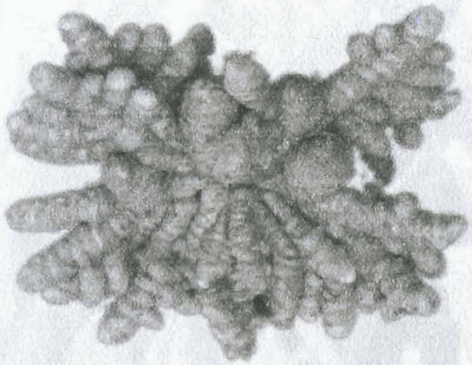
Investigations on the Biosynthesis of Curcumin in Turmeric (*Curcuma longa* L.)

Thesis submitted to

The Faculty of Science, University of Calicut

In partial fulfillment of the award of

Doctor of Philosophy (Biochemistry)



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CERTIFICATE

I herby certify that the thesis entitled **"Investigations on the Biosynthesis of Curcumin in Turmeric (*Curcuma longa* L.)"** submitted to the University of Calicut by Ms. Neema Antony in partial fulfillment for the award of the degree of Doctor of Philosophy in Biochemistry is a bonafide record of the research work carried out by her at Indian Institute of Spices Research, Calicut, Kerala, under my guidance. No part of the work has formed the basis for the award of any other degree or diploma previously. All sources of help received by her during the course of this investigation have been duly acknowledged.

Calicut,
Dated 28 May 2005

B CHEMPAKAM

DECLARATION

I, Neema Antony hereby declare that the thesis entitled **"Investigations on the Biosynthesis of Curcumin in Turmeric (*Curcuma longa* L.)"**, submitted by me for the award of the degree of **Doctor of Philosophy** in Biochemistry to the University of Calicut is an authentic record of the research work carried out by me at Indian Institute of Spices Research, Calicut, Kerala, under the guidance of Dr. B Chempakam, Head, Division of Crop Production and Post Harvest Technology, Indian Institute of Spices Research. This thesis or part of it has not been submitted to any university for the award of any degree or diploma.

Calicut,
Dated 28 Mar 2005

NEEMA ANTONY

ACKNOWLEDGEMENTS

Primly I thank Almighty for his enormous blessings and affection, which gave me strength, and support during all phases of my life. I submit myself and my achievements before him.

I am deeply indebted to Dr. B Chempakam for her guidance, encouragement and support and moreover for her affection and love for me during the course of this work.

I am grateful to the present Director of the Indian Institute of Spices Research, Dr. V A Parthasarathy for extending all facilities to carry out my work. I am equally grateful to the previous Director Dr. Y R Sarma.

I sincerely thank Dr. K Vasu, Head, Nuclear Hydrology Division, CWRDM, Calicut for providing me guidance and all the laboratory facilities at his disposal in carrying out the radio tracer studies there.

Special mention is to be made about Dr. Suparna Sengupta, Rajiv Gandhi Institute of Technology, Trivandrum who extended lab facilities and guidance for enzyme purification studies.

I wish to thank Dr. T John Zachariah, Dr. N K Leela, Dr. Ishwara Bhat and Dr. K S Krishnamurthy, IISR, Calicut and Dr. Shahul Hameed CWRDM, Calicut for their valuable suggestions and timely help in various ways, which enabled me to complete this work.

I place on record my gratitude to Mr. K Jayarajan, Statistical assistant for his help in statistical analysis of data. Thanks to Mr. A Sudhakaran, Artist cum Photographer for photography. The library support rendered by Mr. P Azgar Sheriff is thankfully acknowledged.

I am greatly indebted to many of my friends and colleagues especially, Ms. Maya, Ms. Nisha Elizebath Thomas, Ms. K S Shiny, Mr. P Gobinath, Mr. P Rajesh, Mr. Syam Kumar S, Ms. S Sindhu, Mr. Sinu P John, Ms. K Meera Bhai, Mr. Sandeep Varma, Ms. Ganga and my friends at Rajiv Gandhi Institute of Technology, Trivandrum and Central Institute of Fisheries Technology, Cochin.

I acknowledge the technical help given by Mr. T T Soman, Mr. P Ravindran and Ms. Smitha, IISR and Mr. Velayudhan, CWRDM with thanks.

I am very much thankful to Ms. T V Sandhya and Ms. Sreeshma for helping me in preparation of this thesis.

I am very much indebted to my family for their immense patience, encouragement support and prayer throughout the period of this work.

