Genetic Transformation of Capsicum with Reference to Capsaicin and Carotenoid Production

A thesis submitted to the University of Mysore
in fulfillment of the requirement for the degree of

Doctor of Philosophy

in **Biotechnology**

by

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M.Sc.

Under the supervision of

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.

May 2009

Affectionately Dedicated

to

Maa Saraswati & my Gurus

SPECIALLY MY MENTOR

DILAWAR SINGH SANDHU

&

MY SWEET HOME

INDEX

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DECLARATION

I hereby declare that this thesis entitled **"Genetic Transformation of Capsicum with Reference to Capsaicin and Carotenoid production"** submitted to the University of Mysore, Mysore, for the award of the degree of **Doctor of Philosophy** in **Biotechnology**, is the result of research work carried out by me in the Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore, India, under the guidance of Dr. G.A. Ravishankar during the period September 2003 - September 2008.

I further declare that the results of this work have not been previously submitted for any degree or fellowship.

Place: Mysore
Date:

ASHWANI SHARMA

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CERTIFICATE

This is to certify that the thesis entitled **"Genetic Transformation of Capsicum with Reference to Capsaicin and Carotenoid Production"** submitted to the **University of Mysore** for the award of **Doctor of Philosophy** in **Biotechnology** by **Mr. Ashwani Sharma** is the result of work carried out by him in Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore-570020, under my guidance during the period September 2003-September 2008.

Place: Mysore
Date**:**

G.A.RAVISHANKAR
Research Supervisor

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Guru Brahma, Guru Vishnu, Guru Devau Maheshwara Guru Sakshat Parambrahma tasmaya Sri Guruve namah

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(Ashwani Sharma)

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LIST OF ABBREVIATIONS

c DNA	Complementary Deoxyribonucleic Acid
2,4, D	2, 4, Dichloro Phenoxy Acetic Acid
2iP	2-Isopentenyl adenine
ACC	1-aminocyclopropane-1-carboxylic acid
ADC	Arginine decarboxylase
BA	N^6-Benzyladenine
BAP	Benzylaminopurine
BIM	Bud Induction Media
DFMA	α- DL – Difluro methyl arginine
DFMO	α- DL – Difluro methyl ornithine
EDTA	Ethylene diamine tetra acetic acid
DTT	Dithiothreitol
GA_3	Gibberellic acid
GUS	β-glucuronidase
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IPTG	Isopropyl- β □Di □thiogalactopyranoside
Kin	Kinetin
LB	Luria- Bertani (medium)
MES	2 -(N-morpholino) ethanesulfonic acid
MJ	Methyl Jasmonate
MS	Murashige and Skoog (medium)
NAA	Naphthalene acetic acid
mRNA	Messenger RNA
NAA	Naphthalene acetic acid

PAA	Phenyl Acetic Acid
PAL	Phenylalanine Ammonia Lyase
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
Put	Putrescine
QTL	Quantitative Trait Loci
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SA	Salicylic acid
PAs	Polyamines
SD	Standard Deviation
SDS	Sodium dodecyl sulphate
Spd	Spermidine
Spm	Spermine
TAE	Tris-acetate-EDTA
TE	Tris-EDTA buffer
Tris	Tris (hydroxymethyl) amino methane
w/v	Weight per volume
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
$\mu g mg^{-1}$	Micro gram per milligram

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GENERAL INTRODUCTION
&
REVIEW OF LITERATURE

Content

1.1 Introduction

Chilli, belongs to the genus Capsicum of the family Solanaceae (Macrae, 1993). It is one of the most important crops of the world having distinction of being the first plant to be cultivated in the new world. Chillies have various species of long, hot, mild, fiery and sweet type peppers of which five species are commonly recognized as domesticated viz. *Capsicum annuum*, *C. frutescens*, *C. baccatum*, *C. chinese* and *C. pubescens*. However, the commercially cultivated varieties of Capsicum (Figure 1) are mainly *Capsicum annuum* and *Capsicum frutescens*. Capsicum is principally relished for its pungency and colors. Capsicum varieties are grown mainly for their fruits, which may be eaten fresh, cooked as a dried powder, made into sauces or processed for oleoresin (Poulos, 1993). "Chile", "aji", "paprika", "chili", "Chilli" and ″Capsicum″ are all used frequently and interchangeably for "Chilli pepper" plants under the genus Capsicum (Figure 1). A particular species of Capsicum is called "chile pepper" in parts of Mexico, southwestern United States and parts of Central America generally referred for a pungent variety. The term "bell pepper" is used to refer to a non-pungent, chunky, sweet type, whereas "Chilli pepper" generally refers to a pungent Chilli variety.

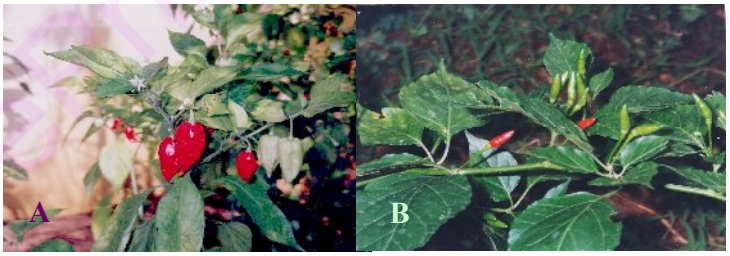


Figure 1 Capsicum plant (A) *C. annuum,* (B) *C. frutescens*

The popular name "chile" or "Chilli" originates from the hot pepper species cultivated in the South American country of Chile (De, 2000). The word comes from a Greek based derivative of Latin "Kapto" meaning "to bite", a certain reference to heat or pungency. The word "chile" is a variation of "chil", derived from the Nahuacl (Aztec) dialect, which referred to plants now known as Capsicum (Domenici, 1983).

1.2 Origin and Diffusion

Capsicum species have been thought to be of Central American origin, but one species has been reported to be introduced in Europe in the fifteenth century. Columbus's discovery of new world resulted in sighting of Chillies, which was found to be an excellent surrogate for black pepper (Kang et al., 2001). By the middle of the seventeenth century, the Capsicum was cultivated throughout southern and middle Europe as a spice and /or medicinal drug. One species was introduced to Japan and about five species were introduced into India by Portuguese, of which *C. annuum* L. and *C. frutescens* L. were cultivated on a large scale (The Wealth of India, 1992). Major Capsicum varieties and their distribution are shown in the Table 1.

Table 1 Major Capsicum varieties and their distribution across the world.

Species/Variety	Distribution
C.annuum var. annuum	America.
C.annuum var. aviculare	Southern borders of U.S.A South through the Caribbean and Northern South America and into low land tropical Peru.
C. chinese	Caribbean, throughout low land, tropical, western, central and eastern south America and as far as Southern Brazil.
C. frutescens	Caribbean, through Northern South America.
C. baccatum var. pendulum	Northern Argentina to Northern Columbia, Coast of Western South America.
C. baccatum var. baccatum	Bolivia, Northern Argentina, South Central Peru, Paraguay and Southern Brazil.
C. pubescens	Throughout Andean South America.
C. eximium	Central and Southern Bolivia to northern Argentina
C. candenasaii	Bolivia

Source: Kang et al., 2001

Original home of the chillies may be tropical South America. There seems to have been diffusion from there to Mexico, or an independent origin in the latter country, where a great diversity of the genus is found. *C. annuum*, which is found in wild state and *C. frutescens* doubtfully found in wild state are now naturalized in the tropics of many countries and are easily disseminated by birds (The Wealth of India, 1992).

The plant was introduced into Spain by Columbus, from where it spread widely. Subsequently, the prolonged viability, easy germination and easy transportation assisted its spread all across the globe. The original distributions of this species appear to have been from the South of Mexico extending into Columbia (The Wealth of India, 1992). "Ginnie Pepper" was well known in England in 1597 and was grown by Gerarde (Evans, 1996). Chilli is essentially a crop of the tropics and grows better in hotter regions. It is cultivated over large areas in all Asian countries, Africa, South and Central America, parts of USA and southern Europe, both under tropical and subtropical conditions. The major chilli growing countries are India, Nigeria, Mexico, China, Indonesia and the Korean Republic. Japan has shown the highest yield of green chillies, followed by India (FAO, 2005).

Chilli peppers grow as a perennial shrub in suitable climatic conditions usually represents glabrous, perennial, woody sub shrubs or shrubs, some tending to be vines, rarely herbs (The Wealth of India, 1992). The majority of the commercial chillies belong either to *C. annuum* or *C. frutescens,* but mostly to the former. All the domesticated forms commonly grown in the old world are within this group. Five or six species are under cultivation, and about 20 wild species have now been recognized in this genus. In addition to *C. annuum*, *C. frutescens*, *C. baccatum* L. var. pendulum (Wild), Eshbough (syn. *C. pendulum* Wild), *C. chinense* Jacq. (syn. *C. angulosum* Mill.) and *C. pubescens,* Ruiz and Pav. have been introduced from South America (The Wealth of India, 1992).

1.3 Chemical composition

Capsicum fruits contain coloring pigments, pungent principles, resin, protein, cellulose, pentosans, mineral elements and a very little volatile oil, while seeds contain fixed (non-volatile) oil. The fruits of most Capsicum species contain significant amounts of vitamins B, C, E and provitamin A (carotene) when in a fresh state. The large type of *C. annuum* is among the richest known sources of vitamin C, which may be present up to 340 mg / 100g in some varieties (Purseglove et al., 1987) and reviewed by Govindarajan (1985) and Pruthi (1999).

Several pungent compounds found in nature are derivatives of o-methoxyphenol, It was in 1846 that Thresh isolated for the first time the pungent principle from Capsicums. Nelson and Dawson (1923) declared it to be an amide of vanillylamine and isodecanoid acid. The major principles naturally present in Capsicums are capsaicin and dihydrocapsaicin (Kulka, 1967). The degree of pungency and the character of taste sensation vary markedly with different varieties of chillies. Further work on the chemistry of capsaicin has been reviewed by Newman (1953), Rogers (1966) and Pruthi (1980). The chemistry of pungent principles has been reviewed by Pruthi (1980, 1999), Govindarajan (1985) and Anu and Peter (2000).

The pungency is caused by a group of vanillyl amides named capsaicinoids located in the placenta of the fruit. The heat of Capsicum powder is measured by Scoville heat units (Scoville, 1912). One Scoville unit of capsaicinoids is measured as 15 parts per million concentrations. The nature of pungency has been established as a mixture of seven homologous branched-chain alkyl vanillyl amides, named capsaicinoids (Anu and Peter, 2000) (Figure 2).

Chemical Formula	**Capsacinoid**
$(CH_3)_2$. CH. CH=CH $(CH_2)_4$ - CO-R	Capsaicin
$(CH_3)_2$. CH. $(CH_2)_6$ - CO-R	Nordihydrocapsaicin
$(CH_3)_2$. CH. $(CH_2)_9$ - CO-R	Dihydrocapsaicin
$(CH_3)_2$. CH. $(CH_2)_9$ - CO-R	Homodihydrocapsaicin
$(CH_3)_2$. CH. CH=CH. $(CH_2)_5$ - CO-R	Homocapsaicin
$(CH_3)_2$. $(CH_2)_7$ - CO-R	Nonanoic acid vanillylamide
$(CH_3)_2$. $(CH_2)_8$ - CO-R	Decanoic acid vanillylamide

where R is

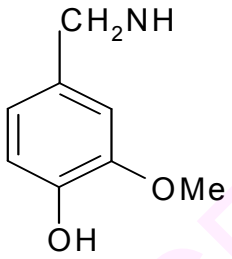


Figure 2 Composition of various capsaicinoids

CAPSAICIN

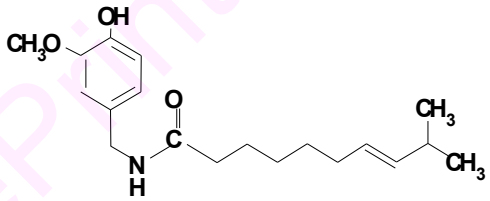


Figure 3 Structure of capsaicin

IUPAC NAME

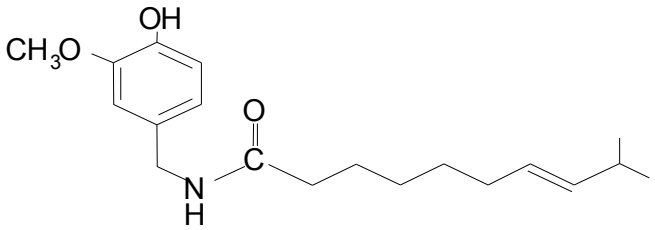
8-Methyl-N-vanillyl-trans-6-nonenamide $(C_{18}H_{27}NO)$

$(CH_3)_2CHCH=CH(CH_2)_4$ $CONHCH_2C_6H_3$-4-(OH)-3-(OCH_3)

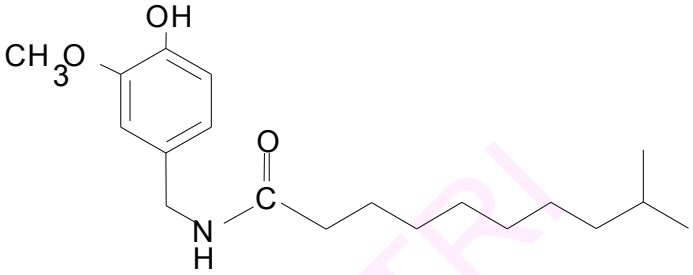
Capsaicin (Figure 3) is an alkaloid, produced by the placenta of pepper fruit makes chilli peppers very hot; however, its applications are numerous. Capsaicin is produced where the placenta and pod wall meet at the top of a pepper, which explains why the bottom half of a pepper is less spicy than the top. Pungency of capsaicin is measured in Scoville units, with Bell and sweet peppers generally rate less than 100 units. Habanero peppers, on the other hand, have 200,000 to 500,000 Scoville units. Pure capsaicin, however rates 16,000,000 units (Anu and Peter, 2000).

Capsaicin is a stable alkaloid seemingly unaffected by cold or heat, which retains its original potency despite time, cooking, or freezing. The precise amount of capsaicin present in chillies can be measured by high performance liquid chromatography (HPLC). Although it has no odor or flavor, it is one of the most pungent compounds known, detectable to the palate in dilutions of one to seventeen million. It is slightly soluble in water, but very soluble in alcohols, fats, and oils. Evidently, all of the capsaicinoids work together to produce the pungency of peppers, but capsaicin itself is still rated the strongest (Anu and Peter, 2000). The structure of major capsaicinoids that are present in chilli are given in Figure 4.

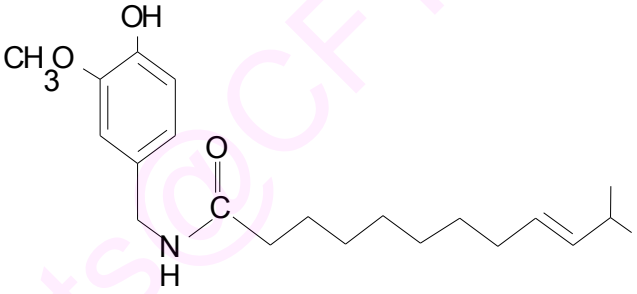
CAPSAICIN (C)



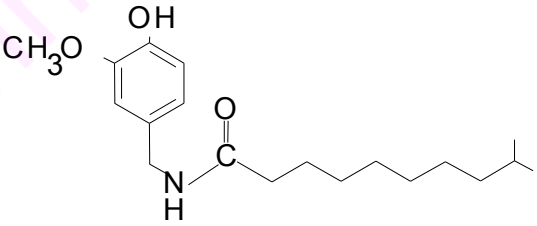
DIHYDRO CAPSAICIN (DHC)



HOMO CAPSAICIN



HOMO DIHYDRO CAPSAICIN



NORHYDRO CAPSAICIN

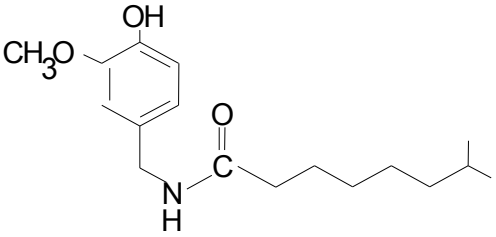


Figure 4 Structure of the major capsaicinoids

1.4 Trade production and Market of Capsicum

Of the total world consumption, chillies proper account for one third, while paprika comprises two thirds. World production of chillies is leaping year after year which is mainly attributed to ethnic food nature of this crop. Chilli production in the world has been estimated to be 2,500,000 tonnes (FAO, 2005), of which India ranks first with the production of 850,000 tonnes (Figure 5) which is the one third of the world production followed by China, Indonesia, Korea, Pakistan, Turkey, Morocco, Sri Lanka, Nigeria, Ghana, Tunisia, Egypt, Mexico, the US, Yugoslavia, Spain, Romania, Bulgaria, Italy, Hungary, Argentina, Peru and Brazil. Around 90% of India's production is consumed within the country (Thampi, 2003). Chillies are mainly traded in dried or powder (ground) form. The British Standards Institution specifies that dried chillies, whole or ground, should contain not more than 11 per cent moisture, 10 per cent total ash and 1.6 per cent maximum of total ash insoluble in hydrochloric acid (Table 2). India ranks first in consumption of Chilli also (Terry, 2002).

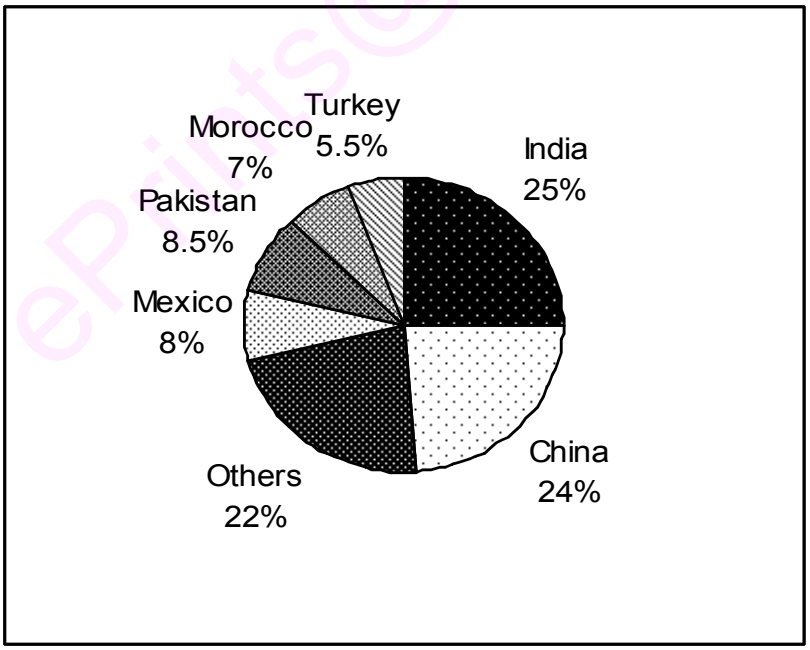


Figure 5 Chilli productions across the globe

Table 2 Comparison of Chemical Composition of Paprika and Chilli

Quality characteristics (units in bracket)	Paprika	Pepper (Chilli)
Chemical composition		
Moisture (g)	7.90	6.50
Food Energy (cal)	390.00	415.00
Protein (g)	13.80	14.00
Fat (g)	10.40	14.10
Total carbohydrate (g)	60.30	58.20
Fiber carbohydrate (g)	19.00	15.60
Total ash (g)	7.60	7.20
Minerals		
Calcium (g)	0.20	0.10
Phosphorous (g)	0.30	0.32
Sodium (g)	0.02	0.01
Potassium (g)	2.40	2.10
Iron (mg)	23.10	9.90
Vitamins		
Thiamine (mg)	0.60	0.59
Riboflavin (mg)	1.36	1.66
Niacin (mg)	15.30	14.20
Ascorbic acid (mg)	58.80	63.70
Vitamin A (IU)	4,915	6,165

Based on the analyses performed by 900 American Laboratories, St. Louis, MO, USA (2000). Adapted from De, 2000

The major Chilli growing states in India include Andhra Pradesh, Karnataka, Maharashtra, Madhya Pradesh, Orissa, West Bengal, Rajasthan and Tamil Nadu. The most popular varieties among these are, *Sannam*, *LC* 334, *Byadgi*, *Wonder Hot*, *Pusa Jwala* etc (FAO, 2005).

1.5 Biosynthesis of capsaicinoids

The biosynthetic pathway of capsaicinoids has been studied in terms of organic chemistry and biochemistry using radiotracer technique. It has been proposed that they are synthesized by the condensation of vanillylamine with C_9 to C_{11} isotype branched chain fatty acids (Bennet and Kirby, 1968; Iwai et al., 1979; Suzuki et.al., 1981) (Figure 6).

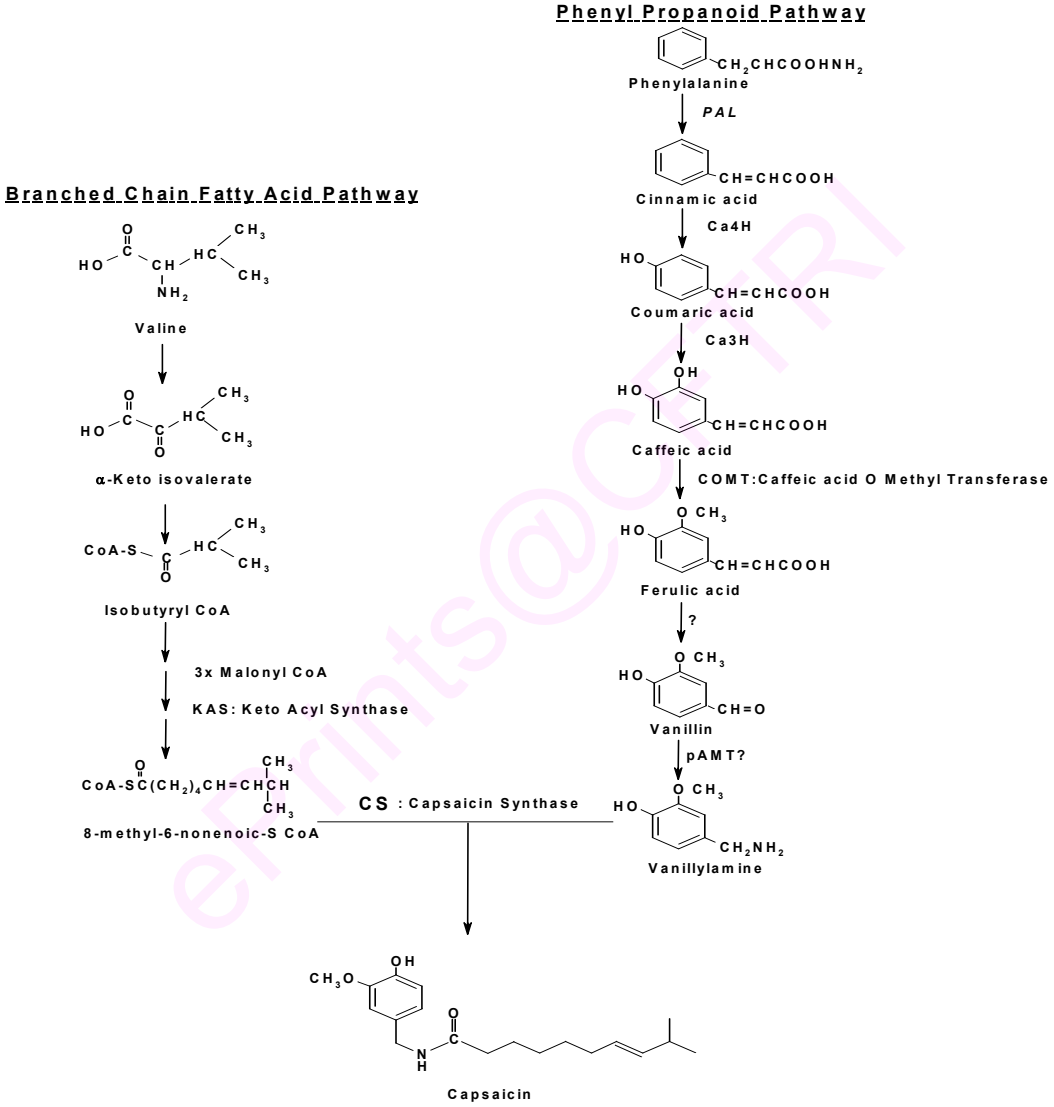


Figure 6 Proposed biosynthetic pathway of capsaicin and vanillin from Blum et al., 2002

The biosynthesis of capsaicinoids starts as early as first week of anthesis as reported by Ohta (1962). The decrease in the capsaicin content is related to increase in peroxidase activity as peroxidase may degrade capsaicin (Zewide and Bosland, 2001). Iwai et al.,

(1979) reported that inter conversion of capsaicin to dihydrocapsaicin or *vice versa* does not occur. The capsaicinoids are accumulated in vesicles or blisters of epidermal cells of chilli placenta (Suzuki et al., 1980; Zamski et al.,1987). The genetics of capsaicin biosynthesis is poorly understood with an exception being C locus, which is mapped on chromosome 2 (Andrews, 1995). This dominant allele is essential for capsaicin production. The homozygous recessive condition *cc* results in complete lack of capacity to synthesize capsaicinoids (Blum et al., 2002). Capsaicin is the major metabolite in *Capsicum* sp. and is produced mainly in the placenta of the fruits. A degree of variability of capsaicin in the varieties of the same species has been recorded by several researchers (Quagliotti, 1971). The various intermediate steps of capsaicinoid and biosynthesis through phenyl propanoid metabolism have been well studied. The biosynthetic pathway of Capsaicinoids has been thoroughly evaluated (Figure 6). Capsaicin and dihydrocapsaicin are the major analogues occupying more than 90% of the total capsaicinoids, whereas homocapsaicin, homodihydrocapsaicin and nordihydrocapsaicin are the minor analogues (Iwai et al., 1979). All these analogues are biosynthesized from L-phenylalanine and L-valine or L-phenylalanine and L-leucine in the placenta of Capsicum fruits by phenyl propanoid metabolism (Iwai et al., 1979). *Trans*-cinnamic acid, *trans*-*p*-coumaric acid, *trans*-caffeic acid and *trans*-ferulic acid were also reported to be involved in the biosynthesis of capsaicin and its analogues (Bennet and Kirby, 1968).

There is a great variation in capsaicinoid content among different pungent pepper varieties and the role of capsaicin in biological processes is controversial (Hans et al., 2001). Capsaicinoid biosynthesis continues throughout fruit development until the end of the growth phase. During the ripening stage of fruit development, however, some decrease in capsaicinoid content may occur (Iwai et al., 1979; Estrada et al., 2000), possibly because of degradation caused by enzymatic oxidation. Peroxidases may be involved in this process because expression and activity of a peroxidase enzyme is positively correlated with capsaicinoid degradation (Diaz et al., 2004).

Capsaicinoids are synthesized by the condensation of vanillylamine with C9 to C11 isotype branched-chain fatty acids; the former is derived from the phenylpropanoid pathway, the latter from valine and leucine (Bennet and Kirby, 1968). In spite of studies on its biosynthesis, many of the enzymes involved in capsaicin biosynthesis are not well characterized and the regulation of the pathway remains elusive. Recently, it has been found that a single dominant gene, *C*, is required for pungent genotypes to produce capsaicinoids, and this gene is mapped to pepper chromosome 2, where several markers that co-segregated with *Pun1* were identified (Blum et al., 2002). In addition, AT3 (Acyl transferase), an acyltransferase, was identified as a strong candidate gene product for *Pun1* based on its map position and its hybridization pattern that correlates it with pungency (Stewart et al., 2005). The recessive allele, *pun1*, is present in the homozygous condition based on the lack of production of capsaicinoids. However, no information is available on the action of specific genes that control the degree of capsaicinoid accumulation in the genus Capsicum (Lee et al., 2006). Lee et al., (2006) also elucidated about some biological clues for unraveling the biosynthetic pathway of Capsaicinoids and its regulation through proteomic approach using the placental tissues of pungent and non pungent pepper.

