CHAPTER 3

Initiation and Establishment of Cell Suspension Cultures of Capsicum chinense

3.1 Introduction

For decades plants have played an important role as a source of raw materials for all kinds of products. Mankind is totally dependent on plants as source of carbohydrates, proteins and fats for food and shelter. In addition, plants are a valuable source of a wide range of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavours, fragrances, colours, biopesticides and food additives. The number of known chemical structures is estimated to be nearly fourfold greater than that in the microbial kingdom (Ramachandra Rao and Ravishankar, 2002). Callus tissue is an essential material in plant cell culture systems. When introduced into a liquid medium and agitated, the cells disperse throughout the liquid to form a cell suspension culture. Such cells are, in theory, totipotent and should also have a potential to synthesize any of the compounds normally associated with the intact plant (Allan, 1996). As new cells are formed they are dispersed into the liquid medium and become clusters and aggregates. Cells in suspension can exhibit much higher rates of cell division than do cells in callus culture. Thus, cell suspension offers advantages when rapid cell division or many cell generations are desired, or when a more uniform treatment application is required (Philips et al., 1995). Several approaches can be used for the in vitro
cultivation of plant cells, including the derivation of hairy roots, immobilized cells and suspension cell cultures. Even so, researchers have focused their attention on suspension cells because these are the most amenable to Good Manufacturing Practice (GMP) procedures and they can be cultivated relatively easily in large-scale bioreactors. Suspension cell cultures have been prepared for several different plant species, including Arabidopsis thaliana, Taxus cuspidata, Catharanthus roseus and important domestic crops such as tobacco, alfalfa, rice, tomato and soybean (Hoehl et al., 1988; Van der Heijden et al., 1989; Archambault, 1991; Nagata et al., 1992; Chen et al., 1994; Wen 1995; Desikan et al., 1996; Schlatmann et al., 1996; Kieran et al., 1997; Seki et al., 1997; Sharp and Doran, 2001; Daniell et al., 2002; Kwon et al., 2003). Cell and tissue cultures of chili pepper have been used as models to study different physiological, biochemical, or molecular processes as well as systems for the isolation of variant cells exhibiting specific biochemical or agronomical characteristics. Fine suspension cultures are usually necessary for several purposes. Because of the industrial value of capsaicinoids, different efforts have been carried out to try to produce them using chili pepper cell or tissue cultures. The biosynthetic capacity of in vitro-cultured cells and tissues to produce capsaicinoids has been investigated by different groups (Ravishankar et al., 1988; Salgado-Garciglia and Ochoa-Alejo, 1990; Ochoa-Alejo and Salgado-Garciglia, 1992). Most of these reports have been attempted on C. annuum and C. frutescens. There is only one report existing on induction of capsaicinoid in C. chinense by salicylic acid or methyl jasmonate (Gutierrez-Carbajal et al., 2010).

The present chapter discusses the establishment of cell suspension cultures from white and friable callus of C. chinense using different concentrations of 2,4-D
and KN either singly or in combinations. The growth patterns of the cultures were examined during a range of culture durations. The optimized culture conditions obtained from the present study were then used to develop strategies to enhance capsaicin biosynthesis in suspension and immobilized cell culture experiments.

3.2 Materials and methods

3.2.1 Plant material and callus induction

Seeds of *Capsicum chinense* Jacq. were collected from a local field at Rüziephema village, Nagaland, India (Fig. 3.1). The seeds were thoroughly washed under running tap water for 5 min, to reduce the level of surface organisms and dirt, then treated with 2% Labolene (v/v) as laboratory detergent, for 10 min and finally rinsed five times with distilled water. These were then surface sterilized with 0.1% HgCl₂ for 5 min followed by several washes with sterile distilled water. The sterilized seeds were cultured on Murashige and Skoog, medium (MS, medium 1962) containing 3% (w/v) sucrose and 0.8% (w/v) agar, the pH of the medium was adjusted to 5.8 before autoclaving at 121°C, 1.05 kg cm⁻² pressure for 15 min. For callus growth rate, two weeks old explants viz., hypocotyl, cotyledon and leaf were excised from *in vitro* germinated seedlings and implanted in MS medium containing various concentrations of 2,4-D (0.5-4mg/l) and KN (0.5-2mg/l) for callus induction and data were recorded after 30 days of incubation (Table 3.1). The callus induction frequency was determined as follows:

\[
\text{Callus induction} (\%) = \frac{\text{No. of explants showing callus}}{\text{No. of explants inoculated}} \times 100
\]