NEEMA ANTONY

DEDICATED TO MY FAMILY & GUIDE

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LIST OF ABBREVIATIONS

Abbreviation	Expansion
@	at the rate of
°C	Degree Celsius
^{15}N	Radioisotope ^{15}N labeled nitrogen
$^{15}NH_4^+$	Radioisotope ^{15}N labeled ammonium ion.
$AgNO_3$	Silver nitrate
AOPP	Alpha- amino-oxy-beta-phenyl propionic acid
APS	Ammonium persulphate
BARC	Bhabha Atomic Research Center
BDMC	Bisdemethoxy curcumin
bp	base pair
BRIT	Board of Radiation and Isotope Technology
BSA	Bovine serum albumin
CAB	Chlorophyl a/b binding protein
CAH	Cinnamic acid hydroxylase
CDNA	Complimentary Deoxyribo nucleicacid
CHS	Chalcone synthase
cm	centi meters
CO_2	Carbon dioxide
CoA	Coenzyme A
Conc.	Concentration
CWRDM	Center for Water Resources Development and Management
Cyt P450	Cytochrome P 450
D	Dextro
DAS	Days After Sowing
DDW	Double distilled water
DEAE	Diethyl Amino Ethyl Cellulose
DEPC	Diethyl pyro carbonate
DHA	Dehydroalanine
DMC	Deoxymethyl curcumin
DNA	Deoxyribo nucleic acid
dNTP	Deoxy nucleotide phosphate
DPM	Disintegrations per minute
DTT	Dithiothrietol
ER	Endoplasmic reticulum
EXT DNA	Extension deoxyribo nucleic acid
F.W	Fresh weight
$F_1 - F_7$	Fraction one to seven
FA	Ferulic acid
g	Gravitational force
GS	Glutamate synthase
H	Hills coefficient
H_2O	Water
HAL	Histidine Ammonia Lyase
HCHO	Formaldehyde
HCL	Hydrochloric acid
HIV	Human immuno deficiency virus
HPLC	High performance liquid chromatography

hr	Hour
ID	Internal diameter
IR	Infra red
kg	Kilogram
kj mol-1	Kilo joules per mol
K_m	Michaelis-Menten constant
KOH	Potassium hydroxide
L	Laevo
L-Glu	Laevo rotatory glutamic acid
LSC	Liquid scintillation counter
m	meter
M	Molar
mCi	Milli Curie
mg	Milligram
mg-1 FW	Milligram per fresh weight
$MgCl_2$	Magnesium chloride
µ M	Micromoles
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
mp	Melting point
MW	Molecular weight
N	Normality
$Na_2{}^{14}CO_3$	^{14}C labeled sodium carbonate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
NH_4^+	Ammonium ion
NH_4OH	Ammonium hydroxide
Nkat	Nano katal
nm	Nanometer
Nos.	Numbers
ODS	Octa di silane
Oligo (dt)	Oligo nucleotide
PAL	Phenylalanine ammonia lyase
PCR	Polymerase Chain Reaction
Pd-C	Palladium Chloride
pH	Negative logarithm of hydrogen ion concentration
PI	Isoelectric point
POPOP	2,2'-Phenylene-bis-5-phenyloxazole
PPO	2,5-diphenyloxazole
PVPP	polyvinyl poly pyrollidone
R_f	Reference value
RL	Red light
RNA	Ribo nucleic acid
ROS	Reactive oxygen Species
rpm	Rotation per minute
SA	Salicylic acid
SDS-PAGE	Sodium doedesyl sulphate – Poly acrylamide electrophoresis
sec.	Second
t	Trans
TCA	Trichloro acetic acid
TEMED	*N, N, N', N'-tetra methyl ethylene diamine*

TLC	Thin layer chromatography
UV	Ultra violet
v/cm	Voltage per centimeter
v/v	Volume per volume
v/w	Volume per weight
VA	Veratryl alcohol
Vmax	Maximum velocity
W	Week
w/w	Weight per weight
µCi	Micro Curie
µl	Microliters

INTRODUCTION

Neema Antony "Investigations on the Biosynthesis of Curcumin in Turmeric (Curcuma longa L.)" Thesis. Indian Institute of Spices Research , University of Calicut, 2005

INTRODUCTION

INTRODUCTION

Spices are high-value, export-oriented crops, which play an important role in the agricultural economy of the country. India is the leading producer of black pepper, large cardamom, ginger and turmeric. They are indispensable in the culinary art. Spices are well known as appetizers and large quantities of the spices are consumed for flavouring foods and their extracts are used in medicine, pharmaceutical, perfumery, cosmetics and several other industries. Their functional properties as preservatives, antioxidants, antimicrobial, antibiotic and anticarcinogenic have been well recognized.

