2°C under 1000 lux light intensity on a rotary shaker at 90rpm.
range of 5.0-5.5 although the maximum biomass yield was on pH 5.5 and alkaloid yield on pH 5.0.

5.3.8 Inoculum concentration

From the results presented in fig.5.6 it was inferred that an inoculum concentration of 5% was ideal for optimum growth of culture although inoculum concentration more than 5% showed slight increase in ephedrine yield.

5.3.9 Growth and production profile of S.rhombifolia in suspension culture

From the growth profile of S.rhombifolia in suspension culture (Fig.5.7) it is evident that the cells grew well in suspension culture (Plates 6 & 7), with a 4 fold increase in cell dry wt. over the growth cycle. The growth curve showed a typical sigmoid pattern with well defined lag (about 5 days), exponential (about 9 days) and stationary phases. The maximum growth was observed on 14th day of culture. The specific growth rate (\(\mu\)) was estimated to be 0.173 day\(^{-1}\) and the doubling time (td) was estimated to be 4 days.

The alkaloid production started at the early logarithmic phase, but alkaloid accumulation was more in the late logarithmic phase (Fig.5.7). The ephedrine production was maximum on the 14th day of culture (649 \(\mu\)g/100 ml culture). Only around 27% of the total alkaloid was released into the medium and the remaining was stored in the cell.
Influence of inoculum concentration on biomass yield of *S. rhombifolia*

Data indicate average of 5 replicates. Cultures were incubated for 20 days at $25\pm2^\circ C$ under 1000 lux light intensity on a rotary shaker at 90rpm.
Fig 5.7

Time course experiment on growth and ephedrine production by *S. rhombifolia* under suspension culture

Data represent average of 5 replicates. The cultures were incubated at 25 ± 2°C under 1000 lux light intensity on a rotary shaker at 90rpm. pH 5.5.
Plate 6  Cell suspension culture of *S. rhombifolia*

A. At early exponential phase

B. At stationary phase

C. Under continuous agitation in orbital shaker
Plate 7  Cells of *S. rhombifolia* under suspension culture

A. At early exponential phase (40X)

B. At late exponential phase (100X)
5.3.10 NMR Spectrum

The $^1$H NMR spectrum (300 MHz) of the isolated product from the cells was run in CDCl$_3$ which gave signals at $\delta = 0.9$, 2.5, 2.8, 4.8 and 7.3 and the spectral data correlated well with that of ephedrine (Fig. 5.8).

5.4 Discussion

Contamination of plant tissue cultures can be subdivided into three types (i) acute contamination at the establishment stage due to incomplete surface sterilization of the explant, (ii) contamination that occurs post establishment, possibly due to an endogenous microflora or poor technique at the subculture stage and (iii) chronic contamination that occurs apparently in batch of cultures after an extended period of supposedly axenic growth (Constantine, 1986). There are conflicting views in the literature as to the seriousness of contamination that occurs post establishment. Constantine (1986) expressed the opinion that latent contamination is the biggest single problem faced by all micropropagation laboratories.

The sudden appearance of visible growth of bacterial contamination at later in vitro stages (after many subcultures) has been attributed to contaminants which had been introduced with the initial plant material. In the present investigation, in sterility test using the sap from the surface sterilized explants in nutrient agar medium indicated the presence of latent bacterial contamination in the original plant material used. The apparent lag period between the introduction and apparent visible growth of contaminants has been explained by the presence
Fig. 5.8 NMR spectrum of the isolated ephedrine from *S.-rhombifolia* cells
of latent bacteria, which need either to adapt to the \textit{in vitro} environment or require a change in the \textit{in vitro} environment for growth (for example transfer to another media and/or a change in growth/room temperature) \cite{ComuMichel1987,Fisseetal1987}. In the present study, the sudden change in the \textit{in vitro} environment while transferring friable callus from static culture to liquid culture might be the reason for the expression of latent bacteria in suspension culture. Moreover, in liquid culture, every cell/small aggregates of cell was in direct contact with nutrient medium which also might have favoured the growth and expression of latent bacteria.

Many investigators have described the antibiotic sensitivity of bacteria isolated from plant tissue cultures \cite{CornuMichel1987,Leggattetal1988} or included antibiotics in the plant growth medium to suppress or eliminate bacterial contaminants \cite{Philipsetal1981,Mathiasetal1987,PodWyzynsaHempel1987}. Further, most of them have observed bactericidal or bacteriostatic effect of their antibiotic treatment on contaminants but were subsequently criticized for not assessing plant cultures for long enough to make certain of the success of their treatment \cite{DeberghVanderscharge1988}. Whereas, some have reported that certain antibiotic treatments had no effect on contamination \cite{Renstletal1988,Leifertetal1991}. In addition, many antibiotics have been found to be phytotoxic to plants \textit{in vivo/in vitro} and can therefore only be incorporated into plant growth media for limited periods of time \cite{CornuMichel1987,Mathews1988,Falkiner1990,Leifertetal1991}.
Several workers have reported the successful administration of comparatively high concentration of antibiotics in the medium to control the contaminants. Administration of gentamycin into plant growth media at a concentration of 200 mg l⁻¹ in combination with rifampicin (100 mg l⁻¹ ) and carbenicillin (200 mg l⁻¹ ) for 72 days were found to be successful and not phytotoxic and were free of contaminants even after two years of treatments (Philips et al. 1981).