The callus cultures induced were maintained on the medium containing 2,4-D (2 mg/l) and KN (0.5 mg/l), hereafter referred as standard medium (SM) and
subcultured every 30 days. Cultures were maintained in a culture room at temperature of 25±2°C, 14/10h photoperiod with an irradiance of 62.2 μmol m$^{-2}$ s$^{-1}$ provided by cool white fluorescent tubes.

3.2.2 Establishment of suspension cultures

*C. chinense* cell suspension cells were obtained by transferring the calli into the liquid medium of the same composition as the semi-solid medium except the gelling agent. About 2 g fresh weight callus was inoculated into 25ml of SM. Fine cell suspension cultures were established by repeated sub-culturing of small cell aggregates. Cultures were incubated on rotary shaker (125 rpm) under the same culture room conditions of temperature and light described above. A time course study on the cell growth, cell viability and product accumulation in batch cultures of *C. chinense* was investigated.

3.2.3 Measurement of cell growth

Cell growth of suspension cultures was evaluated at 5 day interval in the 25 day culture period and expressed as Growth Index (GI). GI is a relative estimation of biomass data at the sampling time to that of the initial condition. It is calculated as the ratio of the accumulated and the initial biomass. The accumulated biomass corresponds to the difference between the final and the initial masses.

$$\text{Growth Index} = \frac{(W_F - W_0)}{W_0}$$

Where GI represents growth index, and $W_F$ and $W_0$, represent the final and initial masses, respectively (Godoy-Hernández, 2006).
3.2.4 Measurement of cell viability

Cell viability assay was carried out using trypan blue exclusion test of cell viability as described by Strober (2001). The assay was based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue whereas dead cells do not. *C. chinense* suspension cells (1ml) were mixed with 1ml of 0.4% trypan blue (prepared in phosphate buffered saline) and incubated for 3 min at room temperature. 1 ml of the trypan blue/cell mixture was put onto Sedgewick Rafter Counting Cell Slide and the stained (nonviable) and unstained (viable) cells were counted separately under microscope. To obtain the total number of viable cells per ml of aliquot, the total number of viable cells was multiplied by 2 (the dilution factor for trypan blue). To obtain the total number of cells per ml of aliquot, the total numbers of viable and nonviable cells was added and multiply by 2. The percentage of the viable cells was calculated as follows:

\[
\text{Viable cells (\%)} = \frac{\text{total number of viable cells per ml of aliquot}}{\text{total number of cells per ml of aliquot}} \times 100
\]

3.2.5 Extraction of capsaicin

Capsaicin was extracted from either cells or liquid medium following the method described by Nunex-Oalenius and Ochoa-Alejo (2005) with some modifications. The suspensions cells were filtered using Whatman paper No. 1 to collect biomasses and culture filtrates following the method described by Nunex-Palenius and Ochoa-Alejo (2005). Cell suspension/immobilized samples were homogenized in methanol (1:10, w/v) and filtered through Whatman paper No. 1 over anhydrous Na$_2$SO$_4$. The extracts were evaporated to dryness at 50°C and the residue suspended in acetone (HPLC grade). In the case of culture medium,
capsaicinoids were extracted with chloroform (7.5× 25 ml) and evaporated the organic phase to dryness as described above. The residue was then redissolved in acetone.

