CHAPTER 1

General Introduction

1.1 Introduction

Plants form an important part of our everyday diet. Plant constituents and their nutritional value have been intensively studied for decades. In addition to essential primary metabolites (e.g., carbohydrates, lipids and amino acids), higher plants are also able to synthesize a wide variety of low molecular weight compounds, the secondary metabolites. Plant secondary metabolites can be defined as compounds that have no recognized role in the maintenance of fundamental life processes in the plants that synthesize them, but they do have an important role in the interaction of the plant with its environment. The production of these compounds is often low (less than 1% dry weight) and depends greatly on the physiological and developmental stage of the plant (Dixon, 2001; Oksman-Caldentey and Inze, 2004; Namdeo, 2007). Biotechnological approaches, specifically plant tissue culture plays a vital role in search for alternatives to production of desirable medicinal compounds from plants (Ramachandra Rao and Ravishankar, 2002). The capacity for plant cell, tissue, and organ cultures to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been recognized almost since the inception of in vitro technology. The strong and growing demand in today’s marketplace for natural, renewable products has refocused attention on in vitro plant
materials as potential factories for secondary phytochemical products, and has paved the way for new research exploring secondary product expression in vitro. It is not only the commercial significance that drives the research initiatives but also the deliberate stimulation of defined chemical products within carefully regulated in vitro cultures provides an excellent forum for in-depth investigation of biochemical and metabolic pathways, under highly controlled microenvironmental regimes (Karuppusamy, 2009). Plant cell and tissue cultures hold great promise for controlled production of myriad of useful secondary metabolites on demand.

1.2 Capsicum species

Chili peppers have been a part of the human diet in the Americas since at least 7500 BC. Archaeological evidence at sites located in southwestern Ecuador showed that chili pepper was domesticated more than 6000 years ago, and is one of the first cultivated crops in the Central and South Americas (Bosland, 1996; Perry et al., 2007). Chili pepper belongs to the genus Capsicum of the family Solanaceae. The term "chili" is a rather confusing terminology; "chile", "aji", "paprika", "chilli" and "Capsicum" are all used frequently and interchangeably for "chili pepper" plants under the genus Capsicum which belongs to a dicotyledonous group of flowering plants. The genus Capsicum consists of approximately 25 wild and 5 domesticated species (Sanatombi and Sharma, 2007). The five domesticated species are Capsicum annuum, C. baccatum, C. chinense, C. frutescens, and C. pubescens. Of the domesticated species, C. chinense is the most pungent fruit type. These domesticated species are derived from different ancestral stocks found in three distinct centers of origin. Mexico is the primary center for C. annuum, with Guatemala a secondary center; Amazonia for C. chinense and C. frutescens; and Peru and Bolivia for C.
*baccatum* and *C. pubescens*. *C. annuum* and *C. frutescens* are widely distributed from Mexico through Central America and throughout the Caribbean region (Greenleaf, 1986). *C. annuum* is the most extensively cultivated species in the world. It is the principal species grown in Hungary, Mexico, China, Korea and the East Indies.

1.3 Capsaicin

One of the main characteristics of chili pepper fruit its pungent taste due to the presence of a group of compounds known as capsaicinoids. Capsaicinoids cause the spicy flavor (pungency) of chili pepper fruit. Kosuge et al. (1965) established that the pungent principle of red pepper consists not of one chemical but actually of the unsaturated and saturated amides-capsaicin and dihydrocapsaicin. The mixture of these two amides was named capsaicinoid, which is cited odourless (Pruthi, 1980). The earliest reported capsaicinoids were capsaicin (vanillylamide of 8-methyl-
trans-6-enoic acid), dihydrocapsaicin (vanillylamide of 8-methylnonanoic acid), nordihydrocapsaicin I (vanillylamide of 9-methyldec-
trans-7-enoic acid) and homodihydrocapsaicin I (vanillylamide of 9-methyldecanoic acid) (Bennett and Kirby, 1968; Leete and Louden, 1968; Masada et al., 1971) (Fig.1.1). Since then, many more naturally occurring capsaicinoids have been identified, namely: homodihydrocapsaicin II, homocapsaicin II, vanillylamides of octanoic, nonanoic and decanoic acids, bishomocapsaicin, trishomocapsaicin, nordihydrocapsaicin II, zucapsaicin (civamide), nonivamide and ω-hydroxycapsaicin (Jurenitsch et al., 1979; Constant et al., 1996; Maillard et al., 1997; Ochi et al., 2003). Of these compounds, capsaicin and dihydrocapsaicin account for approximately 90% of capsaicinoids in chili pepper fruit, these compounds are the two most potent capsaicinoids and their
molecules differ only in the saturation of the acyl group (Bernal et al., 1993; Walpole et al., 1996; Kobata et al., 1998).