India is the land of spices with a strong commercial operation of 25 spices, even though we cultivate around 50 spices. The total area under spices is over 3.2 mega ha. with a production of 37.6 metric tonnes. The important spices and condiments under commercial large-scale cultivation are black pepper, cardamom, turmeric, ginger, chillies, coriander, fennel, fenugreek, cumin and garlic.

India is the largest producer and consumer of turmeric. Today, turmeric, turmeric extracts and oleoresins are commercial products produced in large quantities used as colouring matter in food processing around the world. Curcumin, which belongs to the family of natural diaryl heptanoids, is the major pigment present in the rhizomes of *Curcuma longa* L. and other *Curcuma* species. The rhizomes contain three yellow pigments with a minimum structural variation - Curcumin I (Curcumin), Curcumin II (De Methoxy Curcumin) and Curcumin III (Bis De Methoxy Curcumin). These pigments are now termed as 'Curcuminoids', in place of curcumin.

In the indigenous system of medicine in the orient, turmeric has been used since time immemorial. It enjoys the reputation as an anti inflammatory agent, carminative, diuretic and blood purifier as well as a remedy against jaundice. In fact, curcuminoids I, II and III are found to be the most potent anti carcinogens, inhibiting mutagenesis and carcinogenesis. It has also been identified as the most recent anticancer drug in Chinese literature. The anti ulcerogenic and wound healing effect of curcumin is explained based on its inhibitory effect on thromboxane-2-release. Recently it has been found to be effective against AIDS, by its capacity to prevent HIV- integrase. The blood sugar lowering effect of curcumin, and its anti rheumatic or RES activating property have also been reported. Curcuminoids also show promise for the prevention of Alzheimer's disease, where low doses could lower oxidized protein and interleukin 1-beta and could reduce the insoluble β-amyloid and plaque burden in the brain to 43-50%. In short, during the last ten years, new attention has been given to turmeric and the pharmacological studies on isolated curcumin.

With the growing demand for natural dyes in the West and due to the ban on the use of synthetic dyes in food and drugs, there is an expansion in the market, for turmeric and turmeric products. In this context, it seems worthwhile to find out how the pigment is being synthesized in the plant, so that the information can provide valuable tools for further genetic and biotechnological studies. Further, if the pathway, for the biosynthesis is established, it is possible to enhance the production of the pigment by biochemical manipulation of the rate - determining steps. To achieve this end, the pathways are to be very clearly spelt out.

So far, not much work has been done on the biogenesis of curcumin except for the two schemes proposed by Geissman in 1969 and by Roughley and Whiting in 1973. The confirmation of these pathways has not been

worked out. Thus the two biosynthetic schemes mentioned are not fully accepted and further experimental evidence has not been carried out. Hence the major objective of this study is to evolve a clear picture as to the biogenesis of curcumin, which has been done with the help of biochemical and tracer studies.

The immediate objectives of the study are

i) To study the nature of precursors and intermediates in the pathway.

ii) To assay and localize the key enzymes involved in biogenesis.

iii) To evolve a suitable pathway utilizing the data generated from the study.

The identification of the pathway will ultimately give a lead in the following aspects:

1. Locating the site of synthesis of curcumin
2. The distribution of curcuminoids in rhizomes during plant growth
3. *In vitro* production of curcuminoids by biochemical manipulation of the rate-limiting enzyme
4. To elucidate analogous pathways of biogenesis of secondary metabolites in other spice crops (Black pepper, Ginger, Chillies)

Hence the topic selected for the present study will provide valuable information on the biosynthesis of the pigments in turmeric, which is highly worthwhile in the present scenario, where the demand for natural colouring matter is on the increase and synthetic pigments are being banned as food colourants due to their adverse effect on health. Moreover, the pharmacological and 'bioprotectant' properties of curcuminoids, with its multifaced clinical uses add importance to the choice of the present investigations.

REVIEW OF LITERATURE

Neema Antony "Investigations on the Biosynthesis of Curcumin in Turmeric (Curcuma longa L.)" Thesis. Indian Institute of Spices Research , University of Calicut, 2005

REVIEW OF LITERATURE

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REVIEW OF LITERATURE

PART A: Chemistry and Pharmacology of Turmeric

1. Introduction

Turmeric rhizomes, *Curcuma longa* L. (*Zingiberaceae*), commonly used as spice, are well known for its medicinal values in the Indian traditional system of medicine and have been a recipe for several common ailments. It is an ancient spice, a native of South East Asia, used from antiquity as a dye and a condiment. It is cultivated primarily in India, China, Taiwan, Sri Lanka, Java, Peru, Australia and West Indies.