3.2.6 Preparative thin-layer chromatography of capsaicin

Thin layer chromatography (TLC) was carried out for the separation of capsaicin from other impurities as described by Perucka and Oleszek (2000) with some modifications. TLC plates were prepared by mixing 30 g of silica gel G containing calcium sulphate as a binder, with 60 ml of distilled water. The slurry was poured onto a TLC spreader adjusted to a thickness of 0.5 mm and spread over glass plates of 20 X 20 cm. The plates were then air dried for 4-5 h, and later activated by drying them in an oven for about 30 min at a maximum temperature of 105°C. Extracts were applied onto silica gel TLC plate and developed in Benzene: Ethyl acetate: Methanol (75:20:5 v/v/v) in a chamber pre-equilibrated with the above solvent system for 2 h. The run was continued until the solvent reached the upper edge of the plate. The plates were removed and the solvent was evaporated. Capsaicin standard (1 mg/ml) was also developed in parallel. In order to locate the capsaicin on the TLC plate, the plate containing the standard was exposed to iodine vapour. For improvement of quality separations, commercially manufactured pre-coated TLC ready to use plates (Silica gel 60 GF254) were also purchased from Merck and used. The presence of capsaicin was indicated by the formation of brown spot on the plate (Fig. 3.2). The retention value (Rf) of capsaicin was calculated by the following formula:

\[ \text{Rf value} = \frac{\text{Distance from baseline travelled by solute}}{\text{Distance from the baseline travelled by solvent (Solvent front)}} \]
The Rf value of capsaicin (Rf 0.56) was determined from the standard plate. The bands corresponding to capsaicin were scooped out from the plates not exposed to iodine vapour and the compounds were washed out of silica gel with methanol.

3.2.7 Spectrophotometric determination of capsaicin

Capsaicin gives characteristics absorptions in the ultraviolet (UV) region. The TLC purified capsaicin was measured by absorbance at 280nm using Perkin Elmer Lambda 35 UV/Vis Spectrometer by the method recommended by the Joint Committee of the Pharmaceutical Society and the Society for Analytical Chemistry (Report of the Joint Committee, 1959) on the determination of the capsaicin content of Capsicum and its preparations. A standard curve was also obtained by linear regression from absorbance reading at 280nm, using pure capsaicin (sigma) diluted in HPLC grade methanol. Capsaicin concentrations in the sample were determined by comparing the absorbance reading with the standard curve.

3.2.8 High performance liquid chromatographic analysis

The HPLC analyses were carried out using Waters M515 series equipped with a µBondapak C₁₈ column (10µm particle size, 300 x 3.9 mm). The mobile phase consisted of a solvent phase consisting 6.6% acetic acid in water (phase A) and a methanol-acetic acid (50:1) mixture (phase B) pumped isocratically (73% phase A and 27% phase B) at a flow rate of 0.5 mL/min. Detection was at 280nm. All reagents used were HPLC grade. Capsaicin and dihydrocapsaicin (Sigma) were used as standard. Capsaicin and dihydrocapsaicin identities in the samples were confirmed using HPLC by comparing their retention times with that of the standard as described by Nunex-Oalenius and Ochoa-Alejo (2005) (Fig. 3.2).
3.3 Results

3.3.1 Callus induction

The frequency of callus induction and callus growth rate were studied in different explants of *C. chinense*. The explants were cultured in MS medium with varying concentrations of 2,4-D (0.5-4mg/l), and KN (0.5-2mg/l) either singly or in combination (Table 3.1). Callus induction (callogenesis) was obtained from hypocotyl, cotyledon and leaf explants. Among the two plants growth regulators tested, 2,4-D was found to be more effective than KN in callus induction. Amongst the three different explants studied, hypocotyl had higher potentiality of callus induction than cotyledon and leaf. The maximum callogenesis was recorded in the SM wherein, the induced callus resulted in maximum proliferation rate and was friable in nature (Fig. 3.3a, b). The friable callus grew well in liquid SM when subcultured and incubated on rotary shaker (125 rpm) in culture room (Fig. 3.3c, d, e). The medium containing either 2,4-D or KN also showed callogenesis, however the callus proliferation rate was low as compared to the SM.

3.3.2 Growth profile of *C. chinense* in batch cultures

Characterization of cell growth and cell viability in *C. chinense* cell cultures is important for medium optimisation and development of culture strategy to achieve higher biomass and capsaicin yield. *C. chinense* cell growth showed typical pattern as seen in microbial and plant cell suspension growth (Fig. 3.3e). The lag phase could be seen in the first 5 days. This indicated that the cell needs a short time to adapt to the new environment. The cells entered the exponential phase on day 5-15 (Fig. 3.4a, d). As the cells entered stationary growth phase, rapid cellular division ceased but the cell increased in size (Fig. 3.4b, e). At the late stationary phase and
death phase, cellular autolysis and cell debris could be observed (Fig. 3.4c, f). The cell viability was found to be more than 95% throughout the culture period (Fig. 3.5a).