1.4 Chemistry

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is a naturally occurring alkaloid. It is a member of the vanilloid family of compounds such as vanillin from vanilla, eugenol from bay leaves and cloves, zingerone from ginger and capsaicin from hot peppers. The vanilloids possess a vanillyl (4-hydroxy-3-methoxybenzyl) moiety and this confers their biological activity. Structurally, like other vanilloids, capsaicin has a benzene ring and long hydrophobic carbon tail with a polar amide group (Hayman and Kam, 2008). Capsaicin is a crystalline, lipophilic, pungent tasting, colourless and odorless alkaloid with the molecular formula C_{18}H_{27}NO_{3} and melting point of 62–65 °C. Its molecular weight is 305.40 g/mol, and is soluble in fat, alcohol and oil. As it is not water soluble, alcohols and other organic solvents are used to solubilise capsaicin in topical preparations and sprays (Kasting, 2001). Capsaicin was first crystallized and named by Tresh in 1846, and its molecular structure was resolved by Nelson and Dawson (1919). Resiniferatoxin which is a potent naturally occurring analogue of capsaicin, extracted from the cactus Euphorbia resinifera has also been used clinically (Szallasi, 1999).

1.5 Biosynthesis of capsaicinoids

Capsaicin is an amide derivative of vanillylamine and 8-methylnon-trans-6-enoic acid (Bennett and Kirby, 1968). The capsaicin biosynthetic pathway is relatively well characterized. Capsaicin biosynthesis in plants is defined by two pathways: phenylpropanoid, which determines phenolic structure; and fatty acid metabolism, which determines the molecule’s fatty acids (Ochoa-Alejo and Gomez-
The vanillylamine moiety of capsaicinoids is derived from phenylalanine synthesized by the shikimate/arogenate pathway, whereas the branched fatty acid moiety is derived from valine (Díaz et al., 2004) (Fig. 1.2). It is usually assumed that the vanillylamine moiety is synthesized via the phenylpropanoid pathway. This assumption comes from the work of Zenk (1965), who proposed such pathway for the biosynthesis of vanillin in *Vanilla planifolia*. The phenylpropanoid origin of the vanillylamine moiety has been supported in the case of the *Capsicum* species, wherein transcript accumulation has been reported for the genes *Pal, Ca4h* and *Comt*, corresponding to the phenylpropanoid pathway (Curry et al., 1999). The branched fatty acid moiety is derived from valine, and its biosynthesis was verified by Leete and Louden (1968), who carried out tests with labelled capsaicin precursors. The genes *Kas, Acl* and *Fat*, which code for the enzymes involved in fatty acid metabolism has been reported by Aluru et al. (2003) in *Capsicum* fruit. So far, the most elusive point in the pathway is capsaicinoid synthase, which catalyzes the final condensation of vanillylamine and the branched-chain fatty acid. An assay method for the capsaicinoid synthesizing enzyme activity has been described, in addition to the substrate specificity of this enzyme (Fujiwake et al., 1980), and the levels of enzyme activity have been studied in the developing fruit and in callus culture (Ochoa-Alejo and Gómez-Peralta, 1993), but the enzyme has not yet been purified and characterized.

1.6 Capsaicin pharmacology

1.6.1 Pharmacokinetics: Capsaicin is known to be effectively absorbed topically from the skin. Capsaicin solutions were evaluated simultaneously in a random application pattern on the volar forearms of 12 subjects using a small, single
150 µg dose. Capsaicin was shown to be rapidly absorbed and to quickly reach maximum concentration when capsaicin is applied topically. The half-life of capsaicin was approximately 24 h (Pershing et al., 2004). Capsaicin metabolism is apparently similar in human, rat and dog microsome. Three major metabolites have been identified for capsaicin: 16-hydroxycapsaicin, 17 hydroxycapsaicin and 16, 17-dihydrocapsaicin (Chanda, 2008). Capsaicinoids are metabolised by cytochrome p450 enzymes to macrocyclic, alkyl dehydrogenated, omega, and omega-1 hydroxylated products (Reilly and Yost, 2005). Dihydrocapsaicin is the major metabolite of capsaicin. Dihydrocapsaicin and its metabolites are excreted by the kidney.