Turmeric has a rich history in India and has been used for centuries in various systems of medicine *viz.*, Ayurveda, Unani and Siddha medicine (Dymock, 1972; Anon 1950; Chopra, 1994; Krithikar & Basu, 1935; Ammom and Wahl, 1991; Eigner and Scholz, 1999). Indian woman traditionally used the golden yellow pigment curcumin on many occasions (Shankaracharya & Natarajan, 1973). Later on, curcumin found its way to commercial use as a coloring agent for various items like cotton, silk, paper, wood, food stuffs and cosmetics and also as a food preservative to improve the storage conditions, palatability and presentation of food.

Turmeric, which belongs to a group of aromatic spices, was highly esteemed by the ancient Indo-European people, for its golden yellow dye resembling sunlight. This culture known as 'Arya', worshipped the solar system and attributed special protective properties to those plants, which, like turmeric, contained sun-color yellow dyes. Turmeric continues to be extensively used as a versatile spice throughout India, the middle- east and the far east, with the discovery that its powdered rhizome when added to

food preparations, both vegetarian and non- vegetarian, preserved their freshness and nutritive value.

As with other spices, turmeric, 'the yellow root' made its journey into the West through the Mediterranean regions. The Arabic root *Kurkum* seems to be the origin of the Latin word *Curcuma* by which the genus to which turmeric belongs is known. It is listed as an Assyrian herbal, dating from about 600 B.C and is mentioned in the celebrated *Materia Medica* completed by Diocorides, the Greek physician in the Roman army (ca. A.D.40-90) as an Indian plant, which yielded a yellow color and bitter taste when chewed. Turmeric is noted as a 'vegetable growing in the Fokien region of China- which has all the properties smell, and color of saffron and yet is not really saffron (Parry, 1969).

Curcuma longa L. (syn. *C. domestica* Valet.), which yields the turmeric of commerce, and to a small extent, *C.aromatica* Salisb., *C.amada* Roxb., and *C.zedoaria* Rosc. are predominantly grown in India and to a small extent in other countries (and probably in China) and *C. xanthorrizha* Roxb. in Indonesia (CRC Critical reviews 1980).

Turmeric is typical herbaceous plant with thick fleshy rhizomes and leaves in the sheaths that characterize the family *Zingiberaceae*. (Plate 1, 2, & 3). The plants reach a height of up to 1m. Leaves are alternate, obliquely erect and dark green, where the surmounting leaf sheaths taper near the leaf and broaden near the base, thus enveloping the succeeding shoot. Flowers are seen occasionally on cylindric spikes bearing numerous greenish white bracts, are narrow, and yellowish (Nadkarni & Nadkarni, 1976; Parry, 1969).

The underground rhizome, which is processed into the spice, consists of two distinct parts; the egg-shaped primary or mother rhizome, an