In the present investigation, pretreatment of the callus tissue with the sensitive antibiotics for 30 minutes resulted in the inhibition of the growth of the bacteria but did not eliminate them. Hence in the later generations of subculturing, the contaminant bacteria again appeared whereas incorporation of antibiotics (14 μg ml⁻¹ gentamycin and 18 μg ml⁻¹ ciprofloxacin) into the medium, could attribute to the effective control of the contaminant. A very low concentration of 14 μg ml⁻¹ gentamycin and 18 μg ml⁻¹ ciprofloxacin were adequate for the effective control of the contaminant as against the high dozes of antibiotics reported earlier.

In many cases, the tissue culturists use the antibiotics without screening for antibiotic sensitivity and without determining the MIC and MLC of the antibiotics, which might be the reasons for the low success, need for the use of high dosage and consequent phytotoxicity reported earlier in many instances. The antibiotics are expensive and moreover no one compound is effective against all possible types of contaminating organisms. Further, it is a widely accepted fact
that antibiotics are preferred when explanted material contains concealed microorganisms that are a major obstacle to *in vitro* culture and difficult to eliminate by alternative means.

**Gentamycin** is highly specific in inhibiting the protein synthesis by binding to 30S subunit of the bacterial ribosome and cause misreading of the mRNA. The action of ciprofloxacin is complex but includes inhibition of DNA gyrase, the enzymes that maintains helical twists in DNA and thereby attribute to bactericidal activity. The greater control of bacterial infection by antibiotics in liquid medium may be due to increased surface contact and increased uptake of antibiotics into internal tissues (Leifert *et al.* 1991). Further, the alternative use of the two antibiotics were recommended in order to prevent the development of resistance against a particular antibiotic. Under suspension culture, a lower pH of 5.0-5.5 was ideal for maximum growth and alkaloid production of *S. rhombifolia* compared to solid culture. The pH of a culture varies according to the plant species and stage of culture. In *Helianthus annuus* suspension culture, the pH of the culture was raised from 5.8 in the lag phase to 6.8 in the stationary growth phase (Endress, 1994) whereas, in *Morinda citrifolia* cultures, the pH of the culture was decreased to 4.0 in the log phase from a pH of 5.4 in the lag phase and again raised to 5.1 in the stationary phase (Endress, 1994). As the pH change is not uniform in all the cultures, the optimization of ideal pH for maximum growth and production is highly essential for each and every system.
Unlike microbial cultures, the plant cell cultures require a higher inoculum concentration for satisfactory growth. The minimal amount needed is 2-3 g/100 ml medium (Endress, 1994). The growth of plant cultures depends on a minimum cell density and with Acer sp. it was reported to be 9-15x10^3 cells ml\(^{-1}\) (Street, 1977). A very high inoculum concentration of 10% (w/v) was also reported in some systems (Johnson, 1993). In the present investigation, 5% inoculum level (w/v) using 10 day old actively growing culture alongwith addition of 1 ml of cell free supernatant yielded maximum growth. With further increase, the growth remained almost static. The addition of 1 ml cell free supernatant might have a conditioning effect, as reported earlier (Ludwig et al. 1985; Teasdale & Richards, 1991).

A time course study indicated that the cells followed a sigmoid growth pattern similar to that of a microbial system with a lag phase of 5 days, a logarithmic phase of 9 days followed by a stationary growth phase. Eventhough the alkaloid production was started at the early logarithmic phase, alkaloid accumulation was more in late logarithmic phase and the content in the medium also increased correspondingly. There was no further increase in the alkaloid content during the stationary phase of cell growth. Only 27% of the total alkaloid was excreted into the medium.

From the growth profile, it is evident that S.rhombifolia suspension culture is a slow growing system with an estimated specific growth rate (\(\mu\)) of 0.173 day\(^{-1}\). This suggests that the biomass increased by about 17.3% in one day during
the exponential growth phase. But in fast growing experimental systems such as *N. tabacum* and *Daucus carota*, a very high specific growth rate (μ) of 1.1 day⁻¹ (Noguchi *et al.* 1982) and 0.22 day⁻¹ (Madusudhan *et al.* 1995) respectively were reported. The doubling time (td) of cells in *S. rhombifolia* suspension culture was estimated to be 4 days.

In the spectral data of the isolated product, the signal at 0.9 (3H, d) which appeared as a doublet can be attributed to –CH-CH₃ and –NH-CH₃ appeared at δ2.5 (3H,S). The signal at δ2.8 (1H,m) was due to CH-CH₃ proton and the benzylic Ar-CH-OH appeared at 4.2 (1H,d) as doublet. As expected, the aromatic H’s appeared at δ7.3 (5H,m). These observations confirm that the isolated compound is ephedrine.