3.3.3 Biosynthesis of capsaicin in batch cultures of C. chinense

The time course of capsaicin formation is nonparallel to the cell growth profile. Cell cultivated in the SM initially showed low level of capsaicin yield during active growth. When the cells approached stationary phase, cell growth and cell viability repressed, whereas capsaicin production increased in a continuous pattern (Fig. 3.5b). The accumulation of capsaicin increased gradually in the batch cultures. Product accumulation was slower during the lag growth phase, slightly higher production rate was observed in the exponential growth phase. However, the maximum capsaicin content (104±2.8 μgg⁻¹f.wt) was obtained on day 25 during the stationary growth phase.

3.4 Discussion

3.4.1 Callogenesis

Plant cell cultures are initiated through the formation of a mass of non differentiated cells called “callus”. Induction of callus from explants is an important step for successful plant cell cultures. Assessment on callus induction was studied on three types of explants viz., hypocotyls, cotyledon and leaf. Varying response on callus induction was observed on the explants. The interaction with different concentrations of plant growth regulators on the explants revealed that callus formation potentiality from hypocotyls explants was the highest in the medium fortified with 2,4-D (2 mg/l), and KN (0.5 mg/l). This finding is in consistence with earlier workers on the induction of callus (Phillips and Hubstenberger, 1995;
Ravishankar et al., 1988), who reported that MS medium fortified with 2,4-D ranks as the best callus inducer in Capsicum.

### 3.4.2 Cell growth and viability

The accurate, fast, and reliable determination of cell growth is of critical importance in plant cell and tissue culture. The precise assessment of growth kinetics is essential for the efficient design of bioprocess engineering (Godoy-Hernández and Vázquez-Flota, 2006). Characterization of cell growth is crucial for the optimisation of cell cultures. The growth pattern can be obtained by studying the biomass growth. C. chinense cell cultures showed the different growth phase viz., lag phase, exponential phase and stationary phase. During the lag phase, the rate of cell division was low and the biomass growth was small. When the cell entered the exponential phase, rapid cellular division was observed and the cell biomass growth was increased sharply. In the stationary growth phase, the rate of cell division repressed and biomass growth also decreased. The biomass remained nearly constant till 25 day. Under light microscopic observation, cell debris was observed (Fig. 3.4c, f). This may be due to the cell autolysis by the digestive enzymes (Kong, 2003). Similar growth trend has also been reported in the other plant cultures (Snape et al., 1989; Srinivasan and Ryu, 1993; Pepin et al., 1995; Zhang et al., 1996a, 1997). The cell size decreased as the cell cultures shifted from lag growth phase to exponential growth phase, this might be due to the rapid cellular division in the rapid growth phase. As the cell entered stationary growth phase, the rate of cellular division declined but the cellular size increased. This resulted in an increase in the water holding capacity since the cells no longer grew and the water holding capacity
increased. This increase in cell volume and water content could be related to the drop in osmotic stress during the exponential growth phase.

3.4.3 Formation of capsaicin in C. chinense batch cultures

The time course of capsaicin formation in C. chinense batch cultures suggested a growth associated product pattern (Fig. 3.5b). Capsaicin biosynthesis gradually increased as the cell biomass growth increased showing a paralleled growth curve; however towards the late exponential phase and stationary growth phase where cellular biomass growth repressed, capsaicin accumulation seemed to increase gradually. During the stationary phase, when the cell entered late stationary phase, the cellular biomass decreased. This might be due to the cellular autolysis resulting the leakage of capsaicin in the medium. Apart from the microscopic observation of cell debris, cell lysis could also be reflected by darkening of the culture medium due to release and oxidation of phenolic compounds produced by the cells. These results are consistent to the earlier reports on other cell cultures (Snape et al., 1989; Charlwood et al., 1990; Fett-Neto and Dicosmo, 1992; Zhang et al., 1997; Kong, 2003).