Lee et al., (2006) and Kim et al., (2001) isolated placenta-specific cDNA clones through suppression subtractive hybridization (SSH) which are to be related to pungency of pepper. Capsaicinoids, the alkaloids responsible for pungency in the chilli pepper fruit, are synthesized from phenylpropanoid intermediates and short-chain branched-fatty acids (Rose et al., 2004). In spite of many studies on the biosynthesis of capsaicinoids in the field of genetics, the molecular mechanism of the biosynthetic pathways for capsaicinoid, sub-cellular localization, and cellular structures required for pungency accumulation in peppers remain elusive. Curry et al., (1999), showed that differential patterns of gene product accumulation involved in the phenylpropanoid pathway, *Pal*, *Ca4h*, and *Comt*, were correlated with fruit pungency (Curry et al., 1999). The components of the fatty acid synthase (FAS) complex, Keto 3-oxoacyl-ACP synthase (*KAS*), *Acl*, and *Fat* (Ferulic acid transferase), and *AT3* also showed positive correlation of their transcripts with the pungency (Aluru et al.,

2003). From the results of Prasad et al., (2006), Keto 3-oxoacyl-ACP synthase (*KAS*) expression is directly correlated with the level of capsaicin production and 8-methyl-nonenoic acid pool plays a crucial role in determining the efficacy of capsaicin levels. *KAS* condenses keto acyl-CoA groups with malonyl-ACP, releasing CO_2. It accumulates in the placenta of pungent chilli fruits and it is found in greatest abundance in the epidermal cell layers of the placenta near the capsaicinoid receptacles (Aluru et al., 2003). This gene product may be associated with the Capsicum fruit traits, capsaicinoid biosynthesis, for elongation of the branched-chain fatty acids. Transcript of *pAmt* also appears to be placenta-specific and the transcript abundance is correlated with pepper fruit pungency (Curry et al., 1999). Also this gene product was isolated as a cDNA clone differentially or preferentially accumulated in the placenta of pungent pepper using SSH (Kim et al., 2001).

Up-regulated expressions of two proteins, *Kas* and pAmt, shows that an increase in capsaicin levels is well correlated with the levels of vanillylamine and 8-methyl-nonenoic acid (Blacktock and Weir, 1999). Early genetic studies identified a single dominant gene, *C*, now known as *Pun1*, that in the homozygous recessive condition results in absence of pungency regardless of genotype at other loci throughout the genome that affect pungency level or other aspects of this trait. This gene encodes AT3, a putative acyltransferase (Stewart et al., 2005) on chromosome 2 (Lefebvre et al., 1998; Blum et al., 2002; Ben-Chaim et al., 2006). The only phenotypic variation ascribed to this locus to date, presence/absence of pungency, is a consequence of the loss-of-function allele known as *pun1*, a recessive allele for non-pungency that apparently results from a 2.5 kb deletion spanning the first exon and part of the promoter region thereby preventing expression of AT3 (Stewart et al., 2005). The amount of capsaicinoid produced in hot peppers is a quantitatively inherited trait (Zewdie and Bosland, 2001). Only two reports have focused on this aspect of the trait (Zewdie and Bosland, 2001; Blum et al., 2003), one of which revealed a major QTL for capsaicinoid content, termed *Cap*, on chromosome 7 (Blum et al., 2003). *Cap* was identified in an inter-specific cross between the pungent and the non-pungent pepper for polymorphisms between high and low pungent F2 bulks (Ben-Chaim, 2006). Co-localization was observed between a

set of predicted structural genes from the capsaicinoid biosynthesis pathway and variation in capsaicinoid content (Blum et al., 2003).

1.5.1 Molecular biology of capsaicin synthesis

The capsaicin biosynthetic pathway has two distinct branches, one of which utilizes phenylalanine and gives rise to the aromatic component vanillylamine via the phenylpropanoid pathway (Figure 6). The second branch forms the branched-chain fatty acids by elongation of deaminated valine. The cDNA and in some cases, genomic clones of early phenylpropanoid metabolites have been characterized from many plants for three of these enzymes - Phenylalanine ammonia lyase (*Pal*) (Estabrook and Sengupta- Gopalan, 1991; Pellegrini et al., 1994), Cinnamate 4 - Hydroxylase (*Ca_4H*) (Fahrendrorf and Dixon, 1993; Kawai et al., 1996; Schopfer and Ebel, 1998), and Caffeic acid o-methyl transferase (*Comt*) (Gowri et al., 1991; Lee et al., 1998). Condensation of 8-methyl-6-nonenoic acid with vanillylamine by capsaicinoid synthetase (CS) results in formation of capsaicin. Aluru et al., (1998), and Curry et al., (1999), have isolated a 5-ketoacyl-ACP synthase gene from the Habanero Chilli, *C. chinense,* by screening cDNA libraries of transcripts from placental tissues. The cDNA was synthesized from mRNA isolated from the placental tissue of an immature habanero fruit at approximately 70% of the maximal capsaicinoid accumulation. The hybridizing clones were characterized and the clone with the largest insert was sequenced. Curry et al., (1998), have isolated the cDNA forms of *P*al, *Ca4h* and *Comt* from a library of cloned placental transcripts. These genes encode the first, second and fourth step of the phenylpropanoid branch of the capsaicinoid pathway. Curry et al., (1998), have developed a hypothesis about the regulation of transcription for capsaicinoid biosynthetic enzymes. Transcripts of biosynthetic genes accumulate in the placenta early in fruit development and then decline in abundance; transcript levels of biosynthetic genes are proportional to the degree of pungency, with the hottest Chilli having the greatest accumulation of transcripts. Curry et al., (1998, 1999), have employed these transcript levels as a screening tool of a cDNA library of habanero placental tissue. Using this differential

approach they have isolated a number of cDNA clones and confirmed their differential patterns of expression; two of the clones putatively encode enzyme activities predicted for capsaicinoid biosynthesis, β-ketoacyl synthase and a transaminase. Lee et al., (1998) isolated and characterized o-diphenol-o-methyltransferase cDNA clone in hot pepper. Matsui et al., (1997), have performed purification and molecular cloning of bell pepper fruit fatty acid hydroperoxide lyase. Transcript accumulation of several capsaicinoid biosynthetic genes was correlated with the level of pungency (Curry et al., (1999); Aluru et al., (2003). A recessive allele of the Pun1, responsible for non-pungency within *C. annuum* as a result of a large deletion at Pun1 has been conserved (Stewart et al., 2005). In the allelic state at Pun1, transcript accumulation of capsaicinoid biosynthetic genes and capsaicinoid accumulation are highly correlated (Stewart 2007). Another recessive allele of Pun1, namely $pun1^2$, was identified and sequencing revealed a 4 bp deletion in the centre of the first exon of AT3. Inheritance studies revealed that pun12 co-segregated with the absence of blisters, non-pungency, and decreased expression of two capsaicinoid biosynthetic genes, Kas and AT3 (Stewart et al., 2007).A strong up-regulation of several capsaicinoid biosynthetic genes (pAMT, Pal, Kas, BCAT, FatA) occurs after flowering coinciding with capsaicinoid accumulation in pungent varieties of both *C. annuum* and *C. chinense* (Stewart et al., 2005).

1.6 Carotenoid

Carotenoids are the red, orange and yellow molecules that act as protective agents and accessory light harvesting pigments, and add nutritional and ornamental value to plants (Cunningham and Gantt, 1998). The dietary sources of carotenoids are given in the Table 3.

The change of color in paprika during processing and storage, with subsequent browning, is attributed to oxidative attack catalyzed by light. Oil soluble anti-oxidants are known to retard the color loss of good paprika on storage. Capsanthin and capsorubin are characteristic of the genus Capsicum but other carotenoids such as β-cryptoxanthin, Zeaxanthin and to an extent β-carotene may also contribute to red color (Davies, 1987).

During pepper fruit ripening, selective xanthophyll esterification with fatty acids increases with a gradual decrease of free pigments and is directly linked to the transformation of chloroplasts into chromoplasts (Markus and Biacs, 1999). In the fully ripened stage a balance between free, partially and totally esterified fractions is reached which seems to be largely independent of variety and could be used as indices of physiological maturity of the fruit (Hornero-Mendez, 2000).

Table 3 Dietary Sources of Carotenoids

Source	Main carotenoids	Uses
Annatto (*Bixa orellana*)	Bixin and norbixin	Coloring foods, cosmetics and Textiles
Dunaliella sp.	β-carotene	Feed and food additive; dietary supplement
Haematococcus sp.	Astaxanthin	Feed additive; nutraceutical agent
Marigold (*Tagetus erecta*) and green leafy vegetables	Lutein and Zeaxanthin	Poultry and fishery feed additive; purified oleoresin as food additive
Paprika (*Capsicum annuum*)	Capsanthin and capsorubin	Used as spice in food to add color and flavor
Saffron (*Crocus sativus*)	Crocetin and crocin	Foods and pharmaceutical products
Tomato, red grapes, watermelon, pink Grapefruit, papaya and apricots.	Lycopene and β-carotene	Nutraceutical and food colorant
Vegetables (carrots, pumpkins, sweet potatoes) and vegetable oils	β-carotene	Feed additive

Source: adapted from Delgado- Vargas et al., 2003 ; Rodriguez-Amaya, 2001

The red pigments in Capsicum constitute about 70%-85% and yellow about 15%-23% of the pigment pool (Govindarajan et al., 1987). It is reported that there exists equilibrium between the red and yellow pigment contents in the spice, i.e the percentage of red components increases with increase in total pigment content and that of yellow components decreases correspondingly (Purseglove et al., 1987). The carotenoid content of fruits and vegetables varies greatly in amount, depending on species, variety, time and degree of ripeness (Mangels and Ahuja, 1993). The first studies on the inheritance of mature fruit color in *C. annuum* stated that the yellow fruit color was determined by the 'y′ recessive

allele where as the red color was determined by the 'y′ dominant allele (Hurtado-Hemandez, 1996). Carotenoids can play the role of versatile antioxidants because they are effective biological quenchers as well as chain breakers. β- carotene, shows the remarkable effect of changing its antioxidant to a pro-oxidant behavior at high concentration of β- carotene and in the presence of high oxygen pressure (Burton and Ingold, 1984). β- carotene is an important component in the reaction centers and antenna of the photosynthetic apparatus, it is also a substrate for the biosynthesis of other important carotenoids such as xanthophylls, zeaxanthin, antheraxanthin, violaxanthin and neoxanthin. β- carotene is also precursor of phytohormone abcissic acid (Rock and Zeewart, 1991).

The last step in carotenoid biosynthetic pathway in pepper fruits is conversion of antheraxanthin into capsanthin and violaxanthin into capsorubin which is catalysed by the bifunctional enzyme capsanthin-capsorubin synthase [*Ccs*] (Bouvier et al., 1994). The intense coloration is due to the presence of highly conjugated double bonds capsanthin and capsorubin contribute to red color, where as β- carotene and Zeaxanthin are responsible for yellow-orange color (Phillip and Francis, 1971).

Xanthophylls accumulate in fruits mainly as mono or diesters of fatty acids during ripening process. Evaluation of the carotenoid concentration in fruits has been achieved by measuring the absorbance of benzene extract (Bouvier et al., 1994). The enzyme *Ccs* was detectable only during ripening of red fruits which entails the progressive appearance of transient brown zones yielding the full red color characteristic of the final stage of pepper fruit development (Hugueney et al.,1995). Capsanthin and canthaxanthin have shown better antioxidant activity than lutein and β- carotene respectively. It appears that activity depends on the number of double bonds, keto groups and cyclopentane rings that are on the carotenoid structure.

Apart from pungency principle and pigments more than 125 volatile compounds have been identified in fresh and processed Capsicum fruits (Cuttriss and Pogson, 2004). The significance of these compounds for the aroma is not well known. Over 60 volatile compounds were identified in bell peppers among which 2-isobuty-3 methoxy pyrazine, 2, 6

nonadienal and decadienal are important aroma compounds. This pyrazine and other alkyl-methoxy pyrazines are the character impact compounds of the genus Capsicum (Whitefield and Last, 1991). Peppers contain moderate to high levels of neutral phenolics, flavonoids and phytochemicals that are important antioxidant components of a plant based diet. Carotenoids are a class of hydrocarbons consisting of eight isoprenoid units (Figure 7), joined in a head-to-tail pattern, except at the centre to give symmetry to the molecule so that the two central methyl groups are in a 1,6-positional relationship and the remaining non-terminal methyl groups are in a 1,5-positional relationship.

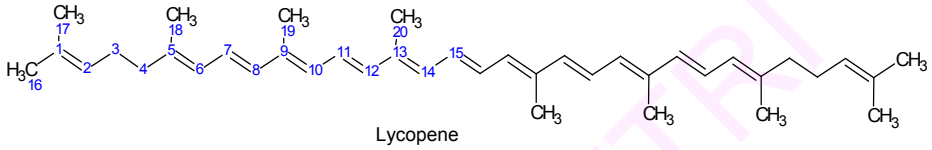


Figure 7 Structure of carotenoid with common numbering system

The nature of the specific end groups on carotenoids may influence their polarity, which may explain the differences in the ways that individual carotenoids interact with biological membranes (Britton, 1995). Some of the characteristics of carotenoids such as their semi-systematic name, number of conjugated double bonds, absorption maxima in different solvents and color are listed in Table 4.

Table 4 Chemistry of Carotenoids

Common name	Semisystematic name	No. of conjugated double bonds	λ_{max} (nm)	*Color*
Antheraxanthin	5,6-epoxy-5, 6-dihydro-β, β-carotene-3, 3'-diol	10	423, 444, 473[a] 421, 443, 473[b] 424, 448, 475[c]	Yellow
Astaxanthin	3,3'-dihydroxy- β, β-carotene-4, 4'-dione	13	478 [b, g]	Red
Bixin	methyl hydrogen 9'- cis- 6,6'- diapocarotene- 6,6'- dioate	9	432, 456, 490[a] 439, 470, 503[d]	Red
Canthaxanthin	β ,β-carotene-4, 4'-dione	11	466[a],477[b]	Red
Capsanthin	**3, 3'-dihydroxy- β, κ-caroten-6'-one**	**11**	**450, 475, 505[a] 468, 483, 518[f]**	**Red**
Capsorubin	**3, 3'-dihydroxy- β, κ-carotene-6, 6'-dione**	**11**	**444, 474, 506[a] 460, 489, 523[f]**	**Red**
α-carotene	β, ε-carotene	10	422, 445, 473[a] 424, 448, 476[e] 421, 445, 473[g]	Yellow
β-carotene	β, β-carotene	11	425, 449, 476[a] 427, 454, 480[e] 432, 454, 480[c]	Yellow
γ-carotene	β, ψ-carotene	11	437, 462, 494[a] 435, 461, 490[g]	Bright orange
ζ-carotene	7, 8, 7', 8'-tetrahydro- ψ, ψ-carotene	7	378, 400, 425[a] 415, 440, 468[g]	Pale yellow
Crocetin	8, 8'- diapo- 8, 8' – dioic acid	7	400, 422, 450[a] 401, 423, 447[b]	Yellow
β-Cryptoxanthin	β, β-caroten-3-ol	11	425, 449, 476[a,c] 428, 450, 476[b]	Yellow/orange
Fucoxanthin	3'-acetoxy-5, 6-epoxy-3, 5'-dihydroxy-6', 7'-didehydro-5, 6, 7, 8, 5', 6'-hexahydro- β-carotene-8-one	9	435, 446, 473[a] 437, 450, 478[g]	Yellow
Lutein	β, ε-carotene-3, 3'-diol	10	421, 445, 474[a] 422, 445, 474[b] 426, 447, 474[c]	Yellow
Lycopene	ψ, ψ –carotene	11	444, 470, 502[a] 446, 472, 503[b] 447, 473, 505[c]	Pink/red
Neoxanthin	5', 6'-epoxy-6, 7-didehydro-5, 6, 5', 6'-tetrahydro- β, β-carotene-3, 5, 3'-triol	8	416, 438, 467[a] 415, 439, 467[b] 414, 437, 465[c]	Yellow
Norbixin	2E, 4E, 6E, 8E, 10E, 12E, 14E, 16E, 18E- 4,8,13,17-tetramethylicosa-2,4,6,8,10,12,14,16, 18-nonaenedioic acid	9	442, 474, 509[d]	Red
Phytoene	7, 8, 11, 12, 7', 8', 11', 12'-octahydro- ψ, ψ –carotene	3	276, 286, 297[a,g]	Colorless
Phytofluene	7, 8, 11, 12, 7', 8' -hexahydro- ψ, ψ –carotene	5	331, 348, 367[a,g]	Colorless
Violaxanthin	5, 6, 5', 6'-diepoxy-5, 6, 5', 6'-tetrahydro- β, β-carotene-3, 3'-diol	9	416, 440, 465[a] 419, 440, 470[b] 417, 440, 470[c]	Yellow
Zeaxanthin	β, β-carotene-3, 3'-diol	11	424, 449, 476[a] 428, 450, 478[b] 432, 454, 480[c]	Orange

a=lightpetroleum, b=ethanol, c=acetonitrile/ethylacetate, d=chloroform;e=acetone; f=benzene, g=hexane, adapted from Cuttriss and Pogson, 2004; Delgado- Vargas et al., 2003 ; Rodriguez-Amaya, 2001

1.6.1 Carotenoid Biosynthetic Pathway

The biosynthetic pathway (Figure 8) involved in carotenoids formation were elucidated in the mid of the last century using various classical biochemical and mutational studies (Bouvier et al., 1994).

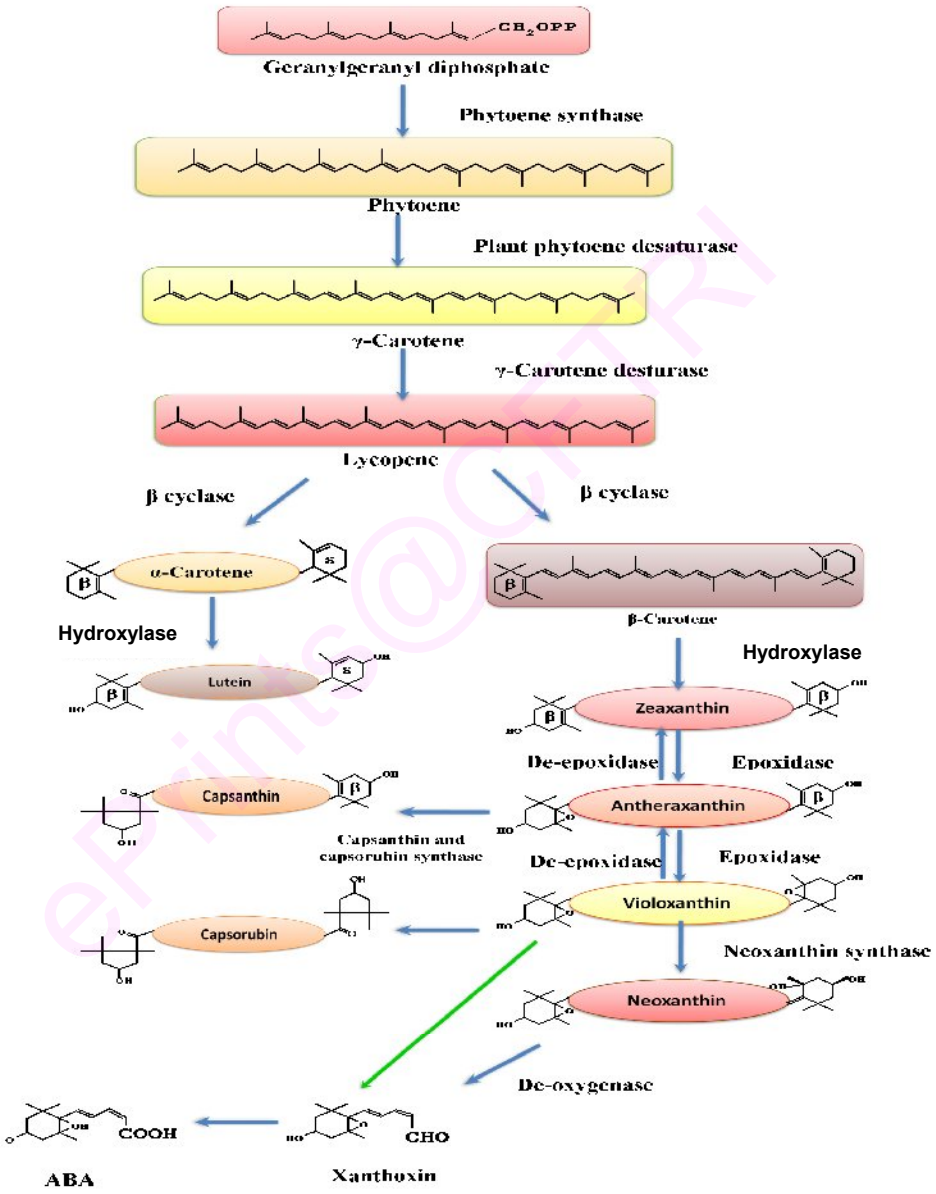


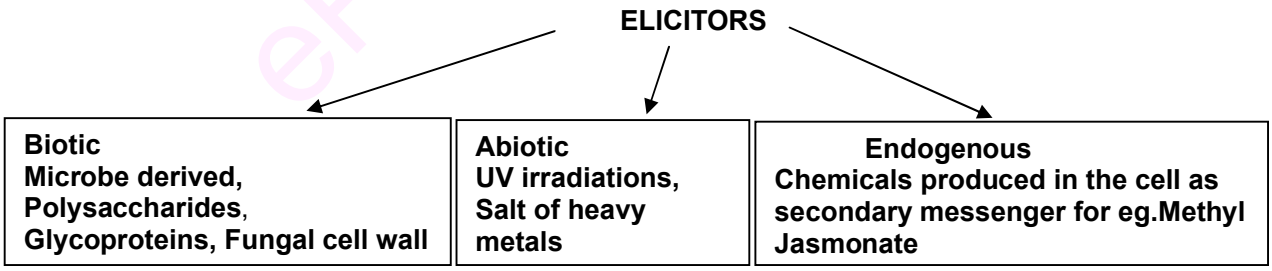
Figure 8 Carotenoid biosynthetic pathway

Various modern molecular and biochemical techniques have facilitated functional complementation of genes leading to the creation of transgenic plants. These studies have enhanced the knowledge of carotenoid biosynthesis, its regulation and the enzymes involved in the pathway**.**

Capsanthin and Capsorubin, two characteristic ketoxanthophylls with an unusual cyclopentane κ-ring are unique to ripened fruits of pepper (*Capsicum annuum*). The pepper chromoplast associated enzyme capsanthin–capsorubin synthase (*Ccs*), transforms antheraxanthin and violaxanthin into capsanthin and capsorubin, respectively. *Ccs* is similar to tomato Cyc-b and posses β-cyclase activity (Bouvier et al., 1994; Lefebvre et al*.,* 1998).

1.7 Elicitation of Secondary Metabolites

Yield of secondary metabolites related to defense pathways in the plants can be enhanced by elicitation. Plants are the source of innumerable secondary metabolites which find use as food additive and ingredients such as flavors, colorants, sweeteners and nutraceuticals. Elicitations is defined as the induction of secondary metabolites produced by molecules or treatments called as elicitors.



Enhancing the levels of plant based economically important secondary metabolic products has become a common practice now. Elicitors are the compounds, which are generally used to enhance the levels of these secondary metabolites. Cell suspension cultures are the main source for *in-vitro* production of secondary metabolites, which could easily be stimulated under the action of elicitors. Elicitors provide important clues for understanding the molecular basis of the transducing pathway through which exogenous signals induce secondary product biosynthesis, involving various signal compounds *viz*. reactive oxygen species, Jasmonic acid, Ca^{++}.

Elicitor based enhancement of secondary metabolites has been successfully carried out in plants such as *Catharanthus roseus* (Tryptamine), *Nicotiana sps.* (Sesquiterpenoids), *Glycine max* (Isoflavonoids) and *Daucus carota* (Anthocyanins). Sudha et al., (2002), have showed the effect of fungal elicitors and calcium channel modulators on accumulation of anthocyanins in callus cultures of *Daucus carota*. Plant defense can be triggered by local recognition of pathogens but, more effective responses include systemic signalling pathways (Conrath et al., 2002). Two of the most important compounds having this ability are salicylic acid (SA) and jasmonic acid (JA). Systemic responses include those dependent on SA signalling and are named Systemic Acquired Resistance (SAR) (Dempsey et al., 1999). The Induced Systemic Resistance (ISR) is known to be dependent on JA (Feys and Parker, 2000). SA, JA and its derivatives like Methyl Jasmonate (MeJ) have been used as inducers in plants and were found to stimulate their secondary metabolism (Thomma et al., 2000; Hahlbrock et al., 2003). The ability of jasmonate to boost plant defenses against fungal pathogens has already been reported (Thomma et al., 2000).

Biotransformation is a growing field of biotechnology which encompasses biocatalysts of plant and microbial origin making the conformational and configurational changes for enhancing the permeability of the cell to bring out a novel compound and to enhance its productivity. The production of high value food metabolites, chemicals, pharmaceuticals can be achieved by biotransformation using biocatalysts in the form of

enzymes or whole cell (Johnson et al., 1991; Johnson and Ravishankar, 1996; Rao and Ravishankar, 2000).

A careful analysis of secondary metabolite profiles during the culture of plant cells indicate that most of the secondary compound are produced during the post-exponential or stationary phase of growth (Lindiey and Yeoman, 1985). The biochemical factors underlying these phenomena are the channeling of precursors from growth related processes to secondary metabolism (Ravishankar et al., 1988). Mathematical modelling of capsaicin production in immobilized cells of Capsicum was studied by Suvarnalatha et al. (1993) to optimize physical parameters, such as the bead strength of calcium alginate used for immobilization and the medium constituents for enhanced yield. Secondary metabolite production in plant cell cultures can be elicited using a range of elicitors (Di Cosmo and Talleri, 1985).

Elicitation is envisaged to overcome the problem of low productivity of plant cells for industrial applications. Treatment of immobillzed cells and placental tissues with various elicitors, such as fungal extracts (*Aspergillus niger* and *Rhizopus oligosporus*) and bacterial polysaccharides (curdlan and xanthan) have been reported. It was found that curdlan was most effective in eliciting capsaicin synthesis; immobilized cells responded more effectively than placental tissues for curdlan treatment (Johnson et al., 1991). Curdlan and xanthan in combination enhanced capsaicin production by nearly 8-fold for curdlan treatment (Johnson et al., 1991).

Suvarnalatha et al. (1993) showed the optimization for capsaicinoid formation of immobilized C. *frutescens* using Response Surface Methodology. The feeding of intermediate precursors to Capsicum cell cultures not only increased the capsaicin accumulation but also shortened the time required to produce high amounts of capsaicin (Johnson et al., 1991; Johnson and Ravishankar, 1996). Prasad et al. (2006) have reported a 6 fold elicitation of capsaicinoids in cell suspension cultures of Capsicum using biotic elicitors. In a similar experiment there was 3-4 fold enhancement of phenyl propanoid intermediates and 6 fold enhancement of capsaicin precursor viz 8-methyl nonenoid acid.

The process of elicitation has been used to identify rate lmiting step in capsaicin biosynthesis.

1.8 Regeneration of Chilli

It is now well established that *in-vitro* responses such as adventitious shoot induction, shoot elongation and plant regeneration are genotype- dependent. In order to develop an efficient plant regeneration protocol, a number of commercial varieties are tried for shoot regeneration potential, shoot elongation and rooting of regenerated shoot (Christopher and Rajam, 1994, 1996; Venkataiah and Subhash, 2001; Venkataiah et al., 2003). Due to genotype differences and culture conditions it is often difficult to repeat the experiments at different laboratories (Fari and Andrasfalvy, 1994; Steinitz et al., 1999; Ochoa- Alejo and Ramirez-Malagon, 2001). Genotypic differences for tissue culture responses may be related to the variation in endogenous hormone levels (Steinitz et al., 1999). Different explants from a single genotype do not respond identically in a culture, most likely due to varying gradients of endogenous hormones (Christopher et al., 1991). Even with similar explant culture response is never 100 % in most experiments. Many of the genetic difference could be circumvented by growing the source plants under optimal condition and also by varying nutrients and hormonal composition in the culture media.