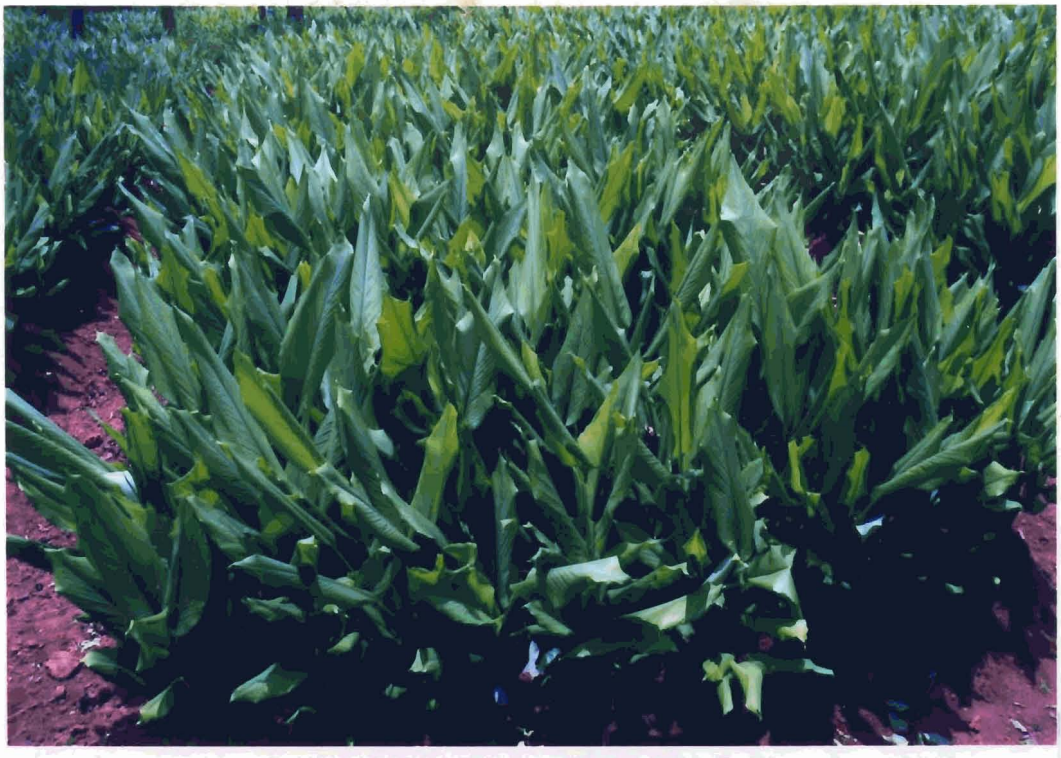


Plate 1. A view of turmeric field

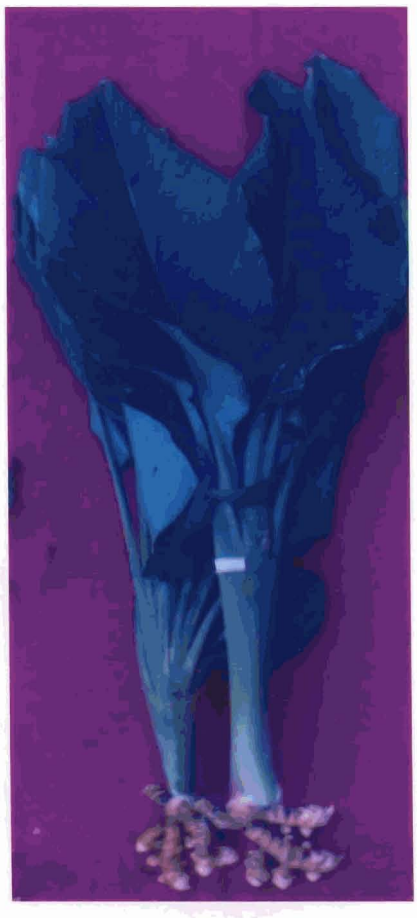


Plate 2. Turmeric seedling with rhizomes

Plate 3. Rhizomes from released turmeric varieties

extension of the stem, and several long cylindrical multi-branched secondary rhizomes growing downward from the primary rhizome. Both forms have transverse rings of leaf scars and dents of root scars. Based on their shape, the two forms used to be differentiated in the western trade, the bulbs as *C. rotunda* and the finger-like, cylindrical forms as *C. longa* though both are from the same plant (Melchier & Kastner, 1974).

In India, turmeric is cultivated in many states, but the bulk of the commercial product comes from Andhra Pradesh and Maharastra and small but notable amounts from Orissa, Tamil Nadu and Kerala (Sholto Douglas, 1973, Spices export promotion council 1977). Sowing is done during June to mid July (Anjaneyulu and Krishnamurthy, 1968; Rao *et al.*, 1975) and harvesting during February to April. Drying up of the plant including the base of the stem indicates maturity. This takes 8-9 months after planting and depends on the variety.

The farmer retains about 15 to 20% of the harvested rhizomes. Well-formed bulbs and finger portion with healthy buds are selected and carefully stored in the shade and covered with turmeric leaves. The matured seed is given fungicidal dip for safe storage and it is also done before planting (Singh, 1976).

There is no published information on a systematic analysis of changes in composition with maturity. Such an analysis will help in deciding the maturity at harvest for optimal yield, curcuminoids, and volatile oil. During the study on the processing and color content of turmeric varieties, it was found that the maximum color content varies with harvest maturity, which in turn varies with varieties and falls to nearly half its value if the harvest is delayed (Krishnamurthy *et al.*, 1975). Compositional difference, particularly, total colored compounds and volatiles between bulbs and fingers are also

unavailable. These variations, as is known from recent studies, are due to varietal differences, and to differences in fertilizer inputs and agricultural practices.

2. Chemistry of Turmeric

2.1. Chemical Composition

Turmeric contains protein (6.3%), fat (5.1%), minerals (3.5%), carbohydrates (69.4%) and moisture (13.1%). The essential oil (5.8%) obtained by steam distillation of rhizomes has *a*-phellandrene (1%), sabinene (0.6%), cineol (1%), borneol (0.5%), zingiberene (25%) and sesquiterpenes (53%) (Kapoor, 1990). Curcumin (diferuloylmethane) (3–4%) is responsible for the yellow colour, and comprises Curcumin I (Curcumin), Curcumin II (Demethoxycurcumin) and Curcumin III (Bisdemethoxycurcumin) (Ruby *et al.*, 1994). Curcumin was first isolated in 1818 by Vogel & Pelletier, and its chemical structure was determined by Roughley and Whiting in 1973. It had a melting point at 176–177°C; formed a reddish-brown salt with alkali and is soluble in ethanol, alkali, ketone, acetic acid and chloroform.