1.6.2 Mechanism of action: The burning and painful sensations associated with capsaicin result from its chemical interaction with sensory neurons. The mechanism of action of capsaicin has been extensively studied over the past decade. It was demonstrated that capsaicin releases substance P from afferent nociceptive neurons (Bevan and Szolcsa´nyi, 1990). Capsaicin activates afferent nociceptive neurons and evokes sensations ranging from hotness to burning. Its analgesic properties are mediated by the depletion of substance P that leads to the desensitisation of small afferent sensory neurons (Hayman and Kam, 2008). Capsaicin and other vanilloids bind to a specific nerve membrane receptor, the Transient Receptor Potential V1 receptor (TRPV1) (previously known as vanilloid receptor, VR1). The TRPV1 contains a heat-sensitive subunit responsible for the burning sensation caused by capsaicin. The TRPV1 receptors also respond to temperature, acidosis, painful stimuli, and osmolarity (Clapham, 2003). Bonding of capsaicin to TRPV1 increases intracellular calcium, triggering release of
neuropeptides such as substance P and the calcium gene-related peptide (CGRP). Contact between capsaicin and sensory neurons produces pain, inflammation and a localized heat sensation (Reyes-Escogido et al., 2011). TRPV1 is located predominantly in the cell membranes of small fibre peripheral nociceptor neurons. These TRPV1 endoplasmic receptors regulate intracellular calcium levels and are activated independently by endovanilloids, leading to reversible phosphorylation by kinases and phosphatases (which play a role in sensitisation), formation of heteromers in association with regulatory proteins, and gene expression (Cortright and Szallasi, 2004). The binding of capsaicin to TRPV1 receptor in small fibre sensory afferent nerve endings activates the receptor and this leads to an influx of calcium and the release of inflammatory neuropeptides (Holzer, 1991). This mediates the pungent properties of capsaicin and limits its tolerability. Following the receptor activation these neurons are functionally desensitised to further painful stimuli and this leads to analgesia (Karai et al., 2004; Baamonde et al., 2005). In addition there may be degeneration of nociceptive fibres (Nolano et al., 1999). This mechanism has served as a base for studies of the structure-activity relationship which are aimed at development of new synthetic ligands for the TRPV1. Capsaicin’s effects in the nervous system are not exclusively analgesic. A number of studies include results showing that it participates in release of somatostatine, CGRP and endothelium (Reyes-Escogido et al., 2011).

1.6.3 Clinical application of capsaicin: Capsaicin is mainly used as a spice, as food additive, and in pharmacological applications. As a medicine, capsaicin is currently used in topical ointments, as well as a high-dose dermal patch (trade name Qutenza), to relieve the pain of peripheral neuropathy such as post-herpetic neuralgia
caused by shingles. It also provides relief in arthritis and respiratory ailments (Mazzone et al., 1999). It is a counterirritant and an analgesic agent (Fusco et al., 1997). Capsaicin is also known to kill some types of cancer cells (Min et al., 2004). Capsaicin and other members of the capsaicinoids group produce a large number of physiological and pharmacological effects on the gastrointestinal tract, the cardiovascular and respiratory system as well as the sensory and thermoregulation systems. These effects result principally from the specific action of capsaicinoids on primary afferent neurons of the C-fiber type (Reyes-Escogido et al., 2011). Capsaicin is also currently available as a topical cream in either 0.025% or 0.075% preparation. The recommended dose is application 2–4 times daily to the affected areas (Hayman and Kam, 2008). Capsaicin has a wide application in the food, medicine and pharmaceutical industries. Due to its high demand and high price (approx. US $ 1000 kg$^{-1}$), the continuous production from cell cultures has been the subject of intensive research.

*Capsicum chinense* Jacq. is an important spice crop of India belonging to the family Solanaceae. It is known by various names in different regions such as ‘Bhoot jolokia’ or ‘Bih jolokia’ in Assam, ‘Naga King Chili’ in Nagaland, ‘Omorok’ in Manipur and ‘Ghost pepper’ by the western media. It is also known by the names, ‘Saga jolokia’, ‘Indian mystery chili’ and ‘Indian rough chili’ (after the chili’s rough skin) (Meghvansia et al., 2010). It has been acknowledged as the hottest chili in the world measuring 1,001,304 Scoville Heat Units (SHU) (Guinness World Records, 2006). Nagaland government has patented this chili and registered as the proprietor with the Government of India, under Geographical Indication Registry. Nagas are known to have used this chili as biological weapon to get rid of enemies, fox and
rodents in their fields. Due to its extra-ordinary pungency level and irritating properties it has also been used as lachrymatory agent. This chili has received the attention of world scientific community due to its extremely high pungency and unique aroma (Meghvansia et al., 2010).

With the above background information, and considering the importance aspects in perspective, it was envisaged to study the enhancement of capsaicin production by manipulating the culture strategy and the use of physical and biochemical stimuli. Thus, the objectives of the present research study were as follows:

1) To initiate and maintain callus for cell cultures of *C. chinense*.

2) To study the influence of precursors and intermediates on capsaicin accumulation in cell cultures of *C. chinense*.

3) To enhance capsaicin biosynthesis in cell cultures of *C. chinense* under nutrient, pH and osmotic stress conditions.