Regeneration in tissue culture is a genetically controlled trait in several plants including *Capsicum* sps. (Venkataiah et al., 2001). Gunay and Rao (1978), reported adventitious shoot bud formation and plant regeneration from seedling explant of *C. annuum* cv California Wonder and Pimento and *C. frutescens* cv Bharath. In hypocotyl segments of *C. annuum* cv Hatvani, induction of adventitious shoots and complete plant regeneration are reported and the gradual decline in the morphogenetic potential has been demonstrated (Fari and Czako, 1981). The correlation between endogenous content of phytohormones and the morphogenetic responses has been well documented by Fari et al. (1982). Shoot proliferation and plant regeneration from mature embryo culture of *C. annuum* cv Mathania

was reported by Agrawal and Chandra (1983). Philips and Hubstenberger (1985) studied the *in-vitro* organogenesis of four pepper varieties. Induction of shoots and plantlet formation has been reported in *C. annuum* cv G-4 from mature zygotic embryo culture (Christopher et al., 1986). Shoot bud induction and plant regeneration from cotyledonary explant of *C. annuum* cv Yutsufusa has been reported (Sripichitt et al., 1987). Successful shoot bud induction and plant regeneration from excised cotyledon explant of *C. frutescens* has also been reported (Subhash and Christopher, 1988). Among various species and genotypes of Capsicum tested, *C. baccatum* var. baccatum and *C. baccatum* var. pendulum gave rise to regenerated shoots but *C. annuum*, *C. chinensis* and *C. frutescens* did not show any shoot bud formation after Seedling Decapitation Method (Fari et al., 1995). Valera-Montero and Ochoa-Alejo (1992) reported successful regeneration in decapitated seedling explant of *C. annuum* cv chilede Anqua, Salvatierra and Tampiqueno 74. The influence of chilli pepper cultivar on the capacity of hypocotyl tissues to form adventitious shoots is also reported (Ochoa-Alejo and Ireta-Moreno, 1990).

Ebida Aly and Hu (1993) reported the morphogenetic response; Hyde and Phillips (1996) showed organogenic regeneration of chilli pepper plants from cotyledon explants of *C. annuum*. An improved and reliable plant regeneration system for *C. annuum* based on bud formation and shoot bud elongation from wounded hypocotyls was reported (Ramoarez-Malagoan and Ochoa-Alejo, 1996), Hyperhydricity in chilli pepper plants regenerated *in-vitro* was studied by Fontes et al., (1999). Growth regulators play a pivotal role in regeneration as shown by Hussain et al., (1999) using phenylacetic acid (PAA) to improve chilli pepper shoot bud elongation. Binzel et al., (1996a) incorporated gibberellic acid in the culture medium to proliferate callus cultures of *C. baccatum* as well as in *C. annuum*. Ramage and Leung, (1996) showed the dependency of Benzyl Adenine and sucrose for shoot formation from the hypocotyl in *Capsicum annuum*. Shoot organogenesis for chilli pepper using hypocotyl explants (Valera-Montero and Ochoa-Alejo, 1992), adventitious shoot bud induction and proliferation by half seed explant (Ezura et al., 1993; Binzel et al., 1996b), zygotic embryos (Christopher et al., 1986), cotyledon, hypocotyl, leaf (Fari and Andrasfalvy, 1994; Steinitz et

al., 1999; Ochoa-Alejo and Ramirez-Malagon, 2001) and petiole explants (Christopher et al., 1991) have been reported. Among the various explants tested, leaf explants were found to be more pronounced for adventitious shoot bud formation followed by zygotic embryos, cotyledons, hypocotyl and petiole explants (Venkataiah et al., 2003). According to Ezura et al., (1993) the presence of radicle cells in the mature embryo axis explant was critical for organogenesis of adventitious buds which was contradicted by Valera Montero and Ochoa-Alejo (1992) by suggesting that roots do not influence bud formation in hypocotyl tissues of chilli pepper but rather have a stimulatory effect on the bud elongation, which is an important step in shoot production.

The process of adventitious regeneration in pepper cotyledon explant has been widely established (Fari and Andrasfalvy, 1994; Steinitz et al., 1999; Ochoa-Alejo and Ramirez-Malagon, 2001). Direct and indirect *in-vitro* plant regeneration from chilli pepper (*C. annuum* L. cv Soroksari) was reported by Berljak (1999). Phillips et al., (2000) investigated all reported regeneration systems for chilli pepper and attempted improvements for many of them. Key results of this study include shoot organogenesis from cotyledon explants. Most of the factors become critical at the time of regeneration and morphogenesis for the tissue culture (Table 5) and it becomes crucial in case of *Capsicum sps.* as it is highly recalcitrant and genotype specific (Binzel et al., 1996a).

Table 5 Main factors tested for *in-vitro* organogenesis and plant regeneration in chilli pepper

Topics	Tested factor	Factors commonly used	References
Species	*C. annuum, C. frutescens, C. baccatum, C. pendulum, C. Praetermissum, C. annuum*	---------	Gunay and Rao (1978); Fari et al., (1995a); Christopher and Rajam (1996); Phillips et al., (2000)
Chilli pepper type	Bell peppers, long green peppers, and hot peppers, Bell peppers	--------	Ochoa-Alejo and Ireta-Moreno (1990); Arrollo and Revilla (1991); Szasz et al., (1995)
Explant	Cotyledons, hypocotyls, mature seeds, leaves, shoot tips, stem segments, and roots, Nodal explant, Anther culture	Cotyledons, hypocotyls, mature seeds, Inverted mode polarity, somatic embryogenesis	Fari and Czako (1981); Agrawal et al., (1989); Ahmad et al., (2006), Ezura et al., (1993); Ramirez-Malagon and Ochoa-Alejo (1996); Phillips et al., (2000); Liljana R. Koleva-Gudeva et al., (2007)
Culture medium	MS, MS/B5 vitamins and MS/L2 vitamins	MS, MES	Hyde and Phillips (1996); Berljak (1999): Vinod Kumar et al., (2006)
Growth regulators for shoot / bud induction	BA,2iP,Zeatin,TDZ,BA/IAA,2iP/IAA and BA/PAA	BA/IAA,BA	Phillips and Hubstenberger (1985); Agrawal et al., (1989); Ochoa-Alejo and Ireta-Moreno (1996); Szasz et al., (1995)
Growth regulators for shoot elongation	NAA, BA, Kin, GA_3, PAA/BA,BA/GA_3/brassinolide and brassinolide/Zea/GA_3, $CuSO_4$	BA/ GA_3, BA/PAA,TDZ	Szasz et al., (1995); Hyde and Phillips (1996); Franck - Duchenne et al., (1998) ; Hussain et al., (1999); Binzel et al., (1996b), Venkataiah (2003), Joshi and Kothari (2007).
Growth regulators for rooting	NAA,IAA and IBA	NAA	Gunay and Rao (1978); Agrawal et al., (1989)
Carbon source	Sucrose and Glucose	Sucrose	Phillips and Hubstenberger (1985); Ramage and Leung (1996)
Ethylene inhibition	$AgNO_3$	$AgNO_3$	Valera-Montero and Ochoa-Alejo (1992); Hyde and Phillips (1996)
Gelling agent	Agar and Phytagel	Agar (Phytagel better)	Hyde and Phillips (1996)
Light regime	Continuous and photoperiod	Continuous light	Phillips and Hubstenberger (1985)
Temperature	25-28.5^{0}C	25 (28.5^{0}C better)	Phillips and Hubstenberger (1985)

1.9 Somatic embryogenesis

Somatic embryogenesis and plant regeneration has been reported in *C. annuum* using immature zygotic embryos. Harini and Lakshmi Sita (1993) first described direct somatic embryogenesis from immature zygotic embryos in chilli pepper. Somatic embryos were also obtained through calli by indirect somatic embryogenesis (Buyukalaca and Mavituna, 1996). The production of artificial seeds, consisting of somatic embryos encapsulated in calcium alginate gel beads has been reported in *C. annuum* (Buyukalaca and Mavituna, 1996). Direct somatic embryogenesis was observed in cotyledon and leaf explants of *C. baccatum*, on the media supplemented with various concentrations of 2,4 -D in combination with 0.5 mg l^{-1} - 2.0 mg l^{-1} Kin (Venkataiah et al., 2006). Liljana et al., (2007) reported the response of anthers from different genotypes to different media, heat-shock and cold-shock pre-treatments regarding the direct somatic embryogenesis. Immature zygotic embryos, and callus derived from immature zygotic embryos are only types of explants which have formed somatic embryos in pepper *C. annuum* L. In the same species somatic embryogenesis was observed either in the presence of 2,4- D + Thiadizuron (TDZ) or BAP or 2,4-D and Coconut milk, but not with 2,4-D alone (Harini and Lakshmi Sita, 1993; Binzel et al., 1996 a; Kintzios et al., 2000). In many protocols it is evident that cytokinins can exert promotive effect on somatic embryogenesis. Several protocols have been reported to induce microspore embryogenesis and plant regeneration in different varieties (Dumas de Vaulx et al., 1981; Mityko et al., 1995, 1999; Dolcet-Sanjuan et al., 1997; Barany et al., 2001). Barany et al., (2005), modified the previous protocols for *in vitro* anther culture in Capsicum (Dumas de Vaulx et al., 1981). Somatic embryogenesis can be used as a tool for producing doubled haploid plants and it will be implemented for routine application in breeding programs for the better production and yield.

1.10 Genetic Transformation

Delivery of appropriate DNA into the cell, integration of introduced DNA into the chromosome for stable transformation, selection of transformed cells (markers) and a good *in-vitro* regeneration system is essential for an effective genetic engineering system that seeks to exploit genetically transformed plants for commercial application. Application of modern genetic manipulation has been limited in pepper due to lack of efficient transformation. Table 6 represents the current status of chilli pepper transformation. Although some advances have been made, the efficiency for recovering transformed plants using *A. tumefaciens* remains low. Liu et al., (1990) worked on *Agrobacterium* based *in-vitro* regeneration and transformation systems in bell pepper, transformed shoots and leaf like structure showed the beta-glucaronidase activity (GUS) in the vasculature without any bacterial contamination. Wang (1991) used cotyledon, hypocotyl and leaf explants for transformation and regeneration, transformants were able to show transient GUS activity. Zhu et al., (1996) obtained transgenic sweet pepper from *Agrobacterium*- mediated transformation using *Agrobacterium* strain GV311-SE harboring cucumber mosaic cucumovirus coat protein (*cms-cp*) gene. Siregar and Sudarsano (1997) obtained shoot regeneration from hypocotyl segments of hot pepper mediated by non disarmed isolates of *Agrobacterium*. Manoharan et al., (1998) established a protocol for regeneration and *Agrobacterium* mediated genetic transformation in hot Chilli (*C. annuum* cv Pusa Jwala) from cotyledonary leaves. Cotyledon segments of hot pepper were used successfully for the regeneration and *Agrobacterium* mediatd transformation by Lim et al., (1999). Dong et al., (1995) obtained transgenic pepper plants containing a CMV (cucumber mosaic cucumovirus) satellite RNA cDNA by *A. tumefaciens* mediated genetic transformation. Kim et al., (1997) investigated RNA-mediated resistance to cucumber mosaic virus in progeny of transgenic plants of hot pepper that expresses RNA.

Table 6 Present status of Chilli Pepper genetic Transformation

Species/cv.	Transformation system	Inference	Reference
C. annuum / Six bell cultivars; *C. glabriusculum* / wild-type accession	*A. tumefaciens*/wild-type strains A281 and C58; pGV33858 plasmid (nptII, gus,35S promoter)	Tumor formation in the absence of growth regulators. Callus and leaf-like structures GUS+.	Liu et al., (1990)
C. annuum / Vegi sweet and California Wonder	*A. tumefaciens*/strain LB4404, p5T35AD (acetolactate synthase gene, gus, 35S promoter); pSLJ1911 (nptII, gus, 35S promoter); pWTT2039 (hpt, gus, 35S promoter)	Shoots and plants GUS+. 0.5±0.7% plant transformation efficiency	Engler et al., (1993)
C. annuum / Golden Tower	*A. tumefaciens*/strain LB4404, pRok1/105 (cucumber mosaic virus I17N-Satellite RNA, nptII, 35S promoter)	Regenerated plants with attenuated symptoms against CMV. 4% plant transformation efficiency	Lee et al., (1993); Kim et al., (1997)
C.annuum var. Grossum /Zhong Hua no. 2	*A. tumefaciens*/strain GV3111-SE (CMV-CP, nptII, 35S promoter)	Regenerated plants expressing CMV-CP	Zhu et al., (1996)
C. annuum/Mulato Bajio	*A. tumefaciens*/strain A208, pTiT37::pMON9749 (nptII, gus, 35S promoter); and strain LBA 4404, pBI121 (nptII, gus, 35S promoter)	Regenerated plants GUS+. 0.1% plant transformation efficiency	Ramirez-Malagon and Ochoa-Alejo, (1996)
C. annuum / Pusa jwala	*A. tumefaciens*/strain EHA105, pBI121 (nptII, gus, 35S promoter)	Regenerated plants GUS+. 2% plant transformation efficiency	Manoharan et al., (1998)
C. annuum L.) Xiangyan 10	*Agrobacterium tumefaciens* strain LBA4404 with plasmid PBI121	40.8% of the regenerated plants transgenic +GUS	Li et al., (2003)
C. annuum PEG mediated protoplast culture	pCAMBIA1302 (hpt, gfp and GUS, 35S promoter)	30% protoplast transformation.	Jeong et al., (2007)

Although efforts towards the stable transformation of Capsicum through *Agrobacterium tumefaciens* is attempted other methods *viz*, particle gun bombardment, electroporation, floral dip, Sonication Assisted *Agrobacteium* Transformation (SAAT) are also being adopted. All these transformation approaches are emerging as the methods of choice for introduction of agronomically important genes for quality improvement, regulation of secondary metabolites, and for the engineering molecular pharming and improvement of the *Capsicum sps.* for the maximum usage in pharmaceutical, nutraceutical and food industry.

1.11 Gene silencing in plants

Silencing of the transgenes at transcription level is referred to as transcriptional gene silencing (TGS); whereas silencing at post-transcriptional level is referred to as post-transcriptional gene silencing (PTGS). TGS involves inhibition of transcription and association with methylation of promoter region. In cases of PTGS, though the genes are transcribed, their mRNA is degraded and it is associated with methylation of the coding region of the transgenes (Veluthambi et al., 2003). TGS can result from the impairment of transcription initiation through methylation and PTGS results from the degradation of mRNA when aberent sense, antisense or double stranded forms of RNA are produced (Fagard and Vaucheret 2000). Napoli et al., (1990) and Smith et al., (1990), demonstrated that introduction of transcribed sense transgenes could down-regulate the expression of homologous endogenous genes, a phenomenon called co-suppression. Post-transcriptional gene silencing (PTGS) known as RNA silencing in plants, is an RNA degradation process through sequence-specific nucleotide interactions induced by double-stranded RNA; is also referred as RNA interference in animals, quelling in fungi (Yu and Kumar, 2003).

This has opened up new avenues for down regulation of genes encoding undesirable traits with special reference to processing characteristics and anti-nutritional traits. The RNA silencing is triggered by the presence of endogenous or exogenously introduced double-stranded RNA (ds RNA), which is further cleaved into small RNAs to become functional in a number of epigenetic gene-silencing processes (Eckardt, 2002). In plants, RNA silencing, as an efficient part of gene silencing, but also plays important roles in the regulation of endogenous gene expression (Voinnet, 2002). The signals of intracellular RNA (Short interfering RNAs (siRNAs), aberrant RNAs, and dsRNAs) can be transmitted systemically from cell to cell over a long distance through the phloem, although the mechanism of their involvement in the process is not clear so far (Palauqui et al., 1997, Voinnet et al., 1998). The mechanism of RNA silencing induced by dsRNA can be simplistically summarized as having two major steps, viz., initiation and effector steps (Cerutti, 2003). The initiation step involves the cleavage of the triggering dsRNA into siRNAs of 21–26 nucleotides with 2-nucleotide 3` overhangs, which correspond to both sense and antisense strands of a target gene (Hamilton and Baulcombe, 1999, Voinnet, 2002). In the effector step, the siRNAs are recruited into a multiprotein complex referred to as the RNA-induced silencing complex (RISC), in which the degradation of target mRNAs occurs with the siRNA as a guide (Hammond et al., 2000, Zamore et al., 2000). The Dicer protein is involved in generating siRNA (Bernstein et al., 2001). The members of the Dicer protein family may be functionally conserved in fungi, plants and animals (Tijsterman et al., 2002). Recent studies reveals that PTG silenced plants produce a sequence specific systemic silencing signal that propagates long distance from cell to cell and triggers PTGS in non silenced tissues of the plant (Palauqui et al., 1997, Voinnet and Baulcombe, 1997).

1.12 Application of RNA silencing in plants

The practical use of RNA silencing to reduce gene expression in a sequence-specific manner promises to be an essential approach in plant functional genomics after the completion of *Arabidopsis* and rice genome sequence. Particularly, the discovery of dsRNA as an inducer of RNA silencing has provided a scheme of dsRNA-mediated interference to direct gene-specific silencing that is more efficient than antisense suppression or co-suppression by over expression of target genes (Fire et al., 1998; Kennerdell and Carthew, 1998; Waterhouse et al., 1998). The dsRNA-mediated silencing was first demonstrated in plants by the simultaneous expression of antisense and sense gene fragments targeted against both an RNA virus and a nuclear transgene (Waterhouse et al., 1998). In this respect, transformation vectors capable of dsRNA formation were constructed by Wesley et al., (2001), linking the gene-specific sequences in both sense and antisense orientation under the control of a strong viral promoter. Thus, the dsRNA interference can generate transformants showing both reduction and loss of function. It is reported that, inclusion of an intron as a spacer between the sense and antisense arm of a dsRNA construct greatly increases the silencing effect.

Biotechnology has come a long way to play its role in the well being of human. The progress in the transgenic research led to the development of genetic manipulation strategies for specific traits. The compiled information provides an idea about the latest developments in Capsicum biotechnology area. There are reports for regeneration and transformation in Capsicum, various regeneration reports are there with various parameters of explant orientation and hormonal regime. However, information is lacking on what are the factors, which determine recalcitrant and genotype specific nature of Capsicum.

Development of rapid regeneration protocols in Capsicum is a prerequisite factor for optimizing genetic transformation. *In-vitro* and *in-planta* transformation experiments were done to transform and regenerate the plants. Capsicum is known for pungency (capsaicinoids), aroma and color (carotenoids). Enhancement of these metabolities by elicitation is one of the approaches and other being genetic manipulations. With this background a series of experiments were designed for plant regeneration, transformation, elicitation and transcriptional studies with the following objectives. The results of these studies form the substance of this thesis.

OBJECTIVES

With the above background information it was envisaged to develop an efficient *in-vitro* regeneration system in Capsicum and also a reliable genetic transformation system and to regulate the capsaicin production in transformants aimed towards genetic improvement by transgenic approach.

With theses milieu, the objectives of the present study are

- To develop *in-vitro* plant regeneration system in *Capsicum* sp.
- To develop an efficient genetic transformation system in Capsicum.
- Elicitation of capsaicin and carotenoids using abiotic and biotic elicitors.
- To identify mRNA transcripts differentially regulated under the influence of elicitors.

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MATERIALS AND METHODS

Content

2.1 ESTABLISHMENT OF *IN VITRO* CULTURES

2.1.1 Plant material

Certified variety of seed samples of *Capsicum annuum viz*. Arka Abhir, Arka Lohitha, 226, Pusa Jwala, G-4, Manipuri with wide range of pungency were procured from Indian Institute of Horticultural Research (IIHR), Bangalore Karnataka, *Capsicum frutescens viz*. KT-OC, BOX-RUB, DARL-SEL from Defence Research and Development Organisation (DRDO) Pithoragarh Uttaranchal (Figure 9).

Figure 9 Various varieties of *Capsicum sps.*

2.1.2 Sterilization and seed germination of *Capsicum sps*

After washing the seeds in running water they were treated with 1% Bavistin (Carbendazim, 50% w/w, BASF India Ltd. Thane) for 10 min; followed by 30 seconds in 70% alcohol immediately later rinsed with copious sterilized distilled water. Subsequently the seeds were sterilized in 0.2 % (w/v) mercuric chloride (Hi-media, Mumbai, India) solution for 2-3 min and washed five times in sterile distilled water. The surface sterilized seeds were inoculated (2-4 seeds/bottle) on to ¼ MS basal medium and vitamins (Murashige and Skoog, 1962) (Table 7) with 3% sucrose.

The medium was gelled with 0.8% (w/v) tissue culture grade agar (Hi-media, Mumbai, India) in 200 ml glass jars, containing 40 ml of the medium. The medium contained additives such as polyvinyl pyrrolidone (PVP) 0.5%, activated charcoal 0.5% in different combinations to prevent browning and contamination. The pH was adjusted to 5.6 using a pH meter (Cyber Scan 510, Oakton, USA) prior to autoclaving at 121º C, 1.2 kg cm^{-2} pressure for 20 min. The cultures were maintained at 25±2^{o} C in the dark. Seed germination was recorded at weekly intervals.

Table 7 Murashige and Skoog (MS) Medium

Stock	Components	Aliquot per liter (in ml)	Final conc. (mg l^{-1})
A	NH_4NO_3	20	1650
B	KNO_3	20	1900
C	KH_2PO_4		170
	$MgSO_4.7H_2O$		190
	H_3BO_4		5.2
	$ZnSO_4.\ 7H_2O$		7.0
D	$CaCl_2.H2O$	5	440
E	$Na_2MoO_4.\ 2H_2O$		0.25
	$CuSO_4.5H_2O$	5	0.025
	$CoCl_2.6H_2O$		0.025
	KI		0.8
F	$FeSO_4.7H_20$		27.8
	Na_2EDTA	5	37.3
G	Nicotinic Acid		0.5
	Pyridoxine HCl		0.5
	Thiamine HCl	5	0.1
	Biotin		0.01
	Glycine		2.0
	Myoinositol		100
	Sucrose		30,000
	Agar		8000
	pH		5.6-5.8

2.1.3 Callus and cell suspension cultures

In vitro seedlings of *Capsicum* sp. were raised in half strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Callus cultures were obtained from seedling explants and also from hypocotyls and leaves. Callus was sub cultured once in 15 days in full strength MS solid medium supplemented with 2,4 D (2 mg l^{-1}) and kinetin (0.5 mg l^{-1}) and 3% sucrose incubated at 12-h photoperiod of 3000 lux at 25 ± 2^{o} C.

The cotyledonary leaf (~5x5mm square explants were cut from leaf blade with a scalpel, excluding the basal and apical portions, mid vein and margins) and hypocotyl explants (~10mm length) were placed on callus induction medium (Murashige and Skoog, 1962) containing MS salts and B5 vitamins (Gamborg et al., 1968), 2,4 D (2 mg l^{-1}) and kinetin (0.5 mg l^{-1}) and 3% sucrose. All hormones were obtained from Sigma (USA).

2.1.4 Regeneration of shoot from nodal explant

2.1.4.1 Explant preparation

Seeds of various pungency level were thoroughly washed in running tap water, subsequently surface sterilized with 0.2% $HgCl_2$ (Hi-media, India) for 3 min, washed copiously with sterile distilled water. The seeds were germinated *in vitro* and 15-day-old seedlings showed well-developed roots, two cotyledonary leaves and an apical meristem. The apical meristem (1–2 mm), cotyledonary leaves and root portion were excised using a sharp scalpel and these entire hypocotyls of 5– 7 cm length were used as explants. The explants were inoculated immediately in order to prevent the drying of cut edges of the explant.

2.1.4.2 Culture medium

MS media (Murashige and Skoog, 1962) with adjuvant and growth regulators were prepared. Shoot Bud induction medium (SBIM) comprised MS salts, vitamins, 1.95 g/l MES [2-(N-morpholine) ethanesulphonic acid] (Sigma USA), 17.74–44.38 mM BA [N^6-Benzyladenine] (Sigma USA), 1.44–4.57 mM IAA [indole -3- acetic acid] (Sigma USA) and 10 mM $AgNO_3$ [silver nitrate]. Media pH was adjusted to 5.8 and autoclaved at 121^o C, 1.2 kg cm^{-2} pressure for 15 min.

2.1.4.3 Experimental conditions

The cultures were incubated at 25 ± 2^o C light under 16/8 h of photoperiod with 45 µmol m^{-2} s^{-1} light intensity. Culture vessels of 150 ml Erlenmeyer flasks with 40 ml medium were used for all the experiments. The cultures were continuously exposed to light only for the bud induction phase. For germination, shoot elongation and rooting, the cultures were exposed to 16/8 h photoperiod.

2.1.4.4 Shoot bud induction

Seedlings of 15-day-old were used as the source of explant, the apical meristem, cotyledonary leaves and the root zone were excised and cultured on various media comprising of MS salts and vitamins, MES, BA, IAA and $AgNO_3$. Four explants were cultured per flask and 10 replicates were prepared for each combination. Seedlings were inoculated in vertical, horizontal and inverted mode in bud induction medium comprising MS salts, vitamins, 1.95 g l^{-1} MES, 26.63 mM BA, 2.28 mM IAA and 10 mM $AgNO_3$ (Table 9). These hormones were selected on the basis of previous reports (Liu et al., 1990; Valera-Montero and Ochoa- Alejo, 1992; Hyde and Phillips, 1996) and optimized during preliminary experiments.

After 1 month, the seedlings were removed from inverted position from the medium. Transverse sections of 2–3 mm were made in the bud-induced region of the

hypocotyl and subcultured for elongation of the shoot buds. Shoot buds elongation was tried on media comprising MS media with BA, Kinetin. 2ip, IAA, PAA, GA_3 (Gibberellic acid) and $AgNO_3$ in various combinations (Table 9 & 11). The shoot buds were cultured under light as well as in dark for shoot elongation for 3 months.

2.1.4.5 Rooting

The elongated multiple shoots (3–4 cm long) were excised individually and placed on half strength MS media for rooting.

2.1.4.6 Transfer to soil

Agar was washed gently and thoroughly from the rooted plantlets. Plantlets were then transferred to micro-pots containing soil: vermiculite (1:1) mixture. Pots were watered regularly and kept in shade for 15 days and then transferred to green house.

2.1.5 Multiple shoot regeneration from leaf margin

For direct organogenesis, explants viz. cotyledonary leaves, petiole, proximal portion of the cotyledonary leaf excised from 15-day-old seedlings of *Capsicum frutescens* var. KT-OC and BOX-RUB were used. After 1 month, the shoot buds proliferated from proximal end of leaf and they were excised and sub cultured for elongation of the shoot buds on MS media supplemented with silver nitrate (10µM) and 2.0 mg l^{-1} phenyl acetic acid (PAA) and 1 mg l^{-1} GA_3. After a month the elongated shoots were transferred to half strength MS media for rooting and finally to soil for establishment. Culture conditions are the same as given in the section 2.1.4.2.

2.1.6 Clonal propogation of Bird eye chilli (*Capsicum frutescens* Mill.)

Shoot tips and nodal explants (cotyledonary node) of 30 days old seedlings were used in this study. For this experiment MS basal medium with 3% sucrose (w/v)

and 1% activated charcoal (w/v) was used. The pH was adjusted to 5.7 ± 0.2 before gelling with 0.8% (w/v) of agar (Hi media, India). Explants were cultured in glass jars (200 ml) and the medium was subsequently autoclaved under 1.06 kg cm^{-2} at a temperature of 121^0 C for 15 min. The growth regulators, N^6-Benzyladenine (BA), 2-isopentenyl adenine(2iP) and kinetin at a concentration of 0.5-3.0 mg l^{-1} were added to the MS basal medium individually or in combination with 0.5- 2.0 mg l^{-1} indole–3-acetic acid (IAA) or indole-3-butyric acid (IBA), 2,4 dichlorophenoxyacetic acid (2,4-D) and naphthalene acetic acid (NAA) to obtain the most suitable level for the proliferation of shoot in established explants. The cultures were incubated at 25 ± 2^0 C and light (45 µmol m^{-2} s^{-1}) for 16 h day using fluorescent lights (Philips India Ltd.) for 45 days. The shoots obtained from explants were later subcultured into the same medium for further growth. Even the shoot tips and the nodal segments of the primary shoots were inoculated into the medium with 1.0 mg l^{-1} each of IBA and kinetin for formation of new shoots within next 30 days and also for simultaneous rooting within 45 days. The experiment was repeated twice with 20 replicates each for both shoot regeneration and *in vitro* rooting. Rooted plantlets were removed from the medium, freed of agar by washing in running tap water and planted in sand: compost mixture (1:2) at about 80% relative humidity under the polyethylene hoods in the green house. The plantlets were hardened for 30 days and then transplanted in the field.