Besides curcuminoids and oils, turmeric contains some polysaccharides as well. Gonda *et al.*, (1990) isolated three acidic polysaccharides from "Ukon" by hot water extraction followed by precipitation with ethanol, and showed that they have remarkable activity on the reticuloendothelial system (RES). The components were purified on a column of DEAE Sephadex A-25. They named the three polysaccharides as Ukon A, Ukon B and Ukon C. Another neutral polysaccharide, Ukon D, was isolated by the same group having activity on the reticuloendothelial system (RES), which was composed of L-arabinose, D-galactose, D-glucose, D-mannose in the molar ratio 1:1:12:2 (Gonda *et al.*, 1992).

Srinivas *et al.*, (1992) isolated a novel water-soluble peptide 'turmerin' from turmeric with antioxidant activity. The amino acid composition of this aqueous turmeric peptide was aspartic acid/aspargine, glutamic acid/glutamine, serine, glycine, arginine, proline, alanine, tyrosine, valine, methionine, leucine, isoleucine and phenylalanine in the ratio: 1: 2: 3: 8: 1: 1: 1: 3: 2: 6: 3: 4: 5: 3.

2.2. Oleoresin

Oleoresin, as the name implies, are generally mixtures of compounds, volatile oils, and other active ingredients, non-volatile fatty and resinous materials, extractable by solvents, used singly, in sequence, or in combination (Adamson, 1971). Turmeric oleoresin chiefly functions as a food color, and secondarily, in some of the products, to impart a characteristic mild spicy aroma.

Hexane, heptane, acetone, alcohol and ethylene dichloride have generally been used in the extraction of oleoresin of spices of which alcohol and acetone are good extractants. Acetone appears to be the choice solvent for extraction of good quality turmeric oleoresin and slightly superior to alcohol and ethylene dichloride (Krishnamurthy *et al.*, 1976). Soxhlet extraction of turmeric powder with acetone for 4 to 5hrs gave a yield of about 5.0% containing 42% curcuminoids. Prolonged extraction up to 24hrs gave only fractionally higher yield of curcuminoids. Extraction by batch and counter current cold percolation in columns gave a slightly higher yield of 5.7 to 5.9% with 42.2% to 46.0% curcuminoids and extraction efficiencies varying from 72.9 to 81.7%.

An earlier study on extraction of turmeric with alcohol on a pilot plant scale has shown a higher yield, 6 to 8%, where mild heat had been employed during extraction (Kapur *et al.*, 1963). The yield of oleoresin and efficiency of

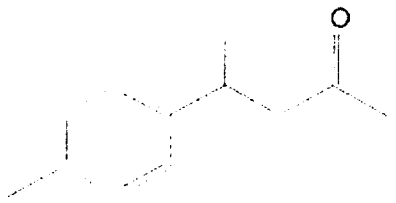
extraction of curcumin are higher with the finer ground than with the coarse ground, in both Soxhlet and cold percolation methods (Krishnamurthy *et al.*, 1976).

The product from industrial practice using good, clean turmeric, with curcuminoids content of 4.5 to 5% is a highly viscous, deep brownish- orange product, and has a yield of about 12%. This analyzes 30 to 40% as curcumin, 15 to 20% volatile oil and has a characteristic fresh, clean, mildly pungent, woody pungent, woody-spicy aroma of turmeric.

2.3. Volatile Oils

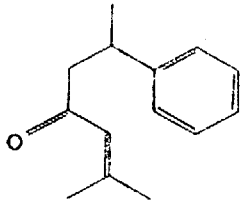
Turmeric owes its aromatic taste and smell to the oil present in the rhizome. Analysis of the oil by Kelkar and Rao (1933) obtained by steam distillation of the powdered rhizome followed by fractional distillation and derivatisation showed that the components were a mixture of predominantly sesquiterpene ketones and alcohols. The residue on steam distillation yielded mainly sesquiterpene alcohols. These were shown to be tertiary in nature by the absence of the reaction with phthalic anhydride. These alcohols gave curcumone (1) on boiling with alkali. The ketones on alkaline degradation gave also 70% yield of curcumone, thus indicating that the ketones and alcohols should have a related sesquiterpene structure. A carbonyl value of, 150 for the oil indicated around 57 - 58% of the ketones. The major fraction distilling at 158-165°C (60%) was treated with semicarbazide to fix the ketones as semi carbazones.

Besides these major components, a mixture of low boiling terpenes, α-sabinene, α-phellandrene, cineole, borneol and the higher boiling sesquiterpene and zingeberene in substantial amounts (25%) were also identified.

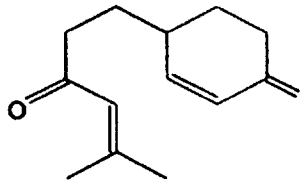


(1) Curcumone

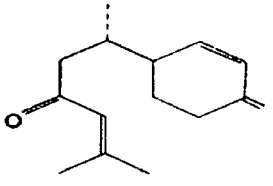
Rupe *et al.*, (1934) simultaneously recognised two major ketonic sesquiterpenes ($C_{15}H_{20}O$ & $C_{15}H_{22}O$) responsible for the aroma of turmeric. They were named ar-turmerone and turmerone. The structure of the former was shown to be (2). The structure of the latter was determined later by Mima (1959) as shown in (3).



(2) ar-Turmerone



(3) Turmerone



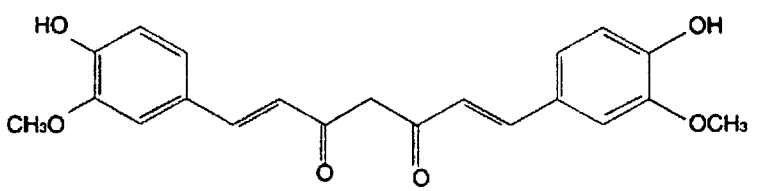
(4) Curlone

Kiso *et al.*, (1984) isolated, from the crude drug "Ukon" prepared from the rhizomes of *Curcuma longa*, a related sesquiterpenoid and named it 'curlon'. It had a molecular formula of $C_{15}H_{22}O$ and on dehydrogenation with Pd-C, yielded ar-turmerone indicating that curlone had bisbolane skeleton. On the basis of UV, IR and ^1H NMR spectral data, they assigned structure (4) for curlone.

2.4. Curcuminoids

Curcuminoids belong to the dicinnamoyl methane group and are present to the extent of 3-5%. It is an important active ingredient responsible for the biological activity of *Curcuma longa*. Curcumin, $C_{21}H_{20}O_6$, m p 184-185°C was isolated as early as 1818 (Vogel & Pelletier) as mentioned earlier. It is insoluble in water but soluble in ethanol and acetone. Daube in 1910 obtained it in crystalline form. The structure of curcumin as a diferuloylmethane was confirmed by the degradative work (Lampe, 1910) and synthesis by Lampe and Godlewska in 1918.

The main colored substance in the rhizomes is curcumin, [1, 7-bis (4-hydroxy-3-methoxy prenyl)-1, 6-heptadiene-3, 5-dione] and two related demethoxy compounds, demethoxy curcumin and bis demethoxycurcumin, which belong to the group of diarylheptanoids (Plate 4).



(5) Isomer of curcumin with diketone structure

Heller (1914) had isolated an isomer of curcumin, with a diketone structure (5), while the chemical synthesis of bisdemethoxy curcumin and demethoxy curcumin were carried out by Srinivasan (1952) and Lampe and Melobedzka (1913). Besides these major constituents, three minor constituents were also isolated by Srinivasan (1952), which were supposed to be the geometrical isomers of curcumin. One of these (6) was assumed to be cis-trans geometrical isomer of curcumin based on its UV spectrum, lower m.p. and

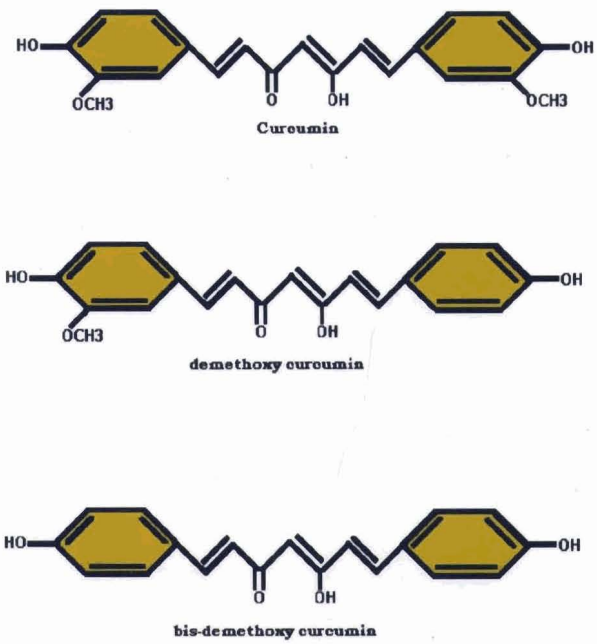
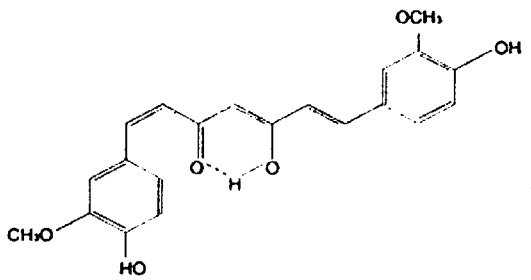