2.1.7 *In-vitro* flowering and shoots multiplication

Fifteen day old nodal explants were aseptically inoculated on modified MS basal medium supplemented with silver nitrate ($AgNO_3$) and cobalt chloride ($CoCl_2$) with concentration varying from 0 to 50 µM respectively (Table 15 &16).The pH of the media was adjusted to 5.8 ± 0.2 before gelling with 0.8% agar (Hi media Mumbai, India). The gelled media was autoclaved at 1.06 kg cm^{-2} pressure and 121°C for 15 min. The cultures were incubated at 25 ± 2^o C and at 16hr photoperiod under cool

light (4.41 Jm^2 s^{-1} 18 h day^{-1}) using fluorescent lights. Data for shoot length and *in-vitro* flowering were recorded on 15th, 25th and 45th day after inoculation.

2.1.8 Quantification of capsaicinoids

Capsicum fruits/callus of different genotypes were harvested after anthesis and dried at 60^0C till it attained constant weight. The dried fruits were homogenized in a mortar containing quartz sand with acetonitrile (1:10 w/v). The extract was centrifuged at 10000 x g at 4^0C for 15 min and pellet was discarded. The aliquots were evaporated to dryness *in vacuo* and extracts were resuspended with 1.0 ml HPLC grade methanol. The mobile phase for HPLC consisted of linear gradient of 0-100 % acetonitrile in water of pH 3.0 for 35 minutes and 100% was maintained for 2 more minutes and run up to 35 min. The detection was at 236nm and flow rate was maintained at 1ml min^{-2}.Coloumn C-18 of 250 x 4.6 mm and 5 µm was used. The reagent used was of HPLC grade. capsaicinoids *viz* Capsaicin, Vanillylamine and Dihydrocapsaicin were purchased from Sigma. A standard stock solution of 1 mg l^{-1} was prepared in methanol, from this 5 and 10 µl of standards and samples were injected to HPLC for three times and the mean value was calculated. Standard deviations of samples were calculated according to Tukey's method.

The samples were centrifuged at 6000 x g for 15 min before injecting to HPLC (Shimadzu, CR-7A). Capsaicinoids were quantified by HPLC (Johnson et al., 1992). Mean area was calculated by injecting 5 and 10µl of samples and standards thrice. Based on the retention time and peak area of standard compounds, the capsaicinoids were identified in samples and quantified.

***2.1.9* Pigment extraction**

All extraction was done in the dark or in subdued light. Dried and finely powdered *Capsicum* elicited fruit samples (100 mg) were repeatedly extracted under stirring at room temperature with diethyl ether (2X20 ml, for 1 and 0.5 h,

respectively), followed by methanol (3x20 ml, for 1, 0.5 and 0.5 h, respectively) until colorless extracts were obtained. The suspensions were filtered on sintered glass funnels and the combined filtrates were made up to 100 ml with methanol, in volumetric flasks. The extract was used further for color estimation and quantification.

2.1.10 Color measurement and quantification

Extracts of the matured fruits (1 mg dry tissues per ml of the methanol) were taken for color measurement using color measurement system (Hunter Lab color measuring system Lab Scan XE, USA) with cuvett assembly 40mm X 50mm, port size 1.2 inch, C illuminant and 2° view angle. L stands for lightness, a dimension of + red - green and b represents +yellow - blue. Finally, color differentiation is measured represented as DE.

2.1.11 Thin Layer Chromatography (TLC) of carotenoids

For qualitative and quantitative analysis TLC for capsanthin and capsorubin was conducted, preparative Kieselgel-60 plates (Merck) were made and Developing mixture: Petroleum Ether: Benzene: Acetone: Acetic acid (90:10:15:5) was added in subdued light. One gm of commercial chilli sample was shaken for 30 min with 3.0 ml acetone. The suspension was centrifuged and the supernatant was separated. This procedure was repeated, as the solid rest was nearly white. The collected supernatants were evaporated to 1.0 ml. This extract was further used for TLC and HPLC. Pigment solution (3 ml) was spotted onto the plates. The developed plates were allowed to dry at room temperature. *Rf* values were compared with earlier reports of Vinkler and Richter (1972). Bands were identified & eluted in methanol and were further used for HPLC.

2.1.12 HPLC determination of carotenoids

Two different types of samples were adopted, firstly acetone extract and other being TLC eluted fraction. HPLC was used for quantification of carotenoids. Aliquots (2 ml) of the saponified acetone extract were evaporated gently to dryness in a stream of nitrogen and the residue was dissolved in mobile phase (1 ml), filtered

through a 0.45-1xm membrane disc (Schleicher and Schiill, Dassel, Germany) and injected. The total extract as well as the fractions separated from TLC plates were analyzed by HPLC (Shimadzu LC-10AT) using reversed phase C-18 (Supelco) 25 cm × 4.6 mm column. For HPLC Isocratic Mobile Phase Acetonitrile: 2-propanol: Ethylacetate (80:10:10) was adapted and Flow rate was set at 0.8ml / min detecting at 450nm. The quantification was based on the reports of Vinkler and Richter (1972), where peak areas have been defined and same has been adopted for determination of pigments composition and carotenoids.

2.2 ELICITATION STUDIES

2.2.1 Application of elicitors and analysis of secondary metabolites

The abiotic elicitors *viz*., Salicylic acid, Ibuprofen, and Methyl Jasmonate were purchased from Sigma, USA. Stock solution was prepared at 1M and later used at different concentrations (1mM, 2.5mM and 5mM) for spraying to the whole plant containing flowers, completely opened flowers or branches containing flowers. The flowers of *C. frutescens* sprayed with water were taken as control. The fruits of *C. frutescens* plant which received different treatments were harvested 25, 30, 35 and 40 days after anthesis and extracted with 1:2 (w/v) of acetonitrile followed by *in-vacuo* evaporation and re-suspended in 1ml methanol.

The extracted samples were centrifuged at 4000 rpm for 10 minutes. The samples were subjected to High Performance Liquid Chromatography (HPLC) for quantification of phenyl propanoid intermediates and major Capsaicinoids.

The biotic elicitors used for this study were fungal stock cultures *viz; Aspergillus niger* and *Rhizopus oligosporus* (obtained from Food Microbiology Department, Central Food Technological Research Institute, Mysore). Fresh cultures were made on Potato Dextrose Agar (PDA) slants and incubated for 7 days. Spores

of the respective fungi were used to prepare spore suspension or inoculum in 0.1% sodium lauryl sulphate and diluted ultimately to get a spore density of ~ 2.5 X 10^6 spores / ml. Later the same was inoculated into the 50 ml of PD broth contained in 250 ml Erlenmeyer conical flasks (10 replicates) and the cultures were incubated in dark for 10 days. After incubation the cultures were autoclaved at 1.06 kg / cm^2 pressure and 121^oC for 15 min and later the mycelium was separated from the culture broth by filtration. The mycelial mat was washed several times with distilled water and an aqueous extract was made by homogenizing the dried fungal mat in mortar and pestle using acid washed neutralized sand. The extract was filtered through a Whatman no. 1 filter paper and 1% (equivalent to 1 g dry mycelium in 100 ml distilled water), 2.5 % and 5.0% filtrate was used for spraying to the flowers for elicitation.

2.2.2 Sample preparation and Chromatography condition for pigment separation

Chromatographic separations of the carotenoids from matured and ripened fruits of control and elicited *Capsicum* fruits were done on a Tracor 985 liquid chromatograph equipped with a Model 970A variable-wavelength UV-Vis detector and a Model 951 pump.

A Milton Roy LDC (I-10B) integrator was employed to record retention time and chromatograms and to evaluate peak areas. Reversed-phase columns (either Merck LiChrospher 100 RP-18, 5 lxm, 25x0.4 cm I.D. or Merck Superspher RP-18, 4 lxm, 12.5×0.4 cm I.D.) were used at ambient temperature and were protected with precolumns (Merck, LiChrospher 100 RP-18, 5 lxm, 4X0.4 cm I.D.). Chromatograms were monitored at 450 nm; the mobile phase was acetonitrile: 2-propanol: ethyl acetate (80:10:10, v/v); the flow rate was 0.8 ml/min; the pressure was 850-1050 p.s.i. and the recorder chart speed was 0.5 cm/min.

2.2.3 Extraction and estimation of endogenous polyamines

The extraction of endogenous polyamines (PAs) was carried out by acid hydrolysis of perchloric acid. PAs were analyzed according to Flores and Galston (1982). Callus tissues were grounded in 5% cold perchloric acid at a ratio of about 100mg/ml perchloric acid. Samples were incubated for 1h in ice bath and centrifuged at 10,000 rpm (Hettich D-78532, Germany) for 20 min and the supernatant containing the free polyamines were benzoylated. To 0.5 ml PCA extract, 1ml of 2 N NaOH and 10 μl benzoyl chloride was added, vortexed for 20 sec, incubated for 20 min at room temperature. Saturated NaCl (2 ml) was added to the mixture. The benzoylated polyamines were extracted in 2 ml diethyl ether after centrifugation. Ether phase was collected, evaporated to dryness and re-dissolved in 100 μl methanol. The standards were prepared in the same way and subjected to HPLC analysis.

2.3 TRANSFORMATION STUDIES

2.3.1 Pollen transformation

Anthers were dissected from the *in-vitro* flowers and pricked with a sterile needle soaked in *Agrobacterium tumefaciens* strain EHA 101 inoculum. *A. tumefaciens* EHA 101 containing the binary vector pCAMBIA 1301 was used in the experiments. The vector pCAMBIA 1301 contains the selectable marker gene hygromycin phosphotransferase (*hpt* II) under the control of the CaMV 35S promoter and CaMV 35S terminator; β-glucuronidase (*uid* A) gene with a catalase intron under the control of CaMV 35S promoter and NOS terminator. *A. tumefaciens* harbouring the binary vector was maintained in Luria Bertani (LB) medium with 50 mg l^{-l} kanamycin solidified with 1.5% Agar. Cultures were grown overnight in LB medium, supplemented with 50 mg l^{-l} kanamycin at 28^0 C and at 120 rpm (OD_{600} - 0.5-1.00), prior to transformation. The cells were harvested by centrifugation at 4,000 rpm for 5 min, resuspended in infection medium comprising half strength MS salts with 1.0 mg

l^{-1} niacin, 1.0 mg l^{-1} pyridoxine HCl, 10 mg l^{-1} thiamine HCl, 2% sucrose and 200µM acetosyringone (Sigma, USA) and used for co-cultivation. The anthers were co-cultivated for 6 h and 12 h duration in independent experiments.

2.3.1.1 GUS assay

GUS assay was performed by immersing the anthers for 12 h at 37^0 C in a GUS assay buffer containing 100mM sodium phosphate (pH 7), 20mM EDTA, 0.1% triton X-100, 1mM potassium ferrocyanide, 1mM potassium ferricyanide, 20% methanol, and 1mM X-Gluc (5-bromo 4-chloro indolyl-D-glucuronide cyclo-hexamonium salt) from Sigma, USA. Methanol was added to the reaction mixture to suppress endogenous GUS like activity. The results were expressed in terms of percentage pollen transformation frequency.

$$\% \text{ Transformation frequency} = \frac{\text{Number of pollen showing blue GUS staining}}{\text{Total number of pollen in microscopic field}} \times 100$$

2.3.2 *Agrobacterium tumefaciens* mediated genetic transformation studies on *Capsicum* sp.

2.3.2.1 Sensitivity tests for selection of transformed tissue

The seedling tissues were wounded and inoculated into callus induction medium supplemented with different concentrations of hygromycin (1, 3, 5, 10, 20, 40 and 50 mg l^{-1}). Filter sterilized hygromycin was added to the sterilized medium. The cultures were maintained under dark for a period of 2 months. The minimum concentration of hygromycin required for complete inhibition of regeneration response was determined and overall data was recorded as percentage regeneration response. The experiment was carried out in triplicates and data was represented in terms of mean and standard deviation.

2.3.2.2 *Agrobacterium culture and transgene expression cassette*

Agrobacterium tumefaciens agropine type wild strain EHA 101 (Obtained from Dr. Juan B Perez, Instittuto Canaro Investiganes Agrswas, Spain) and binary vector pCAMBIA 1305.2 (Obtained from Center for the Application of Molecular Biology to the International Agriculture, Canberra, Australia) was used in the experiments.

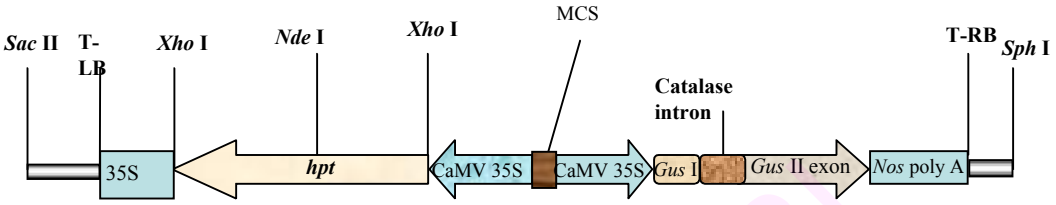


Figure 10 T-DNA region of pCAMBIA 1305.2

2.3.2.3 T-DNA region of pCAMBIA 1305.2

The vector pCAMBIA 1305.2 contains the selectable marker gene hygromycin phosphotransferase (*hpt* II) under the control of the CaMV 35S promoter and CaMV 35S terminator; β-glucuronidase (*uid* A) gene with a catalase intron under the control of CaMV 35S promoter and NOS terminator (Figure 10).

2.3.2.4 Maintenance of binary vector

The binary vectors were maintained in *E. coli* DH5α. The vectors were introduced in *E. coli* competent cells by $CaCl_2$ mediated transformation.

Luria-Bertani broth (LB) (Ingredients)	Concentration
Bacto-tryptone	10.0 g l^{-1}
Bacto-Yeast extract	5.0 g l^{-1}
Sodium chloride	10.0 g l^{-1}

The pH was adjusted to 7.0 with 2N NaOH and the total volume was made to 1 liter with deionized water.

SOB medium (Ingredients)	Concentration
Bacto-tryptone	20 g l^{-1}
Bacto-Yeast extract	5 g l^{-1}
Sodium chloride	0.6 g l^{-1}
Potassium chloride	0.19 g l^{-1}
Magnesium sulphate	10.0 mM
Magnesium chloride	10.0 mM

First four components were autoclaved and sterilized magnesium salt solutions were added separately and then mixed to constitute the SOB medium.

SOC (per 100 ml) medium

To 1.0 ml of SOB added 7 μl of filter-sterilized glucose solution (50%w/v)

0.1 M $CaCl_2$ stock solution

Dissolved 1.47 g of $CaCl_2$ in 100 ml of deionized water. The solution was filter sterilized and stored as 20 ml aliquots at -20°C.

Kanamycin stock solution

Kanamycin sulphate (Sigma USA) was dissolved in water, filter-sterilized and stored at -20°C. The stock solution was of 10mg ml^{-1}. Kanamycin was used at a working concentration of 100 μg /ml

2.3.2.5 Mobilization of binary vectors to *Agrobacterium tumefaciens*

Binary vectors were mobilized to *Agrobacterium* strain EHA 101 by freeze-thaw method. *Agrobacterium* sp. was grown in 5 ml of LB medium overnight at 28°C in a shaker. Two milliliter of the overnight culture was added to 50 ml LB medium in a 250 ml conical flask and shaken vigorously (200 rpm) at 28°C until the culture grew to an OD of 0.5-1 at 600 nm. The culture was chilled on ice and the cell suspension was centrifuged at 3000 rpm for 5 min at 4° C. The supernatant was discarded and the cells were resuspended in 1 ml of ice-cold 20 mM $CaCl_2$ solution. This was

dispensed as 0.1 ml aliquot into prechilled microcentrifuge tubes. About 1µg of plasmid DNA was added to the cells. The cells were frozen in liquid nitrogen for 2 min. The cells were thawed by incubating the tubes in 37^{O} C water bath for 5 min. LB medium, 1ml was added to the tube and incubated at 28^{O} C for 2-4 h with gentle shaking. This period allowed the bacteria to express the antibiotic resistance genes. The tubes were centrifuged for 5 min at 5,000 rpm. Supernatant was discarded and the cells were resuspended in 100 µl of LB medium. The cells were spread on an LB agar plate containing 100 mg l^{-1} kanamycin. The plates were incubated at 28^{O} C. The transformed colonies appeared in 2-3 days.

2.3.2.6 Isolation of binary vectors from *Agrobacterium tumefaciens*

Agrobacterium tumifaciens was inoculated to 5ml culture and was incubated at 28^{0} C for 24 h. The cells were harvested by centrifugation at 12,000 rpm for 10 min in 1.5 ml microcentrifuge tube. The bacterial pellet was resuspended in 100µl cell suspension solution containing 50mM glucose, 25mM Tris, 10mM EDTA pH 8.0. Subsequently 20µl lysozyme (20mg/ml stock) (Sigma USA) was added, mixed well and incubated at 37^{0}C for 15 min. Freshly prepared 200 µl of cell lysis solution (0.2 M NaOH and 1.0% SDS) was added and mixed completely by repeated inversion. Equilibrated phenol 50 µl was added with 2 volumes of cell lysis solution and mixed thoroughly followed by addition of 200 µl of neutralization solution (3M sodium acetate pH 5.2), mixed completely by repeated inversion of the tube. The tubes were centrifuged at 12,000 rpm for 5 min, the upper aqueous phase was transferred to a fresh micro centrifuge tube, 2.5 volumes of 95% ethanol was added and placed on ice for 10 min. the tubes were centrifuged at 12,000 rpm for 15 min to spin down the DNA/RNA pellet and the pellet washed in 70% ethanol for further purification. It was centrifuged at 12,000rpm for 5 min and the pellet resuspended in 50 µl TE buffer (10mM Tris, 0.1 mM EDTA, pH 7.8).

2.3.2.7 PCR for the detection of binary vector in *Agrobacterium tumefaciens*

Plasmid DNA was isolated from control and transformed into *Agrobacterium* using the above protocol. PCR was performed using primers designed for hygromycin phosphotransferase (*hpt* II) gene.

The PCR mixture (25 µl) contained 50 ng of DNA prepared from *Agrobacterium tumefaciens* , control untransformed *Agrobacterium tumefaciens ,* 1X PCR buffer, 2.5 mM of dNTPs and 1 unit of *Taq* DNA polymerase (MBI Fermentas, Lithuania), 25 p moles of each primer (Genosys, Sigma USA). PCR for *hpt* II gene (Table 8) was performed at initial denaturation at 94^{o} C for 5 min followed by 35 cycles of 1 min denaturation at 94^{o} C, 1 min annealing at 55^{o} C and 1 min extension at 72^{o} C with a final extension of 72^{o} C for 10 min. PCR for *GUS* (Table 8) was carried out as described above with annealing at 52^{o} C for 1 min. The thermal cycler used was Primus 25 PCR system (MWG, AG Biotech, Germany). The PCR products obtained were separated on 1% agarose gel, stained with ethidium bromide and observed under UV light and documented (Hero-Lab Gmbh. Germany).

2.3.2.8 Polymerase chain reaction (PCR) for GUS expression in transgenic *Capsicum sp.*

PCR was performed using primers designed for hygromycin phosphotransferase (*hpt* II) gene and *GUS* gene. The PCR mixture (25 µl) contained 50 ng of DNA prepared from *A. tumefaciens*, control untransformed plantlets and transformed plantlets as the template, condition of PCR are same as above. The PCR products obtained were separated on 1% agarose gel, stained with ethidium bromide and observed under UV light and documented (Hero-Lab Gmbh. Germany).

2.3.2.9 Southern hybridization (Sambrook et al., 1989**)**

The isolated genomic DNA (approx. 40µg quantity) from putative transformants was digested with *Nde* I and *Sac* II enzymes, for Southern blot

analysis. The digested DNA was electrophoresed on 0.8% (w/v) agarose gel, transferred (Sambrook et al., 1989) to Biobond plus nylon membrane (Sigma, USA) and hybridised with the 479-bp *hpt* II gene probe. The probe for *hpt* II gene was prepared using a psoralen biotin labelling kit (Ambion, USA). Hybridisation signals were detected using a Biodetect Kit (Ambion, USA).

2.3.2.10 Restriction digestion of pCAMBIA 1305.2 vector and *Capsicum* sp. genomic DNA

Materials

1. Restriction enzymes: *Pml* I and *Bgl* II (MBI Fermentas, Lithuania)
2. 10 X restriction enzyme buffer (MBI Fermentas, Lithuania)
3. BSA, acetylated, 1 mg l^{-1} (MBI Fermentas, Lithuania)
4. Nuclease-free deionized water (Bangalore Genei, India).

The following were added in a micro centrifuge tube in the order stated:

Nuclease-free water	13.8 µl
Restriction enzyme 10X buffer	2.0 µl
BSA, acetylated (1 mg l^{-1})	0.2 µl
DNA	3.0 µl
Pml I or *Bgl* II	1.0 µl (10 units)
Final volume	20.0 µl

Since the enzymes were compatible the digestion was carried out together. For digestion, the samples were incubated (after brief centrifugation) at 37^0 C for 8 h and the reaction was stopped by heating them at 65^0 C for 20 min. An aliquot of the digested products were fractionated and observed on 0.8% agarose gel.

2.3.2.11 Transfer of DNA to nylon membrane and hybridization

1. Target DNA
2. Primers for probe preparation (Genosys Sigma, USA)

3. Nylon membrane (Sigma, USA)
4. 20XSSC:

NaCl	3M
Sodium citrate pH 7.2.	0.3M

Hybridization buffer:

SSC	5X
N-lauroylsarcosine	0.1% (w/v)
SDS	0.02% (w/v)

Post-hybridization washing buffer I:

SSC	2X
SDS	0.1%

Post-hybridization washing buffer II :

SSC	0.1X
SDS	0.1%

5. Non isotopic (Psoralen biotin) labeling kit (Ambion, USA)
6. Biodetect kit (Ambion USA)
7. 5-Bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium (BCIP-NBT) detection solution (Bangalore Genei, India)

The digested DNA samples (digested with restriction enzymes *Nde* I and *Sac* II) were loaded onto the agarose gel 0.8% (w/v) and run at 80 V until the dye front reached ¾ of the gel. After electrophoresis the gel was stained with ethidium bromide for 15 min and observed on the transilluminator.

After examination, the gel was soaked for 45 min in several volumes of 1.5 M NaCl and 0.5 N NaOH mixtures with constant gentle agitation. The gel was soaked in several volumes of 0.2 N HCl for 10 min and rinsed briefly with triple distilled water. The DNA was neutralized by soaking in 1 M Tris (pH 7.4) and 1.5 M NaCl at room

temperature for 30 min with mild agitation. The neutralization solution was changed three times with 15 min interval.

The transfer tank was filled with 75 ml of 10X SSC buffer on each side. A Whatmann No. 3 filter was placed on the platform of the tank. The side of the filter paper was dipped into the buffer. The filter paper was rolled gently with a glass rod to remove air bubbles. Six Whatmann No. 3 sheets and nylon membrane was cut to the exact size of the gel. The nylon membrane was dipped in deionized water and then incubated in 10X SSC for 5 min. Two Whatmann No. 3 sheets were dipped in 10X SSC and placed in the middle of the platform. The gel was placed on the top of the filter in inverted fashion. The right side of the gel was nicked to serve as an identification mark. Parafilm strips were placed all around the gel to avoid short circuit of buffer during transfer. The nylon membrane was placed with its right side cut over the gel, so that the cut side matches with that of the gel. Remaining four Whatmann No. 3 filter sheets were dipped in 10X SSC and placed over the membrane. Air bubbles were removed by rolling a glass rod in each step. Stacks of paper towels were placed over the filter paper and applied a weight of about 500 g over the entire assembly. The transfer process was allowed for 24 h with intermittent changes of paper towels and transfer buffer.

After allowing the membrane to dry, it was placed inside a polythene bag and placed over a UV transilluminator for 2 min to allow cross-linking of DNA. The membrane was put in a hybridization jar to which 15 ml prewarmed (68^0 C) hybridization buffer was added and incubated overnight at 68^0 C in a hybridization oven (Shell Lab model-1004-2E) at constant and slow rotation. Hybridization buffer was discarded from jar.

The probe was diluted 10 fold with 10mM EDTA, denatured by incubating in boiling water bath for 10 min and snap cooling on ice. The denatured probe was added to the hybridization buffer and mixed immediately. The membrane was incubated with

the probe at 68^0 C for 6 h with mild agitation in a hybridization oven. The membrane was washed twice in 50 ml of post hybridization washing buffer I for 5 min at room temperature. Membrane was washed again in 50 ml of post hybridization washing buffer II for 15 min at 68^0 C under mild agitation.

2.3.2.12 Detection of hybridization signals

Detection of hybridization signals were done with Ambion Biodetect Kit (Nonisotopic Detection Kit, Ambion, USA). Membrane was rinsed twice for 10 min at room temperature in Ambion wash buffer. Subsequently, the membrane was incubated in blocking buffer twice for 20 min duration. Streptavidin-alkaline phosphatase was prepared by gently and thoroughly mixing together 10ml blocking buffer and 1μl Streptavidin-alkaline phosphatase (Ambion USA) (mixed with the blocking buffer before adding to membrane), added to the membrane and incubated for 45 min. The membrane was washed three times (15 min each time) in 1X Ambion wash buffer. The membrane was immersed in 10 ml BCIP-NBT detection solution (Bangalore Genei, India). For the development of color; the membrane was kept in the dark without shaking overnight. Reaction was stopped by washing the membrane for 5 min with 50 ml of deionized water. The results were documented by photography of the wet membrane following which the membrane was air-dried and stored in dry place.

Table 8 Various primers used in transformation experiments

Primer	Primer sequence (5'–3')	Annealing temperature(°C)	amplification cycles
(***hpt* II**)	F - CGGAAGTGCTTGACATTGG R - AGAAGAAGATGTTGGCGA	55	35
GUS	F - AGAATGGAATTAGCCGGACTA R - GTATTAATCCCGTAGGTTTGTTT	52	35
Lycopene cyclase (*Lcy-e*)	F - CCTGCATTGAACATGTTTGG R - AACCTGCAGGGAGTCACAAC	60	35

2.4 TRANSCRIPTION STUDIES

2.4.1 Analysis of transcript levels for carotenoids

Total RNA was extracted from the fruits (Green and Ripened) using a total RNA extraction kit (RNeasy kit, Ambion, USA). All the plastic wares were treated with 0.1% diethyl pyrocarbonate (DEPC) (Sigma USA) and the working area, electrophoresis tank and other required materials were treated with RNase Zap (Ambion, USA). The control and transgenic tissues were harvested, frozen in liquid nitrogen and RNA was extracted immediately. Quality and concentration of RNA were checked on denaturing agarose gel. All the RNA samples were subjected to DNase (Ambion, USA) treatment to avoid possible artifact amplifications from contaminant genomic DNA. Lcy-e gene specific primers were designed across the intron. First-strand cDNAs were synthesized from 2 µg of total RNA in 20 µL final volume, using MuLV reverse transcriptase (Ambion USA) and oligo-dT(18) primer (Sigma USA) following the manufacturer's instructions. To quantify template quantities, the RT-PCR reaction was stopped in the early exponential phase (28^{th} cycle) to maintain initial differences in target transcript quantities. PCR was performed using Forward 5' CCTGCATTGAACATGTTTGG 3' and Reverse 5' AACCTGCAGGGAGTCACAAC 3' (Table 8).

Ten microlitres from each PCR reaction was fractionated on a 1.5% (w/v) agarose gel in Tris-acetate EDTA buffer and stained with 0.5% (w/v) ethidium bromide. The gels were photographed with a Digital Imaging System (HeroLab, GMBH, Germany).

2.4.2 Isolation of specific cDNA and genomic DNA

To isolate placental specific cDNA, total RNA was extracted using total RNA extraction kit (Ambion, USA). To avoid possible RNase contamination, all plastic wares were treated with 0.1% DEPC (Sigma-Aldrich, USA) and the working area,

electrophoresis tank and other required materials were treated with RNase Zap (Ambion, USA). *Capsicum* fruits were harvested and placenta, pericarp and seeds were separated, frozen in liquid nitrogen followed by immediate RNA extraction. Quality and concentration of RNA were checked on denaturing agarose gel and by absorbance measurements at 280, 260 and 320 nm in a UV spectrophotometer. All the RNA samples were subjected to DNase (Ambion, USA) treatment to avoid possible artifact amplifications from contaminant genomic DNA. The genomic DNA was extracted according the Plant DNA extraction kit (Sigma-Aldrich, USA).

2.4.3 PCR conditions for amplification of gene encoding Lcy-e (*Lycopene cyclase*)

PCR amplification for Lcy-e gene was performed following 35 cycles at 94°C 2', 94°C 30 sec, 60°C 30 sec, 72°C 1', 72°C 10' with XT-5 *Taq* polymerase (Bangalore GeNei, India) with initial denaturation at 94^{o}C for 2 minutes and final extension at 72^{o}C for 10 min. An aliquot of 10 µL from each PCR reaction was fractionated on a 1.5% (w/v) agarose gel in Tris-acetate EDTA buffer. Ethidium bromide solution (0.5µg/L) stained gels were photographed with a Digital Imaging System (HeroLab, Germany). The transcript abundance of CS was quantified using the intensity histogram.

2.5 Bio-safety measures

All the work was carried out according to the guidelines of IBSC (Institutional Bio-Safety Committee, CFTRI, Mysore). The transgenic work was carried out with permission from RCGM (Regulatory Committee for Genetic Modifications, Department of Biotechnology, Government of India). The transgenic work was confined to the *in vitro* laboratory level. ISO 14001 guidelines of CFTRI were followed for the disposal of contaminants and transgenic waste.

2.6 Statistical analysis

Experiments were carried out in triplicates and the data was presented in terms of mean and standard error. The mean and standard deviation was calculated according to Tukey's method (Tukey, 1953)

RESULTS AND DISCUSSION

Content

3.1 *In-vitro* regeneration of chilli Capsicum

Capsicum or Chilli is highly recalcitrant and genotype specific so *in-vitro* regeneration is very tough. For the plant improvement and enhancement of the secondary metabolite production; genetic manipulations and genetic engineering approaches are in practice. Metabolic engineering of Capsicum, with regard to capsaicinoid and carotenoids production is of immense importance. Overall focus in Capsicum is for its improvement for pre-harvest management practices and high yield of oleoresin for specific uses. In order to achieve these objectives stable genetic transformation protocol is a pre-requisite. To overcome the constrain of recalcitrance and genotype specificity of Capsicum *in-vitro* regeneration studies were done extensively with great degree of success.

3.1.1 *In-vitro* shoot regeneration of Capsicum plants

The seedlings after 15 days of inoculation comprised of well-developed apical meristem, cotyledonary leaves and profuse roots. Aseptically grown decapitated seedling explants produced higher shoot bud induction in inverted inoculation method (with invert polarity) when compared to upward or horizontal inoculation with normal polarity (Table 9; Figure 11a–d). No morphogenetic response was observed in explants cultured on control medium comprising MS basal salts and vitamins, 2 g l^{-1} MES [2-(N-morpholine) ethane sulphonic acid] and 10 µM $AgNO_3$ [silver nitrate]. Profuse shoot bud induction was observed when the cultures were exposed to continuous light. On an average, 19.4 ± 4.2 shoot buds were produced from each explant and 60–65% explants responded when cultured on medium supplemented with 26.63 µM BA [N^6-Benzyladenine], 2.28 µM IAA [indole-3- acetic acid], 10 µM silver nitrate, 2 g l^{-1} MES (Table 10 Figure 11 a). Interestingly 20–35 roots were observed in bud-induced inverted explants (Figure 11 a). The shoot buds were formed in a circular fashion along the cut edges of the decapitated shoot tip region of the seedling explant. Horizontal and vertical inoculation of the explants did not show good response in terms of adventitious shoot bud formation. Use of other cytokinins like kinetin, 2iP or zeatin

gave only 4–20% response with 2–6 shoot buds per explants (Table 9). It is evident that among the tested cytokinins only BA was efficient to elicit regeneration response in a polarity dependent manner in *C. frutescens*.

Incorporation of exogenous polyamines increased the number of shoot buds and the percentage explant response. The percentage response to shoot multiplication increased up to 83, 75, 70%, respectively, under Putrescine (Put), Spermine (Spm) or Spermidine (Spd) treatments. The addition of 50 mM Put in the bud induction medium with BA resulted in 22.6 ± 2.1 shoot buds per explant (Table 10 Figure 11 b) and 83% explant responded under 24-h continuous light. Incorporation of Spm and Spd did not change the shoot bud induction response (Table 10 Figure 11 c, d).

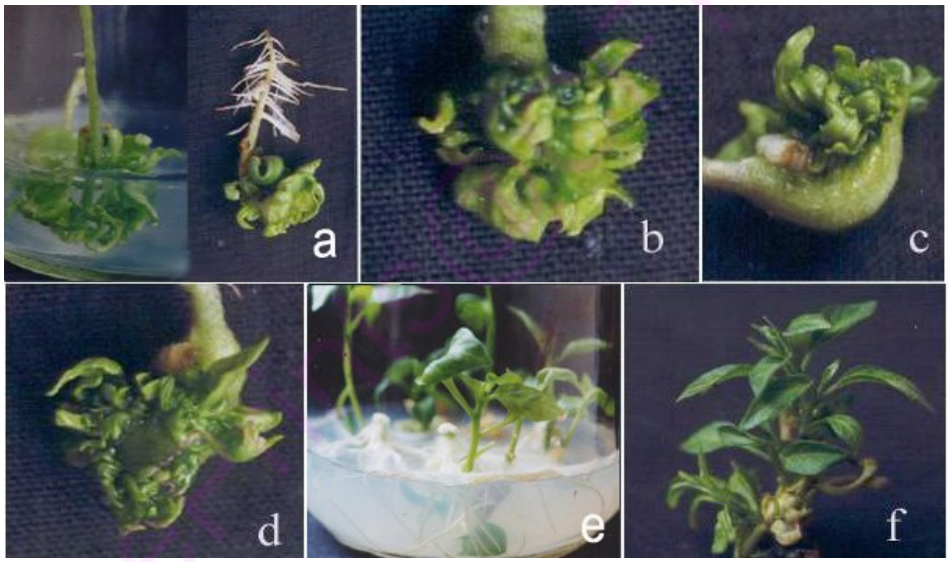


Figure 11 Stages of direct shoot bud induction and regeneration in *Capsicum frutescens* var. KTOC

(a) Shoot bud induction in inverted decapitated seedlings of *C. frutescens*. Induction of shoot buds on the apex and profuse rooting on the hypocotyls can be seen after 2 months of culture. (b) Shoot bud induction under the influence of Put in inverted decapitated seedlings. (c) Shoot bud induction under the influence of Spd in inverted decapitated seedlings. (d) Shoot bud induction under the influence of Spm in inverted decapitated seedlings. (e) Rooted shoots of *C. frutescens*. (f) Mature hardened plant of *C. frutescens*.

Table 9 Influence of inoculation mode on shoot bud induction in *Capsicum frutescence* var. KT- OC

Mode of inoculation	Number of shoot buds per explant (MS basal +2 gl^{-1} MES + 26.63 µM BA + 2.28 µM IAA + 10 µM $AgNO_3$)[a]	
	Under 24/0-h photoperiod	Under 16/8-h photoperiod
Upward shoot polarity	1.0	-
Horizontal mode	2.6 ± 0.8*	-
Downward shoot polarity	19.4 ± 4.2**	10.9 ± 2.2**

Ten decapitated seedling explants were used for each treatment and the experiments were repeated thrice and the mean ± SE values of the results were represented. No response was observed when the explants used under upward and horizontal mode in medium comprising kinetin, 2iP and zeatin as cytokinin source. No response was observed in explants cultured on control medium comprising MS basal salts and vitamins, 2 g l^{-1} MES, 10 µM $AgNO_3$

**P < 0.01, *P < 0.05 compared to respective upward shoot polarity inoculations

[a] Data for optimum concentration of hormones and growth regulators are presented

Table 10 Effect of different cytokinins on shoot bud induction in *Capsicum frutescens* var. KT- OC[a]

Media[b]	Under 24/0-h photoperiod (continuous light)		Under 16/8-h photoperiod	
MS salts and vitamins + 2 gl^{-1} MES + 2.28 µM IAA + 10 µM $AgNO_3$	% response	Mean number of shoot buds	% response	Mean number of shoot buds
+13.31 µM BA	20	4.2±2.1*	15	2.5±0.5*
+26.63 µM BA	65	19.4±4.2**	60	10.9±2.2*
+89.77 µM BA	32	10.4±3.3*	25	6.4±1.4*
+9.29 µM kinetin	2	4.3±2.1*	-	-
+11.6 µM kinetin	6	2.1±1.1*	4	2.4±0.8*
+23.23 µM kinetin	3	2.5±1.2*	-	-
+0.91 µM zeatin	8	4.3±2.4*	-	-
+4.56 µM zeatin	20	6.1±2.0*	12	4.4±0.6*
+9.12 µM zeatin	-	-	-	-
+2.46 µM 2iP	20	4.3±2.0*	-	-
+4.42 µM 2iP	10	1.6±0.5*	12	1.9±0.4*
+9.84 µM 2iP	-	-	-	-

Ten decapitated seedling explants were used for each treatment in inverted inoculation mode and the experiments were repeated thrice and the mean ± SE values of the results were represented; **Significance at P < 0.01; [a] Data recorded after 60 days of culture; [b] significance at P < 0.05; [a] Data for optimum concentration of hormones and growth regulators are presented

To study the influence of polyamines in regeneration of Capsicum, polyamine inhibitors DFMA and DFMO (1 mM) each were incorporated in the bud induction medium comprising BA and IAA. The process of shoot bud induction was completely inhibited in this medium. However, only 2–4 shoot buds were induced when exogenous polyamines along with the inhibitors DFMA and DFMO were incorporated (Table 11). The polyamine incorporation restored the regeneration potential of explants to a certain level. However, response was only up to 26% when compared to the control (up to 60%) (Table 11).

When these shoot buds were inoculated in the elongation media, comprising of MS salts and 10 µM $AgNO_3$ supplemented with 7.34 µM PAA or 2.8 µM GA_3 and incubated in continuous dark and light resulted in a spectacular response of 68% and 10.4 ± 1.2 shoot buds elongated from each explant (Table 12) under incubation in dark. The elongated shoots (3–4 cm length) rooted profusely when cultured on basal medium devoid of hormones (Figure11 e). The plants were acclimatized in greenhouse (Figure 11 f). After hardening, 60–75% of the plants survived in field. All the *in-vitro* regenerated plants appeared normal without any morphological variations. Several biochemical processes and cellular signaling are required for differentiation during shoot morphogenesis in plants. Plant growth regulators play a key role in controlling the differentiation process required for regeneration. However, change in polarity orientation of the explant in culture medium played a key role in regulating the regeneration process. Several factors are involved in regeneration of *C. frutescens*, which is known to be highly recalcitrant in *in-vitro* regeneration system. (1) Shoot buds were induced only in explants cultured in inverted polarity. (2) Polyamine pools, polarity of the explant and continuous exposure to light might be the key factor which triggers shoot bud induction and thereby regeneration. (3) The morphogenetic response appeared to be polarity dependent. Use of optimized concentration of GA_3 under dark incubation resulted in elongation of shoot buds.

Table 11 Effect of exogenously fed polyamine and polyamine inhibitors on shoot bud induction in *Capsicum frutescens* var. KT- OC[a]

Plant growth regulators MS salts and vitamins + 2 gl^{-1} MES + 2.8 µM IAA + 26.63 µM BA + 10 µM $AgNO_3$					Percentage response		Mean number of shoot buds on the hypocotyl	
Put (mM)	Spm (mM)	Spd (mM	DFMA (mM)	DFMO (mM)	16/8-h light photoperiod	24 -h light photoperiod	16/8-h light photoperiod	24 -h light photoperiod
50	0	0	0	0	20	83	12.3 ± 2.0**	22.6 ± 2.1**
0	50	0	0	0	28	75	10.4 ± 2.1*	19.4 ± 2.5*
0	0	50	0	0	20	70	10.6 ± 3.0*	17.6 ± 4.2*
50	0	0	1	0	18	26	1.8 ± 0.4*	2.9 ± 0.5*
50	0	0	0	1	16	22	–	4.3 ± 0.4*
50	0	0	1	1	08	10	–	5.3 ± 0.6*
0	0	0	1	1	00	00	–	–
0	0	0	0	0	60	65	10.9 ± 2.2*	19.4 ± 4.2**

Ten decapitated seedling explants were cultured in inverted mode and used for each treatment and the experiments were repeated thrice and the mean ± SE values of the results were represented in the table **Significance at P < 0.01; *significance at P < 0.05; [a] Data recorded after 60 days of culture.

Table 12 Effect of two different hormonal combinations and light on elongation of shoot buds of *Capsicum frutescens* var. KT-OC[a]

Media	No. of shoot bud elongation per explant		Percentage bud elongation per explant	
MS salts + vitamins + 10 µM $AgNO_3$	Light	Dark	Light	Dark
+ 7.34 µM PAA	–	4.9 ± 0.5*	13	28
+ 2.8 µM GA_3	2.4 ± 0.4*	10.4 ± 1.2**	31	68

There was no response on MS basal hormone-free medium, **Significance at P < 0.01; *significance at P < 0.05; [a] Data recorded after 45 days of culture.

Apart from balance between auxins and cytokinins, other factors like polarity are involved in this complex process of morphogenesis (Sushma and Palni, 2004). Nevertheless we cannot rule out the possibility that polarity reversal interacting with other cellular process triggering the regeneration response in *C. frutescens*. By this study we have developed a regeneration protocol for a high pungent chilli *C. frutescens* var. KTOC. In an earlier study carried out with nodal explants of *C. frutescens*, the combination of kinetin (1mg l^{-1}) and IBA (1 mg l^{-1}) was reported to be useful in clonal propagation through nodal explants (Gururaj et al., 2004). The nodal explants did not produce multiple shoots. Single shoots proliferated from nodal explants giving rise to 3–5 nodes (Gururaj et al., 2004). It was reported that MS

medium containing kinetin and 2iP were less effective for growth of shoot tip explants (Gururaj et al., 2004). Similar response was observed in the present study too wherein, Kn and 2iP did not elicit morphogenetic response when the explants were cultured in inverted shoot polarity.

It was noticed that morphogenetic response of *C. frutescens* was triggered under the influence of polyamines (Table 11). Similar observations have been made by other workers in different plant systems. Polyamines are known to promote shoot multiplication in various plant systems (Chi et al., 1994; Bias et al., 2000). It is postulated that, PA(s) are type of growth regulator or secondary hormonal messenger (Galston 1983; Davies 1987). It is well known that both ethylene and polyamines are metabolically related, and both utilize the same precursor S-adenosyl-L-methionine (SAM) for their synthesis (Evans and Malmberg, 1989). Ethylene is known to inhibit plant morphogenesis (Bayer, 1976). It has been suggested that polyamines and ethylene may regulate each others synthesis. For instance ethylene has been shown to inhibit arginine decarboxylase and SAM decarboxylase activities in pea seedlings (Apelbaum et al., 1985). These enzymes are necessary in the production of polyamines (Smith, 1985). Polyamines were reported to promote somatic embryogenesis in carrot (Feirer et al., 1984), the promotive effect of ethylene inhibitors such as $AgNO_3$ on regeneration was thought to be due to enhanced polyamines synthesis rather than reduced ethylene production. Pua et al., (1996) clearly described the synergistic effect of $AgNO_3$ and Put on shoot regeneration in Chinese radish. Reports on somatic embryogenesis in carrot (Roustan et al., 1990) indicates that the potent ethylene action inhibitor $AgNO_3$ helps in increasing ADC activity, which in turn increases the levels of endogenous polyamines in carrot embryogenic cultures. All these evidences suggest that there may be a strong link among ethylene, polyamines and its effect on plant regeneration in *Capsicum sps*. In the present study, we found that incorporation of polyamine inhibitor treatments resulted in inhibition of morphogenetic response (Table 11). On the other hand,

exogenous administration of polyamines results in the restoration of morphogenetic potential and increases the percentage explant response (Table 11). Attempts were made earlier for direct regeneration as well as for somatic embryogenesis from leaf and hypocotyl explants with various hormonal combinations (Phillips and Hubstenberger 1985; Ebida Aly and Hu, 1993; Harini and Lakshmisita, 1993; Binzel et al., 1996; Kim et al., 2001). Inoculation of explants in reverse polarity was found to enhance regeneration in *Cedrus deodara* (Sushma and Palni 2004). The use of certain key components like MES, silver nitrate, Phenyl Acetic Acid (PAA) were selected on the basis of its reported beneficial effect on Capsicum regeneration by other workers in various genotypes of *Capsicum sps* (reviewed by Ochoa-Alejo, and Ramirez- Malagon, 2001), for shoot bud induction in *C. annuum* (Valera-Montero and Ochoa-Alejo, 1992) and PAA for shoot bud elongation (Hussain et al., 1999).

Silver ion is a potent inhibitor of ethylene action (Bayer 1976) and has been found to enhance plant regeneration in many plant systems (Bias et al., 2000; Reddy et al., 2002) including *C. annuum* (Hyde and Phillips, 1996) and hence, used in the present study also. In general, the elongation and *in-vitro* rooting of regenerated shoots or shoot buds are difficult in *Capsicum* species (Liu et al., 1990; Christopher and Rajam, 1994). Similarly, Ebida Aly and Hu (1993) have first rooted the rosette shoot buds of *C. annuum* and obtained elongation of buds on medium containing NAA. Apart from that, PAA have also been reported for efficiency of shoot bud elongation (Hussain et al., 1999). Elongation of shoot buds in medium supplemented with GA_3 was profuse and the response was much better in the dark (Table 12). The potted tissue cultured plants in field showed normal growth, flowering and yield.

3.1.2 Shoot bud induction from shoot tip explants and regeneration from cotyledonary leaf

From the results it is evident that mode of explant inoculation (Table 13) on to the suitable medium had profound influence on induction of shoot buds in *C. frutescens*.

Moreover, the preconditioning of explants by treating with very low concentration of IAA (0.5 mg l^{-1}) and BA (1 mg l^{-1}) only showed rapid shoot bud multiplication from decapitated shoot tip explants compared to explants without preconditioning, which did not show shoot bud proliferation. Therefore, regeneration data presented here is the one that was obtained from these explants only. In both vertical mode and horizontal mode of inoculation, maximum of 4 shoots were produced in both BOX-RUB and KT-OC varieties respectively. When hypocotyl explants were inoculated in inverted mode on to the Shoot Bud Induction Medium (SBIM), best shoot proliferation was noticed. The combination of 10 mg l^{-1} BA and 1 mg l^{-1} of IAA in SBIM supported 30 and 45 shoot buds in KT-OC and BOX-RUB variety respectively (Figure 12 B, C). The initiation of shoot bud formation from cut edge of the decapitated explants was observed from 15th day of culture and distinct buds were evident by 30 days. Induction of long, thin and white roots was so evident from the surface of hypocotyl extending from the collar region to middle of hypocotyl explant (Figure 12 A). However, the number of roots varied which were 30 and 45 in BOX-RUB and KT-OC variety respectively.

Table 13 Response of the Shoot tip of *Capsicum frutescens* to the mode of inoculation in the SBIM media[a]

Variety	Explant orientation	No. of Shoot buds	No. of roots formed on the hypocotyl
KT-OC	Vertical	1	5 ± 0.3
	Horizontal	2 ± 0.83	4 ± 0.62
	Inverted	30 ± 2.65	42 ± 4.0
BOX-RUB	Vertical	1	7 ± 0.3
	Horizontal	2 ± 0.83	3 ± 0.72
	Inverted	45 ± 2.65	38 ± 4
BOX-RUB	Horizontal	12 -18	7 - 13

All media contain complete MS salts +SBIM media composition. [a]Data recorded on 30th day of inoculation.

The elongation of these shoot buds were very effective on medium containing 3 mgl^{-1} Gibberellic acid (GA_3), 5 mgl^{-1} BA and 10μM silver nitrate (Figure 12 D), wherein 75-80% response was noticed and it was more pronounced. The shoot buds elongation of *C. frutescens* BOX-RUB variety was better than in KT-OC variety. The *in-vitro* rooting of the shoots was effective on medium devoid of growth regulators in both the varieties. Subsequent to hardening of rooted plants, 70-75% of the transplanted ones survived.

Table 14 Response of the various explants on the various media[a]

Varieties	Explant used	media	No. of explant responded	Regeneration frequency(%) Mean ± S.D
KT-OC	Nodal explant	BN media	15	45.4 ± 0.8
	Buds	PB media	19	57.5 ± 1.6
BOX-RUB	Leaf	BK media	13	39.3 ± 2.1
	Buds	PB media	7	21.2 ± 2.1

All media contain complete MS salts +SBIM media composition. [a]Data recorded on 30th day of inoculation

A maximum of 12-15 shoots were induced in presence of BA (5 mg l^{-1}) and Kinetin (1 mg l^{-1}) (BK media) from the cotyledonary explant (Table 14, Figure 13A) Shoot buds (8-10) were obtained from the margins of the proximal portion of the leaf explant when inoculated directly on SBIM within 4 weeks of incubation (Figure 11B). When petiole explants were cultured alone on the same media, 2-4 shoots per explant were obtained from the distal portion (Figure 13 B&C). Differentiated shoots from cotyledonary leaf and petiolar portion grew well and proliferated in the medium having 2 mg l^{-1} PAA, 7 mg l^{-1} BA and 10 μM $AgNO_3$ (Figure 13 D) (PB media). *In-vitro* rooting of these shoots was evident on MS basal medium (Figure 13 E). Regenerated plants were phenotypically normal.

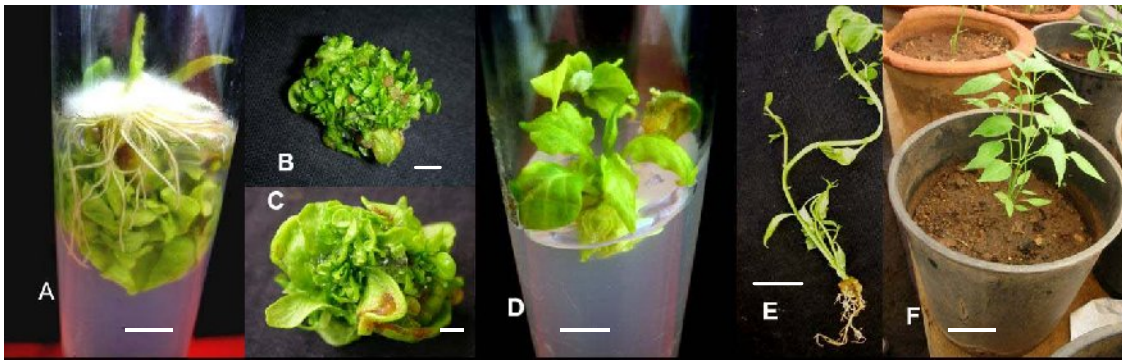


Figure 12 High frequency shoot bud induction from decapitated seedling in *C. frutescens* Mill.

A) High frequency shoot bud induction from decapitated seedling explant in inverted mode on SBIM B) Shoot buds on SBIM medium after 15 days C) Shoot buds on SBIM medium after 30 days D) Elongation of shoot buds into 3-4 cm long shoots E) *In-vitro* rooting of shoot on plain MS medium F) Potted plant for field transfer. Bar A-C 3mm, D-F 6mm.

Figure 13 Direct regeneration from the petiole of the leaf in *C. frutescens* Mill.

A) Direct regeneration from the petiole of the leaf, B) Elongation of the shoots, C) Multiple shoots. D) & E) Elongation of shoot on plain MS media. Bar A-2.5mm, B & C 5mm.

The recalcitrant nature of *Capsicum* sp. for morphogenesis has been well known (Steintiz et al., 1999). The morphogenetic potential was improved by reverse polarity of seedlings after decapitating the apical meristem and root zone. In order to further improve the morphogenesis potential of explants preconditioning has been reported in some other plant systems (Lee et al., 1982; James and Thurbon, 1981) and moreover, these protocols are highly specific for particular variety and not responsive for all varieties (Venkataiah et al., 2003). Containment of apical dominance of decapitated seedlings by reversal of the polarity

of the explants was achieved as in similar report of *Cedrus deodara* (Sushma and Palni, 2004). In the present study, the shoot buds response to differentiation was similar to earlier report on *C. annuum* (Ediba Aly and Hu, 1993). Valera-Montero and Ochoa-Alejo (1992) and Vinod Kumar et al., (2005) earlier reported the beneficial effect of MES and PAA in *C. annuum* organogenesis. Fully organized multiple shoots were obtained in *C. frutescens* by administering GA_3. Both BA and IAA induced direct differentiation of shoot buds from cotyledonary leaf explants in *C. frutescens*. Similar observations were made in *Tagetus* under influence of BA and IAA (Misra & Datta 2001). The GA_3 was used to induce elongation of differentiated shoot buds without the intervention of callus by avoiding callusing from explants (Misra and Datta 2001; Chakrabarty et al., (2000).

Out of the different hormonal combinations tried for *in-vitro* shoot regeneration from both nodal and shoot tip explants of *C. frutescens*, the combination of kinetin (1.0 mg l^{-1}) and IBA (1.0 mg l^{-1}) was found effective (Table 15). Though the nodal explants did not produced multiple shoots but single shoot proliferated into elongated shoots with 3-5 nodes in the combination of 1.0 mg l^{-1}each of kinetin and IBA (Figure 14A). It was found that MS medium containing kinetin and IAA or 2iP and IAA/IBA were less effective for growth of shoot tip explants (Table 15) as compared to kinetin (1 mg l^{-1}) and IBA (1 mg l^{-1}) combination alone which gave rise to long shoots with a maximum number of nodes per culture (7.2 ± 0.37) with 100 % response from shoots after 45 days of culture (Figure 14B). The proliferation of shoots on the medium with kinetin + IBA started after 6-8 days of culture from nodal explants on this medium. The incorporation of BA alone or in combination with either IAA or IBA induces callusing from the base of both nodal and shoot tip explants without differentiation of shoot buds. Similarly the combination of kinetin + IAA or 2iP + IAA/IBA also was not effective for shoot proliferation from both nodal and shoots tip explants. The other two auxins 2, 4-D and NAA were also not effective in combination with cytokinins for shoot proliferation (Table 15; Fig 14). Addition of activated charcoal (AC) played a vital role in

regeneration of plants in *C. frutescens* in this study. The media containing AC prevented browning of explants and resulted in regeneration; this may be due to the adsorption of Fe-chelates as reported earlier or may be due to absorption of metabolites inhibiting morphogenesis. In the present study when media without activated charcoal was used there was very slow response from both shoot tip and nodal explants with very small shoots (< 1.5 cm). The primary shoots when sub-cultured on to MS medium supplemented with kinetin (1.0 mg l^{-1}) along with IBA (1.0 mg l^{-1}), considerable elongation of shoots was achieved with formation of 4-7 nodes and wide green leaves (3-3.5cm length and 2-2.4 cm width). Sub culturing of both shoot tips and nodal segments of primary shoots onto the medium containing axillary buds responded well on medium with 1.0 mg l^{-1} each Kinetin and IBA with 1.0% AC by producing new shoots. Another advantage of this hormonal combination was the simultaneous rooting of the shoots within 45 days (Figure 14C) without the necessity of sub culturing for *in-vitro* rooting. A maximum of 4-5 roots per shoot with a root length of 7-9 cm were produced after 60 days in the same medium used for elongation. The rooted plants were transplanted to soil and raised in pots under green house conditions for one month, followed by their transfer to out side (Figure 14 D). Approximately 80-90% of the plantlets survived. After 4 months, the potted tissue cultured plants showed good yield which was almost equal to normal plants (Figure 14 E).

In general the elongation and *in-vitro* rooting of regenerated shoots or shoot buds in *Capsicum* species is difficult especially in varieties such as Early California Wonder (Christopher and Rajam, 1996). Similarly Ebida Aly and Hu (1993) first reported the rosette shoot buds of *C. annuum* and then obtained elongation of buds on medium containing NAA. In contrast to these reports in *C. annuum*, in the present study we achieved not only the elongation of shoot buds but also simultaneous rooting of the elongated shoots at optimal levels of 1.0 mg l^{-1} kinetin and IBA combination.

Figure 14 *In-vitro* clonal propagation of *Bird eye* chilli (*Capsicum frutescens* Mill)
(A,B) Initiation of shoot formation from nodal explant on MS medium containing kinetin (1 mg l^{-1})+ IBA (1 mg l^{-1}) + AC (1 g l^{-1}) (C) Elongation of primary shoot with simultaneous *in-vitro* rooting on medium containing kinetin (1 mg l^{-1}))+ IBA (1 mg l^{-1}) + AC(1 g l^{-1} (D) Potted plant; and (E) 4 months old tissue cultured plant with flowers and fruits

Table 15 Shoot proliferation from shoot tip (A) and nodal explants (B) of *C. frutescens in-vitro*[a]

MS medium with 1% activated charcoal + hormones (mg l^{-1})			Percentage of explants responded		Shoot length (mm) (±SE)		Number of nodes(±SE)	
Kin	**IBA**	**IAA**	**A**	**B**	**A**	**B**	**A**	**B**
0	0	0	-	-	-	3.2±0.86	-	0.6±0.2
0.5	0	0	30	20	3±0.2	10±0.7**	0.3±0.09	0.6±0.2**
1.0	0	0	30	20	3.2±0.3***	11.4±0.5*	0.4±0.1*	0.6±0.1*
2.0	0	0	20	30	4.1±0.7**	11.4±0.8***	0.6±0.1*	0.8±0.2*
0.5	0.5	0	30	40	10.5±1.2*	23.2±0.6**	1.1±0.4**	3±0.3*
1.0	0.5	0	50	40	9±0.7**	14.8±0.3***	1.3±0.3**	1.8±0.4**
2.0	0.5	0	50	40	8±0.2***	13.8±0.3***	1.1±0.4**	1±0.4**
0.5	1.0	0	60	40	17±0.4***	42.2±0.9***	1.8±0.6**	3.8±0.3*
1.0	1.0	0	100	100	28±0.5**	72±0.6***	4±0.2***	7.2±0.3*
2.0	1.0	0	50	60	12±0.3***	43.4±0.6**	2.1±0.6***	5±0.31*
0.5	2.0	0	60	70	9±1.4*	27.4±0.7***	1.2±0.2**	2.8±0.3**
1.0	2.0	0	50	50	11±1.7*	34.6±0.5***	1.6±0.3***	4±0.3*
2.0	2.0	0	30	50	10±1.1**	31.2±0.5**	1.1±0.3***	2.8±0.3**
0.5	0	0.5	20	10	3.5±1.7*	10.8±0.5**	0.9±0.1**	0.8±0.3**
1.0	0	0.5	20	20	5±0.72**	15.2±0.4**	0.8±0.2**	0.6±0.2*
2.0	0	0.5	30	20	3.6±0.6**	11±0.4**	0.9±0.1**	0.7±0.2*
0.5	0	1.0	20	30	3.5±0.8**	11.8±0.4**	0.4±0.2**	1±0.2*
1.0	0	1.0	30	30	3.4±0.4***	11±0.4**	0.3±0.08*	1±0.3*
2.0	0	1.0	30	20	3.6±0.8**	11.2±0.4**	0.5±0.02*	0.8±0.2*
0.5	0	2.0	30	20	3.4±0.5***	7±0.4***	0.3±0.06*	1±0.2*
1.0	0	2.0	30	20	3.2±0.4**	.2±0.3***	0.4±0.1**	0.8±0.2*
2.0	0	2.0	10	20	3.0±0.1***	.6±0.2***	0.4±0.1*	0.8±0.3**

A= nodal explants, B= shoot tip explants; * $p< 0.05$; * *$p< 0.01$; *** $p< 0.001$; [a]Data recorded after 45 days of culture.

3.1.3 *In-vitro* growth of shoots and *in-vitro* flowering in *Capsicum frutescens* var. KT-OC

The percentage of seed germination of *Capsicum frutescens* var. KT-OC on MS basal medium was 90% and it took 26-32 days to grow up to single node. Nodal explants inoculated on MS medium with 30µM of silver nitrate responded well for shoot growth wherein 2.5 folds increase was obtained (Table 16, Figure 15 A) compared to control. Similarly 30µM of Cobalt chloride supplemented media also influenced shoot growth up to 2.2 folds as compared to control (Table 17, Figure 15 B).

Table 16 Effect of Silver nitrate on shoot growth and *in-vitro* flowering in *C. frutescens* Mill

S.no.	Cobalt chloride (µM)	*No. of flowers*		Shoot length (cm)		
		25 days	45 days	15 days	30 days	45 days
1	0	0	0	1.7±0.3	2.0±0.5	2.4±0.4
2	10	0	1	2.4±0.2	3.5±0.6	4.3±0.5
3	20	2	3	2.5±0.4	3.2±0.2	4.1±0.2
4	30	3	7	2.9±0.2	4.1±0.4	5.4±0.4
5	40	3	4	2.5±0.5	3.6±0.3	4.8±0.6
6	50	2	3	2.8±0.3	3.8±0.4	4.6±0.2

Table 17 Effect of Cobalt Chloride on shoot growth and *in-vitro* flowering in *C. frutescens* Mill.

S.no.	Silver nitrate (µM)	*No. of flowers*		Shoot length (cm)		
		25 days	45 days	15 days	30 days	45 days
1	0	0	0	1.6±0.6	2.1±0.4	2.5±0.4
2	10	0	1	2.1±0.5	3.5±0.2	3.7±0.5
3	20	1	2	2.3±0.5	3.2±0.3	3.9±0.5
4	30	2	4	2.9±0.4	4.9±0.5	6.4±0.4
5	40	4	7	2.7±0.6	3.7±0.4	5.1±0.6
6	50	1	3	2.6±0.5	4.1±0.5	5.8±0.5

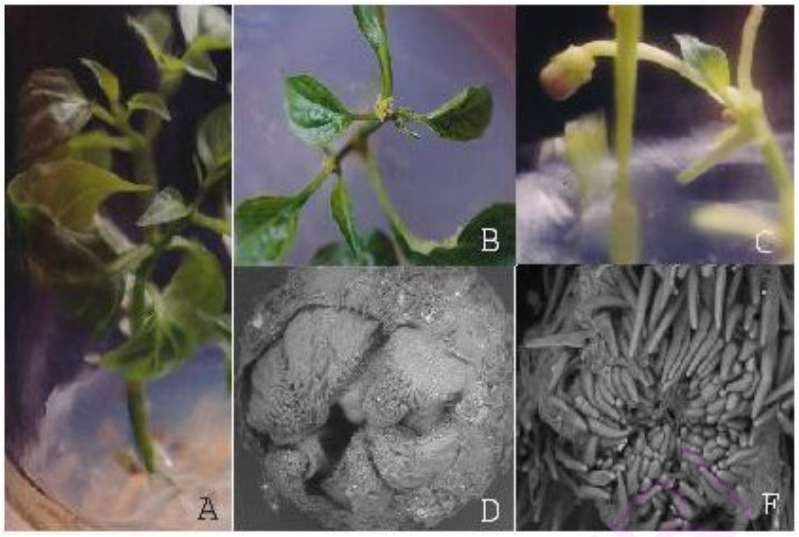


Figure 15 *In-vitro* flowering in *Capsicum frutescens* Mill A) Shoot growth *in-vitro* under the influence of silver nitrate 30 µM on MS basal medium B) Induction of *in-vitro* flower bud under the influence of the 50 µM cobalt chloride on MS basal medium C) Induction of *in-vitro* flower bud under the influence of 40 µM silver nitrate on MS basal medium D) SEM photograph of *In-vitro* flower bud at 40 µM of silver nitrate E) SEM photograph of *In-vitro* flower bud at 50 µM cobalt chloride.

Silver nitrate when used at lower concentration (10µM) did not induce flowering during 25 day of culture, even by 45 days only single flower was noticed (Table 16) but the flower induction was more profuse at 20-50µM of $AgNO_3$, optimal being 40 µM, wherein, a maximum of 7 flowers were noticed on single plant. But, the optimum concentration of $AgNO_3$ was 30 µM for obtaining maximum shoot length (6.4±0.4 cm) after 45 day of culture.

Similarly lower concentration of $CoCl_2$ (10µM) did not support any flower induction during the first 25 days of culture, but was able to induce only single flower after 45 days of culturing. The flower induction was prominent at 20-50µM of $CoCl_2$ with 30 µM as optimum concentration wherein a maximum of 7 flowers were noticed on single *in-vitro* plant (Table 17). Even the shoot length was more at 30 µM $CoCl_2$ (5.4 ±0.4 cm). After 45 days, culture in the media with silver

nitrate (40µM) (Figure 15 C, D) whereas in 30µM cobalt chloride (Figure 15 E) supplemental media seven flowers were produced. Higher concentration of silver nitrate and cobalt chloride resulted in abnormal morphogenetic responses.

In the present study we have reported that influence of $AgNO_3$ and $CoCl_2$ on *in-vitro* flowering of *C. frutescens* KT-OC variety. $AgNO_3$ has been reported to inhibit ethylene action (Bayer, 1976), and cobaltous ions are known to inhibit ethylene production (Lau and Yang 1976). It was found that addition of $AgNO_3$ to the culture media greatly improves regeneration of many dicot and monocot cultures as in case of *Coffea* sp., (Giridhar et al., 2003) and *Vanilla planifolia* (Giridhar et al., 2001) and even somatic embryogenesis in *Coffea* species (Giridhar et al., 2004). In the present study our results with nodal explants are in accordance with above reports. Both Co^{++} and Ag^{++} enhanced the percentage of cultures forming shoots and the number of shoots produced per explant. The exact mechanism of $AgNO_3$ mediated ethylene production and its activity regulation is unclear but it has been explained by an interference of ethylene perception or stress exerted by silver ion.

Silver nitrate is an ethylene action inhibitor and ethylene inhibits S-adenosyl methionine decarboxylase, which in turn promotes polyamine levels, which are implicated in flowering(Bias et al., 2000). Silver (silver nitrate as ethylene action inhibitor) and Cobaltous ion (Cobalt chloride as ethylene biosynthesis inhibitor), are also known to be involved in flower induction and other phenotypic responses (Bais et al., 2000, Reddy et al., 2001). Cobalt chloride effectively inhibits ethylene production and substantially increases shoot regeneration by blocking the conversion of ACC to ethylene (Lau and Yang, 1976).

Capsicum being recalcitrant species and there are lots of variations with in the species for their response in tissue culture studies (Ochoa-Alejo and Ramirez-Malagaon, 2001). According to Bodhipadma and Leung (2002) the *C. annuum* var. sweet banana zygotic embryos were used for *in-vitro* flowering. In fact, the said variety is non-pungent annual variety. In their subsequent report, the same authors Bodhipadma and Leung (2003) used silver thiosulphate in order to

achieve fruit setting in *C. annuum* var. sweet banana. Moreover, report of Tisserat and Galletta (1995) mainly oriented towards obtaining *in-vitro* flowering and fruiting from seedling tips by using Automated Plant Culture System Conditions. The selected explants were obtained from highly pungent *C. frutescens* variety. For obtaining flowering we used *in-vitro* shoots as explants and our results confirmed the requirement of $CoCl_2$ or $AgNO_3$ for inducing *in-vitro* flowering. The ethylene biosynthesis inhibition by $AgNO_3$ and ethylene action inhibition by $CoCl_2$ are well documented in other plant systems (Pua et al., 1996). It was evident that their incorporation into the medium may have similar influence physiologically in *C. frutescens* thus resulting in initiation of *in-vitro* flowering.

3.2 Elicitation of secondary metabolites in *Capsicum* sp.

Secondary metabolities produced during the process of plant cell culture have immense importance as they are responsible for various inter and intraspecific interactions,defence mechanism and regulation of various biosynthetic pathways. Capsicum is known for capsaicinoids and oleoresins; and for the enhancement of these secondary metabolities various abiotic and biotic elicitors are used from bacterial and fungal origin. The present study deals with elicitor mediated enhancement of capsaicinoids (capsaicin) and carotenoids (capsanthin and capsorubin) using various elicitors. This study may have implication in enhancing the secondary metabolities of *Capsicum sps* for use in pharmaceutical, nutraceutical and food industry.

3.2.1 Endogenous pools of phenyl propanoid intermediates, capsaicinoids in different cultivars of Capsicum.

The estimation of capsaicin biosynthetic pathway intermediates revealed genotype specific difference in metabolites related to capsaicin biosynthesis pathway as evident from HPLC (Figure 16), LCMS (Figure 17) profiles

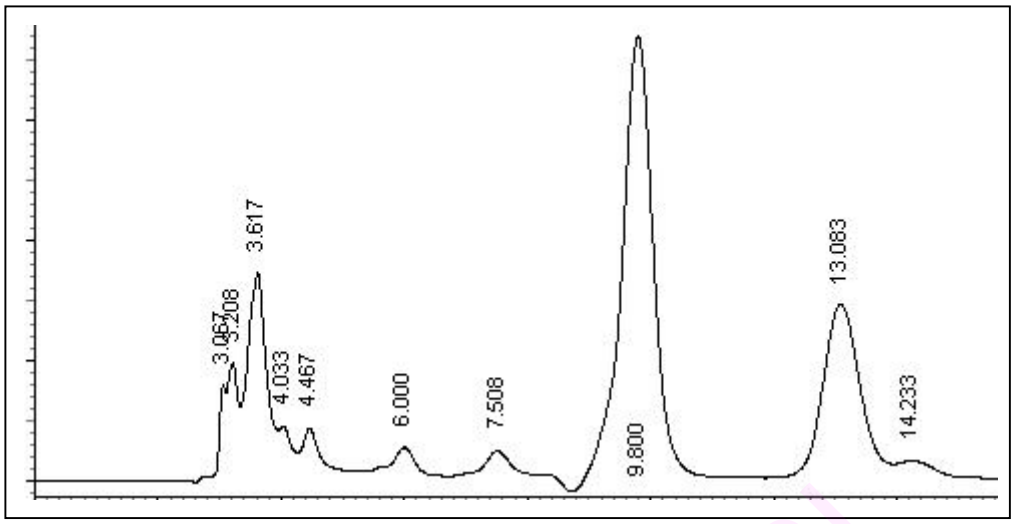


Figure 16 HPLC of the major capsaicinoids from the *Capsicum frutescens* var KT-OC cultivar. Separation of capsaicin from phenyl propanoid compounds by High performance Liquid Chromatography (HPLC) in the cultivar. Retention time of the major metabolites in the sample is vanillylamine -3.61' capsaicin- 9.8' dihydrocapsaicin- 13.08'.

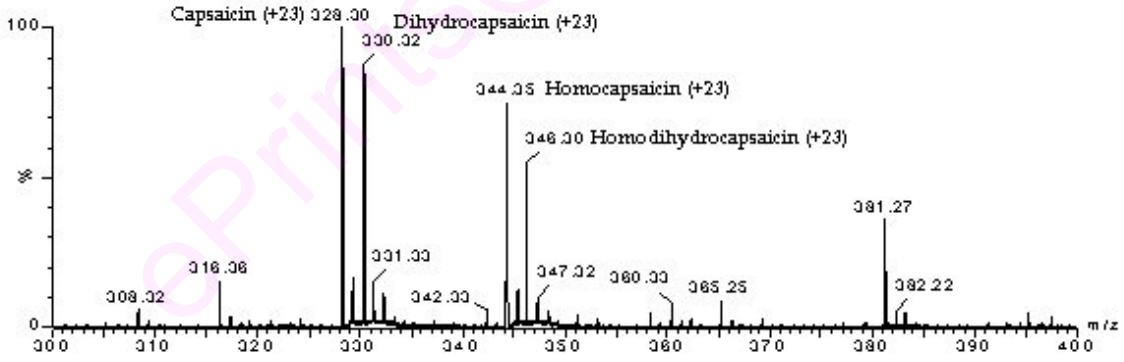


Figure 17 LCMS of the major capsaicinoids from the *Capsicum frutescens*

There was significant difference in the levels of major capsaicinoids among different categories of Capsicum varieties. The capsaicinoid content was highest in M-4 (102 ± 3.4 μgmg^{-1} and 39 ± 1.9 μgmg^{-1} of capsaicin and dihydrocapsaicin respectively) whereas the lowest was detected in Arka Abhir (9± 0.1 μgmg^{-1} and 1.0± 0.01 μgmg^{-1} of capsaicin and dihydrocapsaicin) (Figure18).

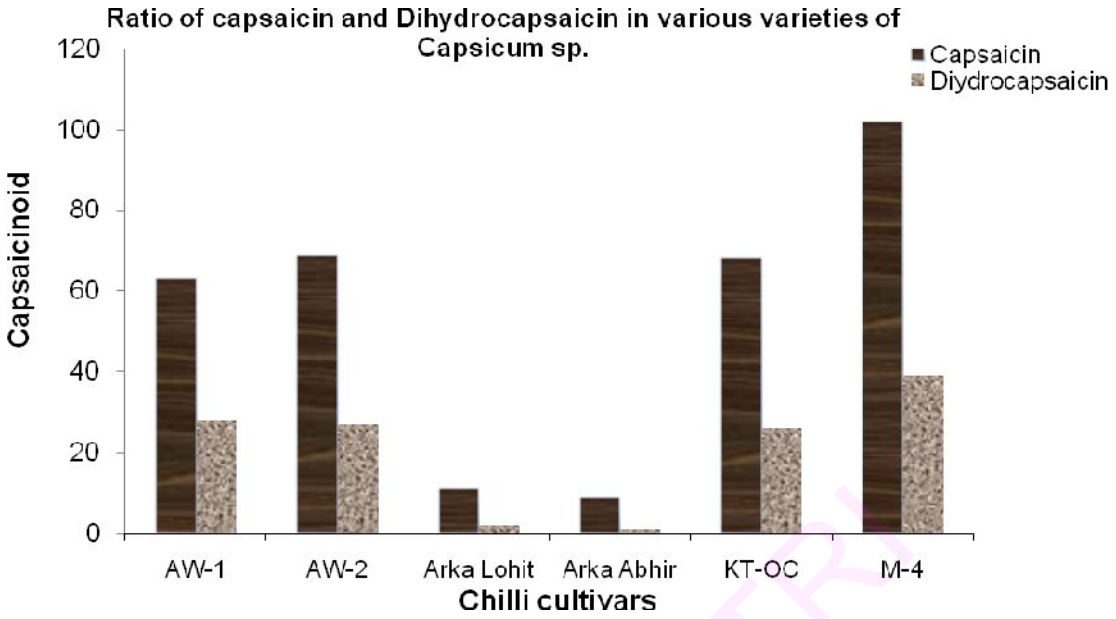


Figure 18 Ratio of Capsaicin and Dihydrocapsaicin in various varieties of *Capsicum* sp.

HPLC (Shimadzu Model LC-7A) separation of phenyl propanoid intermediates and capsaicinoids was done to estimate endogenous pools of metabolites in different genotypes of Capsicum. The mobile phase was a linear gradient of 0-100 % (v/v) acetonitrile in water, with pH 3.0 for 35 min, and 100% acetonitrile maintained till 37th minute. The detection was at 236nm and flow rate was maintained at 1ml min^{-1}. C-18 column (Shimadzu) of 250 x 4.6 mm and 5-µm diameter was used. The retention time of standard compounds was used to identify and quantify the capsaicinoids and phenyl propanoid intermediates in Capsicum fruits and cell suspension cultures, followed by the spiking of the samples.

3.2.2 Effect of elicitors on capsaicinoids in the fruits of *C. frutescens* Mill var. KT- OC and BOX- RUB

Fruits of 35 days old *Capsicum frutescens* Mill var. BOX-RUB plant (both control and elicited) after anthesis was used for the extraction of capsaicinoids. Capsaicinoids that showed immense variation in fruits of elicitor treated plants. Capsaicin content was two and half folds high in methyl jasmonate (MJ) at 2.5 µM treatment. While application of salicylic acid (SA) at 1.0 µM elicited maximum capsaicin by three folds while Ibuprofen (IB) showed moderate influence on capsaicin production. Vanillylamine levels were enhanced almost two and half fold under the influence of salicylic acid (1µM) and methyl jasmonate (2.5µM). Dihydrocapsaicin was enhanced two times in 1µM of salicylic acid (SA) treatment and similarly also at 2 µM of methyl jasmonate (Table 18).

Table 18 Effect of various abiotic elicitors treatment on various capsaicinoids and vanillylamine in *Capsicum frutescens* Mill var. BOX-RUB[a] .

Conc. (µM)	Capsaicin*			Dihydrocapsaicin *			Vanillylamine*		
	MJ	IB	SA	MJ	IB	SA	MJ	IB	SA
0	72.1 ±.06	72.1 ±.06	72.1 ±.06	68.7 ±.05	68.7 ±.05	68.7 ±.05	77.3 ±.05	77.3 ±.05	77.3 ±.05
1	108.7 ±.1	161.2 ±.5	213 ±.9	103.3 ±.1	93.4 ±.08	137.2 ±.3	172.6 ±.7	92.4 ±.09	92.8 ±.8
2.5	175.4±.7	194.5 ±.8	185.6 ±.7	121.4 ±.4	108.6 ±.1	101.2 ±.1	187.4 ±.8	114.3 ±.1	156.5 ±.5
5	124.1±.4	173.5 ±.7	163.6 ±.5	91.6 ±.08	105.3 ±.1	113.7 ±.2	140.9 ±.3	131.0 ±.2	141.8 ±.4

*µgmg^{-1} of the dry weight; [a] after 35 days of anthesis

Table 19 Effect of *Rhizopus oligosporus* treatment on various capsaicinoids and vanillylamine in the fruits of *Capsicum frutescens* Mill var.KT-OC.

Days (after anthesis)	Concentration (%w/v)	Vanillylamine*	Capsaicin*	Dihydrocapsaicin*
25	Control	44± 4.1	14 ± 0.8	6 ± 0.7
	1.0	117± 9.8	30 ± 1.5	11 ± 0.9
	2.5	128± 11.2	31 ± 2.7	12 ± 1.0
	5.0	134± 12.3	84 ± 5.6	16 ± 1.2
30	Control	57± 4.8	29 ± 2.4	9± 0.9
	1.0	131± 12.1	41 ± 4.2	20 ± 1.4
	2.5	139 ± 13.4	52 ± 4.6	25 ± 2.4
	5.0	144 ± 12.4	181 ± 15.1	32 ± 2.7
35	Control	59± 6.0	31 ± 3.0	14 ± 1.2
	1.0	142± 13.5	80 ± 7.4	28 ± 2.1
	2.5	154± 14.2	115 ± 12.4	35 ± 3.1
	5.0	243± 20.1	329 ± 24.5	56 ± 4.5
40	Control	54± 6.2	27 ± 2.1	12 ± 0.8
	1.0	123± 12.0	70 ± 6.5	20 ± 1.5
	2.5	114± 10.1	73 ± 7.1	28 ± 2.4
	5.0	166± 12.4	277 ± 21.5	44 ± 3.4

*$\mu g mg^{-1}$ of the dry weight

Table 20 Effect of *Aspergillus niger* treatment on various capsaicinoids and vanillylamine in the fruits of *Capsicum frutescens* Mill var.KT-OC.

Days (after anthesis)	Concentration (%w/v)	Vanillylamine*	Capsaicin*	Dihydrocapsaicin*
25	Control	47 ± 4.1	15 ± 0.9	5 ± 0.8
	1	49 ± 3.4	17 ± 1.8	5 ± 0.8
	2.5	63 ± 4.5	18 ± 1.2	6 ± 0.5
	5	42 ± 2.9	16 ±1.4	7 ± 0.8
30	Control	59 ± 4.8	30 ± 2.4	10 ± 0.9
	1	61 ± 5.4	41 ± 3.5	13 ±1.0
	2.5	96 ± 6.9	48 ± 3.6	14 ± 0.9
	5	58 ± 5.1	48 ± 6.8	13 ± 0.7
35	Control	61 ± 6.0	31 ± 3.0	14 ± 1.2
	1	111 ± 10.8	36 ± 3.8	15 ± 1.2
	2.5	107 ± 10.2	51 ± 4.1	19 ± 1.3
	5	73 ± 7.1	35 ± 2.9	12 ± 1.0
40	Control	56 ± 6.2	27 ± 2.1	12 ± 0.8
	1	78 ± 6.8	33 ± 2.4	15 ± 1.2
	2.5	98 ± 5.8	42 ± 3.2	18 ± 1.4
	5	61 ± 5.1	28 ± 3.8	11 ± 1.3

*$\mu g mg^{-1}$ of the dry weight

Maximum elicitation of capsaicin (329 ± 24.5 µmoles) was observed at 35 days after anthesis when *R. oligosporus elicitor* was sprayed at the concentration of 5% w/v to the flowers of *C. frutescens* (Table 19) whereas *A. niger* treatment enhanced phenyl propanoid intermediates and capsaicin to the extent of 51 ± 4.1 µmoles when sprayed at the concentration of 2.5% w/v (Table 20). Among the abiotic elicitors, maximum elicitation of phenyl propanoid intermediates and Capsaicin (49 ± 4.5 µmoles) was observed at 35 days after anthesis when Methyl Jasmonate was sprayed at the concentration of 5.0 µM to the flowers of *C. frutescens* (Table 21). In the salicylic acid sprayed plants, capsaicin level enhanced to 44 ± 1.1 µmoles at the concentration of 2.5 µM (Table 22).

Table 21 Effect of Methyl Jasmonate treatment on various capsaicinoids and vanillylamine in the fruits of *Capsicum frutescens* Mill var.KT-OC.

Days (after anthesis)	Concentration (%w/v)	Vanillylamine*	Capsaicin*	Dihydrocapsaicin*
25	Control	46± 4.1	14 ± 0.8	6 ± 0.7
	1.0	50 ± 5.2	19 ±1.2	7 ± 0.8
	2.5	52 ± 4.8	21 ± 1.1	7.2 ± 0.9
	5.0	57± 6.2	24 ± 1.8	8.9 ± 0.8
30	Control	57± 4.8	30 ± 2.4	10 ± 0.9
	1.0	63± 11.0	32 ±2.9.	11.8 ± 1.2
	2.5	69± 11.2	37 ± 3.4	12.8± 1.4
	5.0	84 ± 8.9	41 ± 3.7	14.2± 1.2
35	Control	61 ± 6.0	31 ± 3.0	14 ± 1.2
	1.0	63± 5.9	35 ± 3.1	18.2± 1.8
	2.5	68 ± 8.2	42 ± 3.4	21 ± 1.9
	5.0	98± 7.1	49 ± 4.5	26 ± 1.4
40	Control	57± 6.2	27 ± 2.1	12 ± 0.8
	1.0	61± 8.4	32 ± 3.2	14 ± 1.2
	2.5	69± 6.8	34 ± 3.8	18 ± 1.4
	5.0	89± 8.3	39 ± 3.7	24 ± 2.1

*µgmg^{-1} of the dry weight

Table 22 Effect of Salicylic acid treatment on various capsaicinoids and vanillylamine in the fruits of *Capsicum frutescens* Mill var.KT-OC.

Days (after anthesis)	Concentration (%w/v)	Vanillylamine*	Capsaicin*	Dihydrocapsaicin*
25	Control	47 ± 4.1	15 ± 0.9	5 ± 0.8
	1	48 ± 1.4	16 ± 08	5 ± 0.7
	2.5	49 ± 1.8	17 ± 1.1	5.5 ± 0.6
	5	47 ± 2.1	16 ±1.1	6± 0.7
30	Control	59 ± 4.8	30 ± 2.4	10 ± 0.9
	1	60 ± 1.9	35 ± 2.4	11 ±1.1
	2.5	67 ± 2.1	44 ± 1.1	12± 0.1
	5	65± 4.2	42 ± 2.8	10 ± 0.6
35	Control	61 ± 6.0	31 ± 3.0	14 ± 1.2
	1	68 ± 1.9	38 ± 2.8	15 ± 1.0
	2.5	77 ± 2.8	39 ± 2.8	16 ± 1.0
	5	73 ± 3.6	36 ± 2.1	15 ± 0.9
40	Control	56 ± 6.2	27 ± 2.1	12 ± 0.8
	1	64 ±5.8	31 ± 2.1	14 ± 1.0
	2.5	68 ± 4.8	34 ±2.8	15 ± 1.0
	5	65 ± 6.1	38 ± 3.4	14 ± 1.1

*µgmg^{-1} of the dry weight

3.3 Analysis of carotenoid profile in the fruits of Capsicum

Analysis of two major carotenoids (Capsanthin and Capsorubin) was done. The extracts from the matured fruits were subjected to mass spectroscopy LCMS (Figure 19) and thin layer chromatography (Figure 21). *Rf* value was calculated and it was in congruence with the earlier reports of Vinkler and Richter (1972) HPLC profiles (Figure 20) of various compounds revealed the involvement in carotenoid biosynthetic pathway in Capsicum.

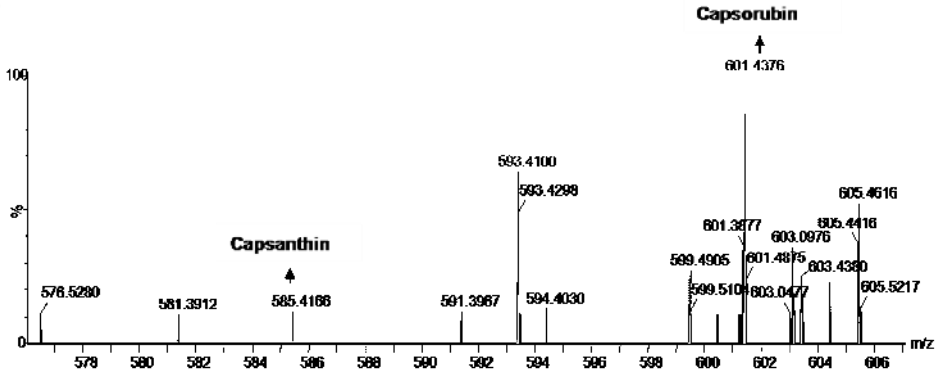
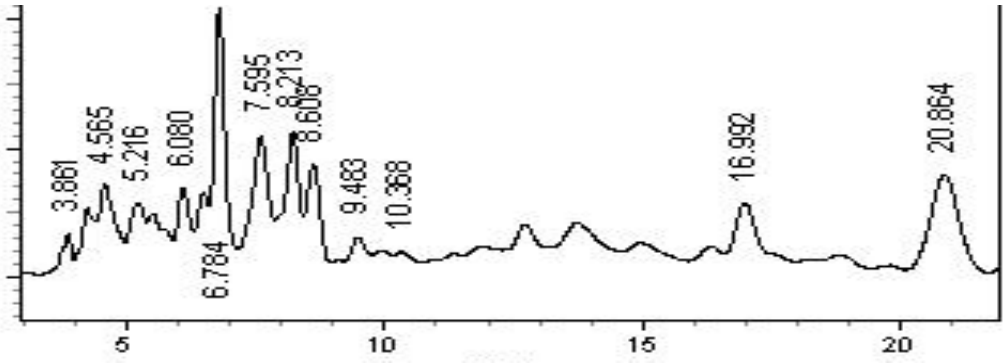
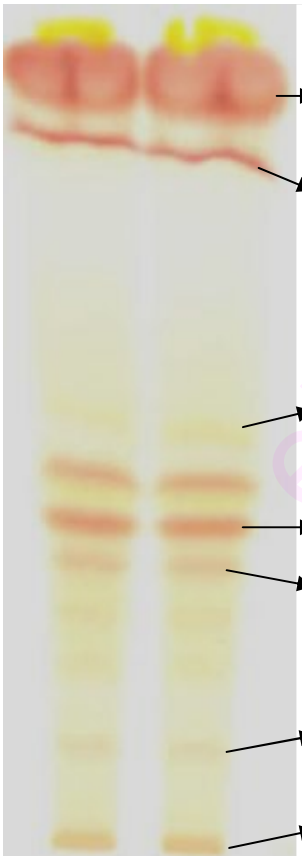


Figure 19 LCMS of the major carotenoids from the cultivar of *Capsicum* sp.



Retention time	Area	Area %	Component
6.784	397849	20.41	Capsorubin
7.595	367588	18.86	Capsanthin
8.213	370444	19.01	Zeaxanthin
8.608	246568	12.65	Violaxanthin
20.864	566415	29.06	β-Carotene

Figure 20 HPLC profile of carotenoids from matured fruits of Capsicum with their retention time.



Component	Solvent system	*Rf* value
Capsanthin	1	0.08
	2	0.37
	3	0.29
Capsanthin ester I	1	0.39
Violaxanthin	1	0.34
Violaxanthin	3	0.34
Capsorubin	1	0.23
	2	0.21
	3	0.31
Cryptoxanthin	1	0.71
Cryptoxanthin	3	0.85
β-Carotene	1	0.95
Zeaxanthin	1	0.46
Zeaxanthin	2	0.69

Solvent system 1: Benzene, Petroleum ether, Acetone, Acetic Acid (10:90:15:5)

Solvent system2: Hexane, Ethyl acetate, Acetone, Ethanol (80:10:7.5:2.5)

Solvent system 3: Acetonitrile, 2-propanol, Ethyl Acetate (85:7.5:7.5)

Figure 21 TLC and *Rf* value of various carotenoids with the respective solvent system from the colored fruits of *Capsicum* sp.

Table 23 Color values of matured fruits of Capsicum varieties.

VARIETY	L	a	b	DE
Pusa Jwala	48.09	25.50	33.41	59.96
G-4	50.54	20.15	35.03	56.97
BOX-RUB	20.03	37.61	13.81	81.63
KT-OC	16.70	37.41	11.53	84.10
Arka Abhir	37.98	19.71	25.65	61.93
Arka Lohit	52.94	11.22	34.33	52.07

The color of the extract (methanol) of Capsicum varieties was measured in terms of 4 parameters namely Hunter 'L', 'a', 'b' values and total color difference 'DE'. The L value of the sample representing the lightness of the samples changed significantly (Table 23). Positive values for 'a' indicates the redness of the sample and the negative value indicates greeness of the sample. Positive values for 'b' indicate yellowness of the sample while negative value indicates blueness of the sample. The 'b' value also showed considerable difference in yellowness; however, the total color difference 'DE' was variable among these cultivars. Maximum color was detected in methanol extract of *C. frurescens* and minimum was in *C. annuum* variety. The analysis of carotenoid profiles of KT-OC variety showed that capsanthin and capsorubin were maximum along with β- carotene as the other major component in ripened pepper fruits.

In the elicitation studies with abiotic elicitors, capsanthin enhanced five fold with the methyl jasmonate (1μM) and three fold increase in 2.5μM of methyl jasmonate. Influence of Salicylic acid at 2.5 μM enhanced capsaicin level by three folds. Ibuprofen at 1μM responded in six fold increase. Capsorubin has enhancement of five folds with the influence of 5μM of salicylic acid and four folds increase with methyl jasmonate and ibuprofen at 2.5μM respectively (Figure 22). Application of all the three abiotic elicitors used in this experiment showed an impact on total carotenoid accumulation, *viz.* capsanthin and capsorubin. The effect of salicylic acid (5μM) on accumulation of capsanthin was very clear as a 12 fold increase in its levels was observed.

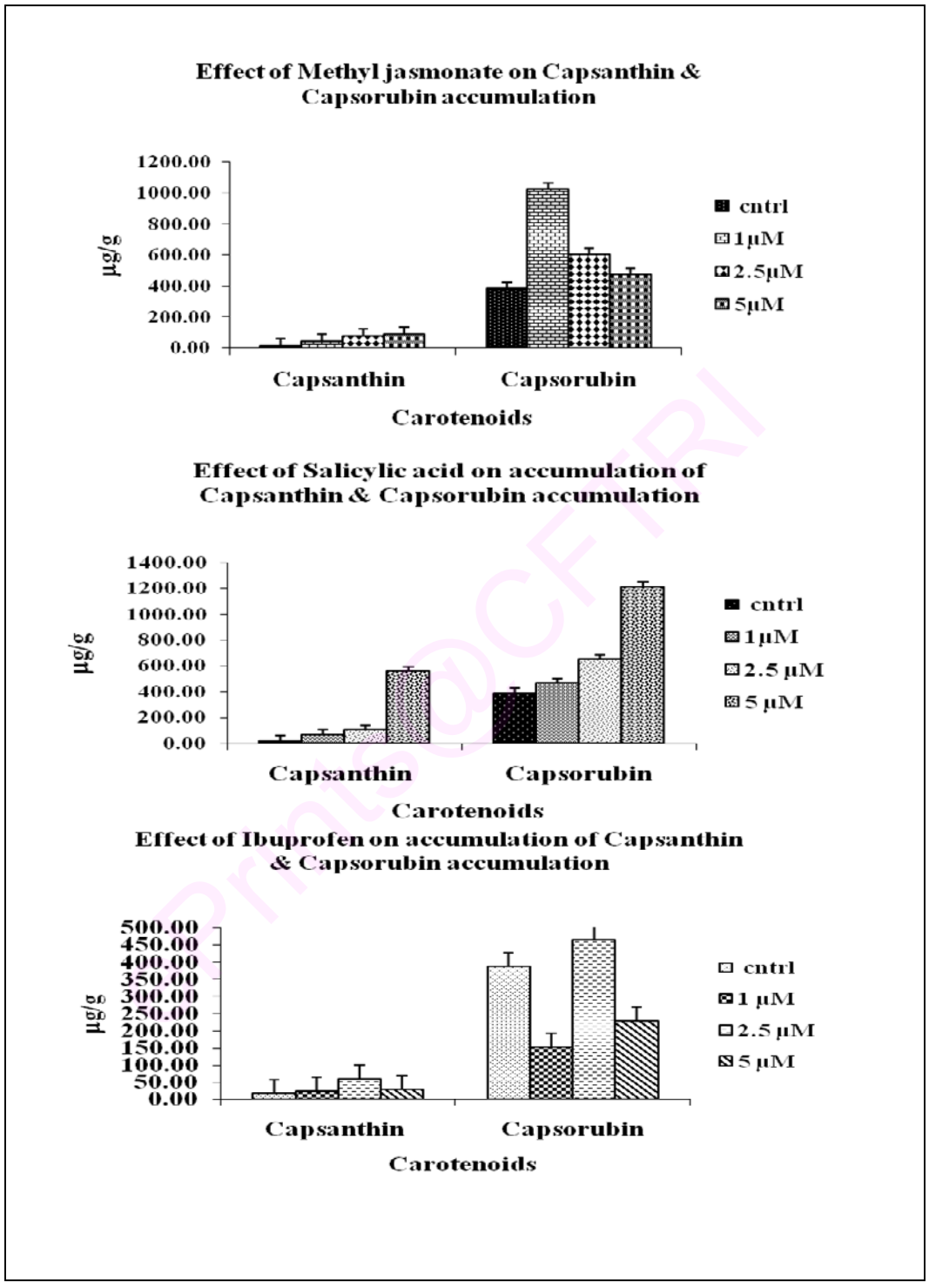


Figure 22 Effect of various abiotic elicitors on the accumulation of capsanthin and capsorubin after 45 days of anthesis in *C. frutescens* sp.

The application of elicitors to enhance secondary metabolite production in plants is like mimicking the production of these compounds naturally in the presence pathogen infection. Plants usually develop a defensive response activated sequentially in a complex multi component network on pathogen attack. These usually include the production of secondary metabolite such as pheonolics. The overproduction of secondary metabolite in *Capsicum* sp is already well studied using *in-vitro* models (Johnson et al., 1991; Johnson and Ravishankar, 1996; Rao and Ravishankar, 2000). These studies usually concentrated on phenyl propanoid components of the capsaicinoid biosynthesis pathway. Capsaicin the major alkaloid of the *Capsicum* sp. is been the product of interest as it is of high economic value in food and pharmaceutical industries. The use of abiotic and biotic elicitors for the production of high capsaicinoid content using the cell suspension cultures is well documented. Prasad et al (2006), has already showed the effect of abiotic and biotic elicitor when sprayed on field grown plants on flowering produced enhanced levels of phenyl propanoid compounds such as Ferulic acid, vanillylamine and capsaicinoids. The same group also developed a spray formulation for pungency enhancement. The current study is an extension of our earlier work on the use of elicitors for the production of economically important secondary metabolites with color component of Capsicum fruit.

Plant defence can be triggered by local recognition of pathogens but, more effective responses include systemic signalling pathways (Conrath et al., 2002). Two of the most important compounds having this ability are salicylic acid (SA) and jasmonic acid (JA). Systemic responses include those dependent on SA signalling and are named Systemic Acquired Resistance (Dempsey et al., 1999). The Induced Systemic Resistance is known to be dependent on JA (Feys and Parker, 2000). SA, JA and its derivatives like MeJ have been used as inducers in plants and were found to stimulate their secondary metabolism (Hahlbrock et al., 2003; Thomma et al., 2000). For this reason we evaluated the possible effects of those molecules on the color composition of Capsicum fruits.

The ability of jasmonate to boost plant defences against fungal pathogens has already been reported (Thomma et al., 2000). The mechanism of action of SA and MeJ (or more general, jasmonates) is still a mater of debate (Felton and Korth, 2000). These two compounds seem to act independently via antagonistic pathways giving rise to different plant responses. Nevertheless, a clear dichotomy does not always exist. In our case, both SA and MeJ were able to induce the priming of the carotenoid accumulation in Capsicum cells, as a response to the abiotic elicitation, at different levels.

3.4 Transformation studies

Genetic transformation is a pre-requisite for crop improvement in a more direct manner. Capsicum being a commercially important crop there is a demand for improvement from economic and utility point of view. Transformation method in *Capsicum* sps has not been successful and isolated reports are also not reproducible due to genetic specificity. Hence various methods of transformation were tried viz. callus, pollen, shoot tip and floral dip.

3.4.1 Callus Induction

The earliest signs of callus formation from cotyledonary leaves as well as from hypocotyls were observed within two weeks in callus induction medium containing half strength MS basal salts with 5 mg l^{-1} BAP, 2 mg l^{-1} 2,4-D and 0.5 mg l^{-1} Kin, producing white to yellow callus (Figure 23). Callus formation was observed in cotyledonary leaf explants in all the cultivars. Maximum response for callus induction i.e. 68% was obtained in medium comprising 2, 4-D and Kinetin (Table 24).

Table 24 Callus initiation in *Capsicum frutescens* var. KT- OC.

*Hormonal treatment			% Explants showing callus initiation
2,4-D mgl^{-1}	BAP mgl^{-1}	Kin mgl^{-1}	
0.5	0.0	2	68
1	0.5	2	36
0	0.5	0	-

*Media: ½ MS salts and B5 vitamins. Data recorded on 15th day of culture.

The callus was grown in callus multiplication medium and sub-cultured on the 25th day. Rapid multiplication of calli obtained in MS medium with 2, 4-D 2 mgl^{-1} and BA 4 mgl^{-1} .

Figure 23 Callus induction and proliferation in *Capsicum frutescens* var. KT-OC in 2,4- D and Kinetin media

3.4.2 Sensitivity tests for selection of transformed tissue

Regeneration was not observed in medium containing 3-50 mg l^{-1} hygromycin. The minimum regeneration inhibition concentration was determined to be 5 mg l^{-1}. However in all the transformation experiments, up to 20 mg l^{-1} hygromycin was chosen as the ideal level for the successful selection of the transformants because it prevents regeneration and also kills the untransformed tissues (Table 25) (Figure 24). Tissue browning was observed under higher concentration of hygromycin. Transfer of transformed tissues to different medium with increasing concentration of hygromycin in the medium gradually gives enough time for the transformed cells to survive and regenerate.

Table 25 Determination of minimum inhibitory concentration of hygromycin for selection of transgenic explants of *Capsicum frutescens* var. KT- OC.

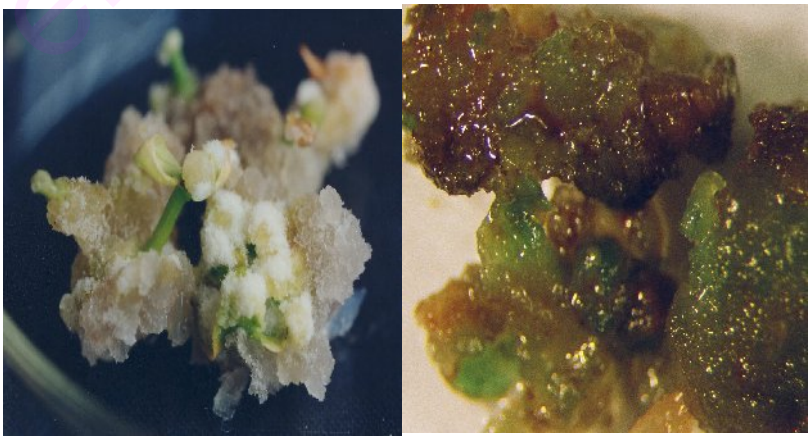
Medium* with hygromycin (mg l^{-1})	Hypocotyl	leaf	Nodal explant	Shoot tip
	2 weeks	2 weeks	2 weeks	2 weeks
Control	0	0	0	0
5	100	0	0	60
10	100	100	40	75
20	100	100	90	95
25	100	100	100	100
40	100	100	100	100

*Half strength MS salts + BA 0.25 mgl^{-1}+ IAA 0.5 mgl^{-1}+ B5 vitamins + 3% sucrose

Figure 24 Minimum inhibitory concentration for leaf sensitivity test

3.4.3 Callus transformation

Leaf explants, approximately 2-3 mm in diameter, were incubated in bacterial suspension for *Agrobacterium* mediated gene transformation for 15-20 min. *Agrobacterium tumefaciens* strain EHA 101 was used bearing pCAMBIA 1305.2 vector with hygromycin as selection marker and β-glucuronidase (GUS) as a reporter gene harboring catalase intron and GRP. The explant was blotted dry and co-cultured on co-cultivation medium comprising MS basal medium with acetosyringone for 48 h. The co-cultivated leaf explants were then selected for 4 weeks on the selection medium, ie. MS media supplemented with 2, 4-D (2 mg l^{-1}) & Kinetin (0.5 mg l^{-1}) with antibiotics and 20 mg l^{-1} hygromycin. The putative transgenic callus obtained from leaf explants was continuously sub-cultured after two months on callus induction media without any antibiotics and finally Gus assay confirmed the transformant calli (Figure 25) which was further confirmed by PCR using *hpt* II and GUS gene (Figure 26).



Control **Gus transformed**

Figure 25 GUS activity shown by the callus of *Capsicum frutescens* var KT OC.

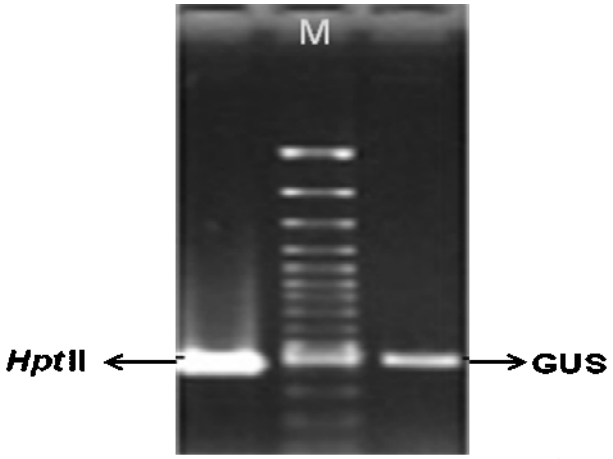


Figure 26 PCR of the transgenic callus using GUS and *hpt II* primers

3.4.4 Pollen transformation

Six hrs of co-cultivation treatment of pollengrain showed 18% transformation frequency (Figure 27) whereas, 12 hr co-cultivation treatment resulted in 4% transformation efficiency indicating that prolonged co-cultivation of anthers leads to decreased transformation efficiency probably due to non viable nature. Transient GUS expression was observed in anthers inoculated with *Agrobacterium tumefaciens* (Figure 28). No GUS expression was noticed in anthers co-cultivated for less than 4 h.

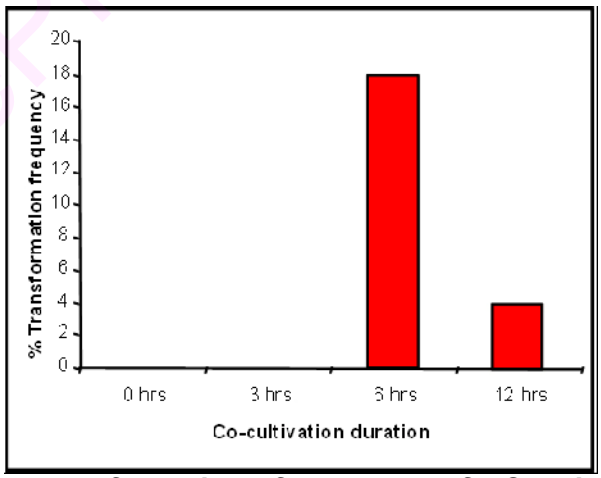


Figure 27 Percentage transformation frequency of *Capsicum frutescens* pollen cocultivated with *Agrobacterium tumefaciens.* Maximum transformation frequency observed in 6 h co-cultivation treatment.

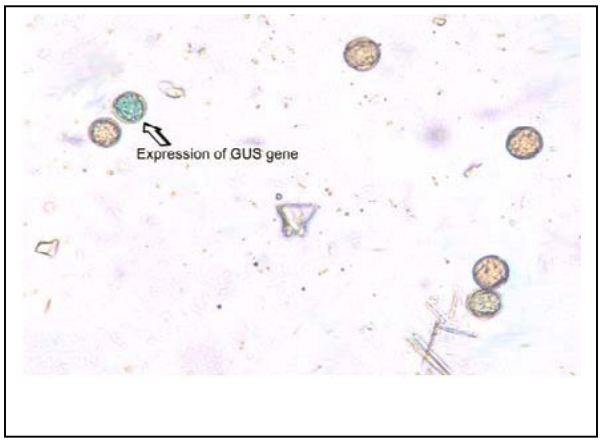


Figure 28 Expression of intron GUS gene observed in *Capsicum frutescens* pollen cocultivated with *Agrobacterium tumefaciens*

3.4.5 Transformation of competent *E. coli* cells

Transformed colonies of *E. coli* strain DH5α was obtained under kanamycin selection. Isolation of plasmid and agarose gel electrophoresis revealed the presence of 12 kb pCAMBIA 1305.2 plasmid in kanamycin resistant colonies (Figure 29)

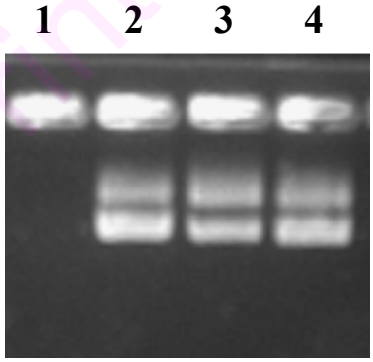


Figure 29 Isolation of plasmid pCAMBIA 1305.2 from *E. coli* strain DH5α and agarose gel electrophoresis

Lanes: 1- Control kanamycin sensitive colonies, which do not receive the plasmid.

2 to 4 – Kanamycin resistant transforments showing 12 kb pCAMBIA 1305.2 vector.

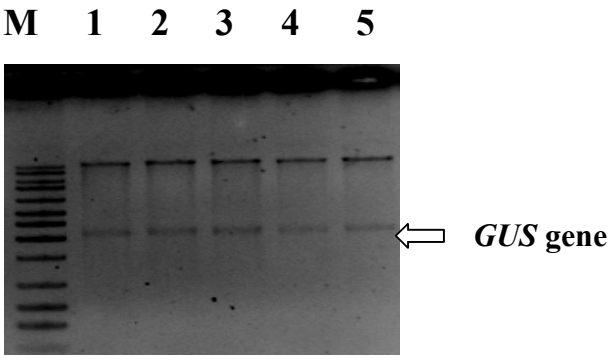


Figure 30 PCR amplification of GUS gene from *A. tumefaciens* 1305.2.

Lanes: M- 100bp marker

1 to 5- DNA from *A. tumefaciens* transformed with binary vector pCAMBIA1305.2

3.4.6 Southern analysis

Though diverse species of bacteria are capable of gene transfer to plants (Broothaerts et al., 2005), *Agrobacterium* sp are widely used for genetic modification of plants. Manners and Way (1989) demonstrated the production of normal plants that contain T-DNA of the binary vector; devoid of T-DNA from the native *A. tumefaciens* mediated transformation of *Stylosanthes humilis*. Integrations occurring at independent loci, segregating through meiosis has been demonstrated in *Agrobacterium* mediated transformation (De Framond et al., 1986).

Southern analysis further confirmed the transgenic nature and stable integration of T-DNA in hygromycin resistant plantlets. The presence of several fragments of variable size in some lines indicates insertion of multiple copies of the T-DNA into the plant genome (Figure 30). The genomic DNA was digested with *Pml* I and *Bgl* II enzymes. *Pml I* cuts once inside the *hpt* II gene and the probe and *Bgl* II cuts just outside the T-DNA left border. No signals were observed in lanes containing untransformed DNA. Plasmid and genomic DNA was digested with *Pml* I and *Bgl* II, separated in agarose gel, transferred to nylon membrane and probed with *hpt* II coding regions (Figure 31)

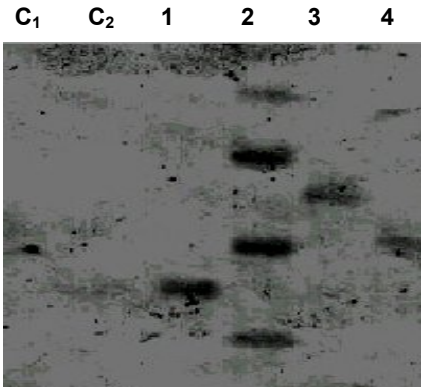


Figure 31 Southern blot analysis of PCR positive transformed plants

C_1 and C_2 untransformed *Capsicum* sp. plants.

1 to 4- DNA from PCR positive *Capsicum* sp T_0 transformants.

3.4.7 *In planta Agrobacterium* mediated transformation studies in *Capsicum frutescens* var. KT-OC Mill. by floral dip method

The most common methods for introduction of DNA into plant cells is use of *Agrobacterium tumefaciens* or rapidly propelled tungsten microprojectiles that have been coated with DNA (Birch, 1997; Hansen and Wright, 1999). Other methods such as electroporation, microinjection, or delivery by virus have also been exploited. For many important species, however, pursuit of the above strategies would be greatly facilitated by the availability of high-through put/non-tissue culture transformation methods. The generation of genetically homogeneous plants carrying the same transformation event in all faces has typically presented two separate hurdles of transformation and regeneration of intact, reproductively competent plants from those transformed cells (Birch, 1997; Hansen and Wright, 1999). Genetic transformation can be transient or stable, and transformed cells may or may not give rise to gametes that pass genetic material on to subsequent generations. Transformation of protoplasts, callus culture cells, or other isolated plant cells is usually straightforward and can be used for short-term studies of gene function (Gelvin and Schilperoort, 1998).

Transformation of leaf mesophyll cells or other cells within intact plants may in some cases broaden the utility of single-cell assays (Tang et al., 1996). Exciting new approaches such as virus-induced gene silencing may also be applicable for some studies (Baulcombe, 1999). However, in many cases it is desirable or necessary to produce a uniformly transformed plant that carries the transgene in the nuclear genome as a single Mendelian locus. Although many successful plant regeneration methods have been developed, these methods often require a great deal of protocol refinement and the focused effort of expert practitioners. It is unfortunate that plant regeneration from single transformed cells often produces mutations ranging from single base changes or small rearrangements to the loss of entire chromosomes (Phillips et al., 1994). It is often necessary to generate and screen a dozen or more independent plant lines transformed with the same construct to find lines that have suffered minimal genetic damage and that carry a simple insertion event (Birch, 1997; Hansen and Wright, 1999). Transformation is feasible in many plant species, but has required acceptance of the above limitations.

Early stages of the revolution that transformed Arabidopsis transformation were carried out by Ken Feldmann and David Marks (1987). They applied *Agrobacterium* to Arabidopsis seeds, grew plants to maturity in the absence of any selection, then collected progeny seeds and germinated them on antibiotic containing media to identify transformed plants (Feldmann and Marks, 1987; Feldmann, 1992). The procedure was difficult to reproduce consistently. Arabidopsis researchers in the mid-1990s focused on empirical transformation protocol improvement and concluded that (a) Plants did not need to be uprooted, treated with *Agrobacterium*, and re-planted as it was followed earlier. Transformants could be obtained by treating only the protruding inflorescences; (b) inclusion of Silwet L-77, a strong surfactant that shows relatively low toxicity to plants, often enhanced transformation reliability; and (c) many different *Arabidopsis* ecotypes were transformable and many different *Agrobacterium* strains could be used, although notable differences in efficiency were observed. In numerous plant transformation systems, the choice of host genotype and/or *Agrobacterium* genotype has been

an important parameter (Birch, 1997). A better understanding of T-DNA transfer and other aspects of *Agrobacterium*/plant interactions (Hooykaas and Schilperoort, 1992; Mysore et al., 2000) may also allow engineering of better host/ bacteria combinations. Other substantially different transformation methods also must be kept in mind (Chowrira et al., 1995; Chen et al., 1998). *Agrobacterium* floral transformation procedures have been a tremendous success with Arabidopsis; such successes, along with the recent information about the targets for Arabidopsis transformation, should inspire a renewal of efforts to adapt these methods to the transformation of other plant species. The benefits are clear: transformation without tissue culture can provide a high throughput method that requires minimal labor, expense, and expertise. Rates of unintended mutagenesis are reduced. More important, simplified transformation protocols facilitate positional cloning, insertional mutagenesis, and other transformation-intensive procedures, reducing the effort required to test any given DNA construct within plants. In the present study we have reported *in planta* transformation by mode of floral dip following the protocol of Clough and Bent, (1998) for Arabidopsis.

Agrobacterium tumefaciens-mediated transformation has been one of the methods used to generate transgenic plants in bell pepper. An alternate transformation method that avoids/minimizes tissue culture would be beneficial for the improvement of bell pepper due to its recalcitrant nature. In this report, transgenic bell pepper plants have been developed by a tissue-culture-independent *A. tumefaciens*-mediated *in planta* transformation procedure.

In the present study, *Capsicum frutescens* Mill. Var KT-OC was used for transformation. *Agrobacterium* strain EHA105 harboring the binary vector pCAMBIA1305.2 that carries the genes for β-glucuronidase (*uid* A) and hygromycin phosphotransferase II (*hpt* II) was used for transformation. GUS histochemical analysis of T_0 and T_1 plants at various stages of growth followed by molecular analysis using PCR

The vector pCAMBIA 1305.2 was introduced into *A. tumefaciens* strain EHA105 by the liquid nitrogen freezing thaw method. For in planta transformation, a single colony of the bacteria

was inoculated in YEB medium supplemented with kanamycin (50 mg l^{-1}) and rifampicillin (50 mg l^{-1}) and cultured overnight at 28^0C. The *Agrobacterium* cells were collected by centrifugation at 10,000g for 30 s at 25^0C and then resuspended in 20 ml (OD_{600} = 0.4–0.5) of liquid MS medium containing 20 mg l^{-1} acetosyringone (AS).

A protocol was developed for the in planta transformation of chilli (*Capsicum frutescens* var. KT-OC). Overnight grown *Agrobacterium tumefaciens* culture was centrifuged at 6000 rpm for 10 minutes and pellet was dissolved in 5% (w/v) sucrose solution containing 0.02% Triton-X-100 and 0.1% Silwet L-77 (Lallah seeds USA). The inoculum was used on unopened flower, partially and fully opened flower in fully grown plant. *β- Glucuronidase* (GUS) histochemical assay showed gene expression in the leaves of 10% of plants when treated with culture containing 0.02% Triton-X-100. *In-vitro* seed germination (T_0 generation) of *C. frutescens* (AW-1) was obtained on MS basal medium**.** GUS-histochemical staining was done for germinated seedlings (Figure 32) which was further confirmed by PCR (Figure 33). About 75% transformation efficiency was obtained. The seeds from the matured fruit were kept for germination but germination percentage was very less. After treatment with 0.2% H_2O_2, germination percentage was 90-95% (Table 26, Figure 34).

Table 26 *In-vitro* germination of putative transgenic *Capsicum frutescens* Mill. seeds

Treatment (H_2O_2)	**Germination percentage**
0.1%	Nil
0.2%	90-95 %
0.3%	Nil
Without H_2O_2	5-10%

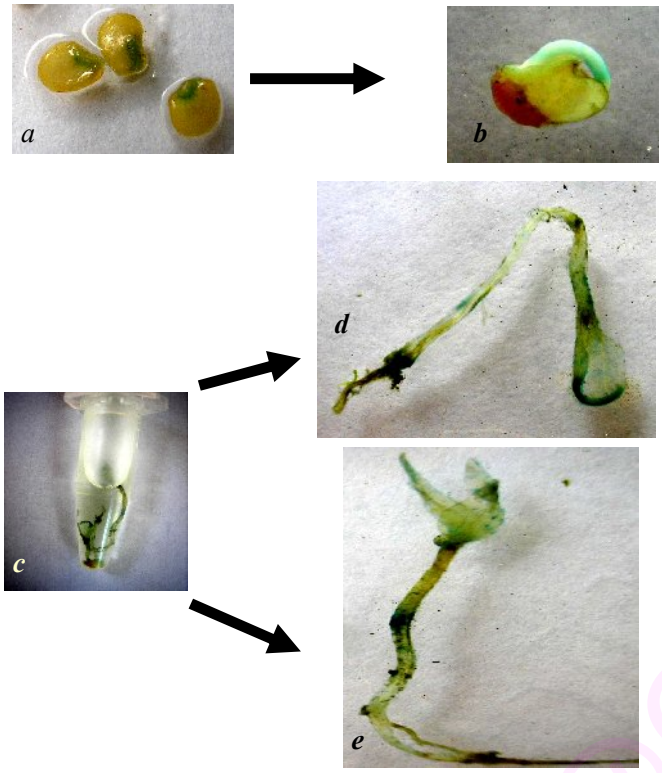


Figure 32 GUS expression through staining of *Capsicum frutescens* var KT-OC transgenic (T_0) seedlings.

a) Transgenic seed.
b) Germinating seed.
c) Transgenic seedling in tube.
d) & e) GUS treated transgenic seedling.

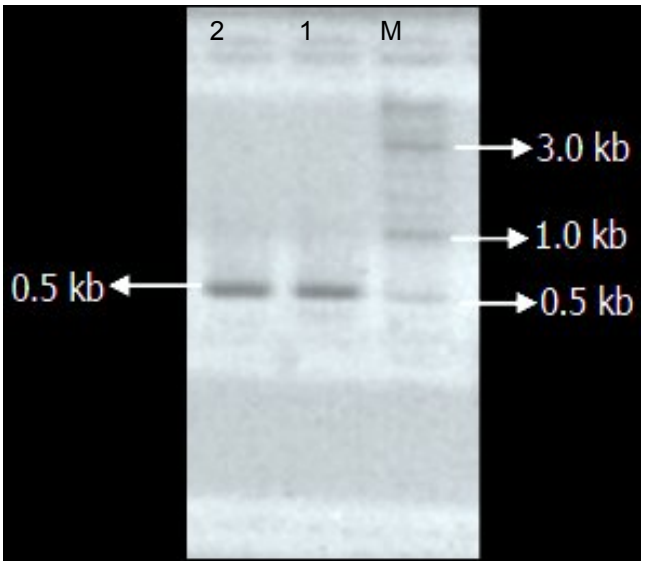


Figure 33 PCR amplification of GUS gene in transformed plant
M= Marker, 1&2= plants having GUS construct

Figure 34 Germination of transgenic seeds of *C. frutescens* var. KT-OC in green house
A) Two day old transgenic plant after hardening.
B) Fifteen days old transgenic plant.
C) Transgenic plant with flowers and fruits.

3.5 Expression analysis by RT-PCR

RT-PCR shows us whether or not a specific gene is being expressed in a sample. If a gene is expressed, its mRNA product will be produced and it also quantifies exactly how active the gene is in the sample. To do this, RT-PCR is performed with the unknown mRNA alongside standardized samples with known mRNA amounts. This approach is used to identify how much mRNA is being produced by the gene. In the present study it is performed to analyze the expression of genes during the ontogeny of carotenogenesis under the influence of elicitors.

Salicylic acid and methyl jasmonate elicited plants were selected for carotenogenic genes expression studies. The expression levels of genes associated with general carotenogenesis in elicited and controlled plant were quantified by reverse transcriptase polymerase chain reaction (RT-PCR) and compared. This genes included lycopene cyclase (*Lcy*, which converts lycopene to β-carotene), the transcript levels of this enzyme was analysed for 30 day.40 day, 45 day and 50 day old fruit. The transcript level of this gene was found to be highest in matured fruit than to

partially red or green fruits. The gene specific primers of *Lcy* were used to study developmental expression. During the ontogeny of the fruit, *Lcy* levels were increased progressively. Increase in expression of *Lcy* correlated with color levels intensity increase. The maximum transcripts of *Lcy* were observed during 45-50 day old fruit (Figure 35). The mRNA transcript abundance of this gene was found significant with different stages of fruit development.

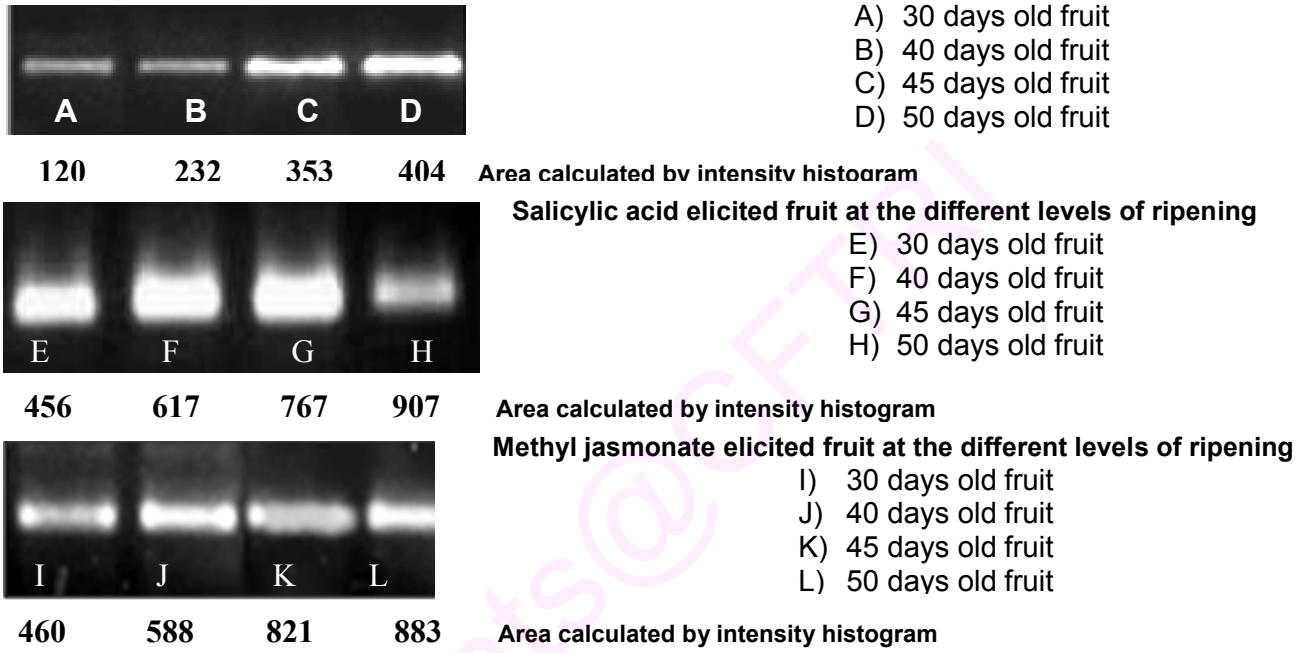


Figure 35 RT-PCR representing mRNA transcriptional abundance of *Lcy-e* gene during the ontogeny of the high pungent Capsicum *frutescens* var. KT-OC fruits

RT-PCR revealed the expression of *Lcy* gene with the ontogeny of fruit from 30th day to 50th day. In elicited fruits (salicylic acid) there was 2.25 fold increases with respect to control at the 50th day, whereas it was 2 fold increase in methyl jasmonate treated fruit. Expression was least in green fruits and it has enhanced during ontogeny and was maximum at maturity.

The control of gene expression at the transcriptional level is a key regulatory mechanism; one or more post-transcriptional control points must be decisive in the regulation of carotenogenesis (Fambrini et al., 2004). There are several studies conducted on the change and the biosynthesis of carotenoid compounds during pepper fruit ripening. Three main factors

contributing to the red color of ripe Capsicum are; A) Disappearance of lutein and neoxanthin that exist in the chloroplast, B) The rise of concentrations of β-carotene, antheraxanthin, violaxanthin C) *de-novo* biosynthesis of zeaxanthin, capsanthin, α-cryptoxanthin, curcubitaxanthin A, capsanthin-5,6-epoxide, and capsorubin (Hornero-Mendez, D et al., 2000).

In capsicum fruit, many ripening-related genes have been characterized especially the carotenoid biosynthesis pathway genes including *phytoene desaturase* (*Pds*) (Hugueney et al., 1992), *ζ-carotene desaturase* (*Zds*) (Breitenbach et al., 1999), *lycopene β-cyclase* (*Lcy*) (Hugueney et al., 1995), *zeaxanthin epoxidase* (*Zepd*) (Bouvier et al., 1996) and *Capsanthin-capsorubin synthase* (*Ccs*) (Bouvier et al., 1994). Although the expression of these genes during Capsicum fruit ripening on the plant has been investigated, there is little information available on their expression in harvested fruit. The biosynthesis and accumulation of several different carotenoids have been previously reported to be promoted by C_2H_4: phytoene synthase-1 and phytoene desaturase genes in apricot fruit (Marty et al., 2005); phytoene synthase, ζcarotene desaturase and β-carotene hydroxylase genes in citrus fruit (Rodrigo and Zacarias, 2007). However, there are very scanty reports on the accumulation of capsanthin and capsorubin, two chromoplast-specific carotenoids of the Capsicum genus. The expression profiles characterised in this study indicate the greater role for *Lycopene-β-cyclase* (*Lcy*) during ripening of Capsicum fruit. β-carotene exists in the chloroplast of young green Capsicum fruit as a light- harvesting pigment (Taiz and Zeiger, 2006). β-carotene is synthesized from lycopene by the action of *lycopene-β-cyclase* which is encoded by *Lcy*, a gene constitutively expressed during fruit development (Hugueney et al., 1995).

Lcy has also been found not to be up-regulated during ripening of Capsicum (Hugueney et al., 1995), tomato (Pecker et al., 1996) and papaya (Skelton et al., 2006). *Lcy*, therefore, may be highly up-regulated during fruit growth to massively synthesize β-carotene for the accumulation of other carotenoids later on. When paprika fruit start to ripen, not only did the level

of β-carotene increase, but other carotenoids which require β-carotene as a precursor also increases such as antheraxanthin, violaxanthin, capsanthin, capsorubin were *de-novo* synthesised (Minguez-Mosquera and Hornero-Mendez, 1994; Hornero-Mendez et al., 2000; Deli et al., 2001). This would suggest that *lycopene β-cyclase* should be synthesised during ripening (or at least be active). Many authors have reported the similarity in nucleotide sequence and conserved motif between *Lcy* and *Ccs* which encodes capsanthin-capsorubin synthase, an enzyme which catalyses the formation of capsanthin and capsorubin in Capsicum fruit (Hugueney et al., 1995; Pecker et al., 1996; Ronen et al., 1999). *Ccs* has been reported to demonstrate the enzymatic activity of *lycopene-β-cyclase* when expressed in *E.coli* and its transcript has been shown to be more abundant than the *Lcy* transcript during ripening of Capsicum (Hugueney et al., 1995). This suggests that the increase in β-carotene level observed during ripening of capsicum fruit may be due to the action of both *lycopene-β-cyclase* and *capsanthin-capsorubin synthase* (Hugueney et al., 1995). However, the role of *Lcy* during ripening of capsicum fruit would be better characterised if the actual enzyme activity is to be measured.

Capsicum being highly recalcitrant and genotype specific is known for pungency (capsacinoids), aroma and color (carotenoids). To enhance these metabolites, genetic manipulations and elicitation studies were conducted. For optimizing genetic transformation regeneration is the prerequisite. *In-vitro* and *in-planta* transformation experiments were done to transform and regenerate the plants. Abiotic and biotic elicitor mediated elicitation studies were conducted and transcript studies for carotenogenesis continued for these elicited plants. This study will be helpful for crop improvement of Capsicum which can be of use in pharmaceutical, nutraceutical and food industry.

SUMMARY AND CONCLUSION

4.1. BRIEF BACKGROUND

The genus *Capsicum*, commonly known as chilli, "red chilli", "chilli peppers", "paprika", is a member of the family Solanaceae; having approximately 22 wild species and 5 domesticated ones namely *C. annuum, C. frutescens, C. baccatum, C. chinese* and *C. pubescence* (Govindarajan, 1985). It is economically important as a spice owing to its pungency and aroma due to its constituent of capsaicinoids. Around 20 capsaicinoids are known; among them most common is capsaicin, dihydrocapsaicin, homocapsaicin, homodihydrocapsaicin, nordihydrocapsaicin. The capsaicinoids content in various varieties had been reported, ranging from 0.2% to 1.0 % (De, 2000). Most of the bigger red colored fruits cultivated and marketed belongs to the species *C. annuum* while the highly pungent ones belong to *C. frutescens*.

The 'hot' pungency of chilli is due to alkaloids called capsaicinoids which share a common aromatic moiety, vanillylamine (VA), and differ in the length and degree of un-saturation of a fatty acid side chain (Gowri et al., 1991). Capsaicin and Dihydrocapsaicin differ in degree of un-saturation of 9-C Fatty acid chain. Pungency is inherited as a single major gene at locus *C*. Non pungency is a recessive trait (Iwai et al., 1979). Capsaicinoids are synthesized by the condensation of VA with a short chain branched fatty acid by Capsaicin Synthase (*CS*) in a *coenzyme A* dependent manner. The site of synthesis and accumulation of synthesis of the capsaicinoids is in the epidermal cells of the placenta (Johnson et al., 1991). Within the cell, *CS* activity has been demonstrated in vacuolar fraction (especially bound to cytoplasmic face C-face), later the capsaicinoids accumulate in vacuoles and then as receptacles (orange yellow droplets on placenta surface) in space between cuticular and epidermal layers.

Plants are known to produce more than one trillion tons of organic compounds and 50,000 different compounds every year (Markus and Biacs, 1999). Secondary metabolites in plants are derived from basic photosynthates with modifications to

produce simple to complex molecules. Plant secondary metabolites are widely classified as phenolics, terpenoids, steroids and alkaloids based on their biosynthetic pathways and are useful as food additives, flavors, colorants, and pharmaceuticals. Paprika and its oleoresin are two of the most commonly used natural colorant in the food industry. Commercial value of the additives is based on their richness in carotenoids which originate in the fruit wall. Major carotenoids in *Capsicum* sp. are capsanthin (35% of total carotenoids), capsorubin (10%) and β-carotene (19%) (Minguez-Mosquera and Hornero-Mendez, 1994).

Elicitation can be one of the modes to enhance the carotenoids for the commercial utility (Lee et al., 1998). The world market for food colors has been assessed to be worth more than $500 million. The market for natural food colors has an annual growth rate of 4-6% compared with 1-2% for artificial colors. Paprika Capsicum color is in demand for its use in nutraceutical and pharmaceutical industries (Dempsey et al., 1999).

For the crop improvement and enhancing the yield, transformation can be the best tool for genetic manipulation. For transformation a well pronounced regeneration is pre-requisite, for chilli crop improvement it is biggest bottleneck as chilli is highly recalcitrant and genotype specific for regeneration. However few reports of regeneration are there which are genotype specific with meager reproducibility. In view of the demand for pungency factor-capsaicinoids and color values-paprika carotenoids, this study is aimed at development of genetic transformation system which could be of use to enhance valuable constituents of food and nutraceutical importance in *Capsicum* through metabolic engineering.

4.2. OBJECTIVES OF THE STUDY

With the above background information it was envisaged to develop an efficient *in-vitro* regeneration system and a reliable genetic transformation system using *Agrobacterium tumefaciens* and to regulate the capsaicin and carotenoid production in transformants aimed towards genetic improvement by transgenic approach in *Capsicum sp*.

1. To develop *in-vitro* plant regeneration system in *Capsicum* sp.
2. To develop an efficient genetic transformation system in *Capsicum*.
3. Elicitation of capsaicin and carotenoids using abiotic and biotic elicitors.
4. To identify mRNA transcripts differentially regulated under the influence of elicitors.

4.3. SUMMARY OF RESULTS

4.3.1. *In-vitro* plant regeneration system in *Capsicum* sp.

For the regeneration of *Capsicum* sp., germplasm rich in carotenoids and capsaicinoids were collected from from DRDO, Pithoragarh, Uttaranchal, and Indian Institute of Horticultural Research, Bangalore. For callus induction, leaf and Hypocotyls were used as explants with different auxin and cytokinin in the various combinations. Callus obtained was white to pale yellow color, regularly subcultured in 2-3 weeks. Reproducible and successful regeneration protocols were established using various hormonal regimes, different plant parts and different mode of inoculation. Most efficient was from posterior end (petiolar region) of the leaves. The maximum response to shoot regeneration (58-65%) was from Shoot Bud Induction Media (SBIM) comprising of MS media + {BAP (10 mg l^{-1}) + IAA (1 mg l^{-1}) + $AgNO_3$ (10μl) +MES (1.98 mg l^{-1})} which gave

rise to 20-40 shoot buds per explants (Figure 12) (Table 9). Later each bud could be elongated in MS media +$AgNO_3$ (10µM) + PAA (5 mg l^{-1}) and GA_3 (1 mg l^{-1}) (elongation media) (Table12) (Figure 12) giving rise to individual plants. In another regeneration protocol developed by this study decapitated (0.5mm) shoot tip was inoculated in inverted polarity in SBIM, which gave rise to 30-40 shoot buds. Later each bud gave rise to individual plants on elongation media. One more protocol was successfully attempted using the nodal explants in media comprising of MS + BAP (2 mg l^{-1}) +NAA (0.5 mg l^{-1}) where it was possible to achieve 45% regeneration frequency. For one more avenues towards pollen-transformation and maintenance of haploid cell lines induction of *in-vitro* flowering in *Capsicum frutescens* was attempted. Maximum numbers of flowers (7) were produced using MS media supplemented with $AgNO_3$ (40 µM) (Table 16) (Figure 15) and same number of flower were obtained with $CoCl_2$ (30 µM) containing media after 45 days of inoculation (Table 17) (Figure 15).

4.3.2. Elicitation of capsaicin and carotenoids using abiotic and biotic elicitors.

In order to achieve elicitation of capsaicinoids and carotenoids in *Capsicum*, fruits of different genotypes were harvested and various abiotic (Salicylic acid, Methyl Jasmonate) of the concentration of 1.0, 2.5 and 5.0 mM respectively and biotic (*Aspergillus* sp. and *Rhizopus* sp.) elicitors were spread and fruits were harvested after regular intervals. Fruit samples were subjected to High Performance Liquid Chromatography (HPLC) for quantification. There was a remarkable enhancement of the capsaicinoids with various elicitors. Capsaicin content was two and half folds high in methyl jasmonate (MJ) at 2.5 µM treatment. While application of salicylic acid (SA) at 1.0 µM elicited maximum capsaicin by three folds while Ibuprofen (IB) showed moderate influence on capsaicin production. Vanillylamine levels were enhanced almost two and

half fold under the influence of salicylic acid (1µM) and methyl jasmonate (2.5µM). Dihydrocapsaicin was enhanced two times in 1µM of salicylic acid (SA) treatment and similarly also at 2 µM of methyl jasmonate (Table 18). Maximum elicitation of capsaicin (329 ± 24.5 µmoles) was observed at 35 days after anthesis when *R. oligosporus elicitor* was sprayed at the concentration of 5% w/v to the flowers of *C. frutescens* (Table 19) whereas *A. niger* treatment enhanced phenyl propanoid intermediates and capsaicin to the extent of 51 ± 4.1 µmoles when sprayed at the concentration of 2.5% w/v (Table 20). Among the abiotic elicitors, maximum elicitation of phenyl propanoid intermediates and Capsaicin (49 ± 4.5 µmoles) was observed at 35 days after anthesis when Methyl Jasmonate was sprayed at the concentration of 5.0 µM to the flowers of *C. frutescens* (Table 21). In the salicylic acid sprayed plants, capsaicin level enhanced to 44 ± 1.1 µmoles at the concentration of 2.5 µM (Table 22).

Two major carotenoids in chilli *ie.* Capsanthin and Capsorubin were separated by Thin Layer Chromatography from the matured fruit pericarp and confirmation of the same was done by the following biochemical analysis *viz.* color instrumentation analysis, mass spectroscopy and photometric analysis. In the elicitation studies with abiotic elicitors, capsanthin enhanced five fold with the methyl jasmonate (1µM) and three fold increase in 2.5µM of methyl jasmonate. Influence of Salicylic acid at 2.5 µM enhanced capsaicin level by three folds. Ibuprofen at 1µM responded in six fold increase. Capsorubin has enhancement of five folds with the influence of 5µM of salicylic acid and four folds increase with methyl jasmonate and ibuprofen at 2.5µM respectively (Figure 22). Application of all the three abiotic elicitors used in this experiment showed an impact on total carotenoid accumulation, *viz.* capsanthin and capsorubin. The effect of salicylic acid (5µM) on accumulation of capsanthin was very clear as a 12 fold increase in its levels was observed.

4.3.3. Efficient genetic transformation system in *Capsicum.*

For the attempt towards transformation, Hygromycin sensitivity test was conducted. For callus transformation, leaf and hypocotyl explant from the plant were adopted. For shoot transformation seedlings, were excised 1-2mm below the cotyledonary node and were cultured in *Agrobacterium tumefaciens* strains EHA 101 harboring binary plasmid pCAMBIA1305.2 with hygromycin as selection marker and β-glucuronidase (GUS) as a reporter gene and finally transferred to selection media containing antibiotics and hygromycin. After two months the putative transformed callus were tested for GUS according to Jefferson et al. (1987). Cultures were maintained in 20 mg l^{-1} hygromycin selection medium. Confirmation of the transgenicity was done by PCR using GUS and hygromycin primers. A protocol was developed for the *in planta* transformation by floral dip method of *Capsicum* sp. using pCAMBIA1305.2. T_0 generation seeds were germinated *in-vitro* as well as in pots (Figure 34) (Table 26). Transgenic nature of seedlings was confirmed by PCR and Southern blotting.

4.3.4. mRNA transcripts differentially regulated under the influence of elicitors.

mRNA synthesis was standardized for the transcription analysis under elicitor application followed by cDNA preparation. Gene specific Lycopene cyclase (*Lcy*-e) primers were designed and standardization of RT-PCR has been done. Results of RT-PCR revealed the expression of L*cy-e* gene with the ontogeny of fruit from 30th day to 50th day. Enhancement of capsaicinoid in elicited fruits under salicylic acid treatment was 2.25 fold increases with respect to control whereas it was 2 fold increase in methyl jasmonate treated fruit at the 50th day. Expression was least in green fruits and with the developmental stages it has enhanced to maximum at maturity.

4.4. LEADS OBTAINED IN THE STUDY

i) So far the reports on *in-vitro* regeneration in *Capsicum* sps are meager and show poor reproducibility. The protocols developed in the present study are very much reproducible and very effective.

ii) An elicitor mediated up regulation of capsaicinoids and carotenoids would be of agricultural importance, which is evident from *in vitro* as well as *in vivo* studies.

iii) Genetic transformation of Capsicum has been developed in this recalcitrant genus.

4.5. FUTURE LINES OF WORK

The extension of studies carried out in this thesis could be in the following lines.

- The study gives an insight to the *in vitro* morphogenetic behavior of *Capsicum* and involvement of polyamines in plant morphogenesis. The results may be useful for further studies on determination of polyamine signaling involved in morphogenesis.
- The study demonstrated the regeneration of transgenic plants from *A. tumefaciens* mediated transformation system. The protocol for transgenic Capsicum plants developed will be useful for crop improvement.
- There is a possibility of taking up studies in metabolic engineering of secondary metabolite pathways in Capsicum for value addition.

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