Plate 4. Curcuminoids

lower stability when compared to curcumin, which has cis-trans configuration.



(6) cis-trans geometrical isomer of curcumin

A new curcuminoid, cyclocurcumin was isolated from the nematocidally active fraction of turmeric, together with the other three known curcuminoids, which did not show any nematocidal activity. Interestingly, cyclocurcumin did not show the expected nematocidal activity but when the four curcuminoids were mixed, a strong nematocidal activity appeared; so it was concluded that the strong nematocidal activity of turmeric is due to a synergistic action of the curcuminoids. Cyclocurcumin has the same molecular formula of curcumin, which is formed by an intramolecular addition of the enol-oxygen to the enone group.

The content of curcuminoids in turmeric ranges from approximately 2.5% to 6%, (Krishnamurthy *et al.*, 1976) of which curcumin accounts for about 49% of the total pigments, p-hydroxy-cinnamoyl-feruloyl-methane (DMC) for about 29% and *p, p'*-dihydroxy-dicinnamoyl-methane (BDMC) for about 22%. The absorption spectra of these three components vary slightly, with their maxima at 429nm, curcumin; 424nm, demethoxy curcumin and 419nm, bisdemethoxy curcumin. (Roughly and Whiting, 1973).

The three curcuminoids also exhibit fluorescence under ultraviolet and after separation on thin-layer plates, can be directly estimated by fluorescence-densitometer when irradiated at 350nm (Jentzsch *et al.*, 1959).

The fluorescence spectra of curcuminoids show distinct excitation at 435nm, and emission at 520 nm (Karasz, 1973). Estimation of the total curcuminoids is done on the alcoholic extract of the powdered rhizome by measurement of optical density at their absorption maxima, 420 to 425nm (Official Analytical Methods, 1958). The three components, curcumin, demethoxy curcumin, and bisdemethoxy curcumin have been variously estimated by thin-layer chromatography and found to be present in the ratio 60:30:10 (Perotti, 1975), 47:24:29 (Jentzsch *et al.*, 1959), 49:39:22 (Krishnamurthy *et al.*, 1976) and 42:24:34 (Govindarajan and Raghuveer, 1980; Govindarajan, 1980).

The effect of maturity on curcuminoids and the ratio in Sri Lankan *Curcuma longa* was studied by Cooray *et al.*, (1988), using TLC-UV spectrometry and TLC-UV-densitometry techniques. Similar results were obtained by both methods.

2.4.1. Structure Elucidation

The constitution of the curcuma color as diferuloyl methane was first suggested by Ciamician and Silber in 1897, and confirmed by the later degradative work of von Kostanecki and Lampe, and its synthesis by Lampe in 1910 (quoted by Mayer). On boiling with alkali, curcumin gave vanillic and ferulic acid whose constitution had already been established. Fusion with alkali yielded protocatechuic acid, and oxidation with permanganate gave vanillin. Reaction with hydroxylamine yielded a characteristic isoxazole derivative. Curcumin was easily acetylated with acetic anhydride giving diacetyl-curcumin, with methlyliodide it gave tetramethyl curcumin, and on hydrogenation a mixture of tetrahydro- and hexahydro-curcumins. Along with molecular formula the structure was thus established as diferuloyl methane.

Chemical synthesis

Lampe and Milobedzka (1913), starting from carbomethoxy feruloyl chloride, synthesized curcumin, which on condensation with ethyl acetoacetate gave the ester. The ester on hydrolysis and loss of carbomethoxy feruloyl chloride gave the diferuloyl compound. The hydrolysis of this compound released the carboxymethyl and acetyl groups and gave curcumin identical with the natural curcumin (quoted by Mayer, 1943).

Another synthesis of curcumin from acetyl acetone and vanillin had been reported early by Pavolini and had been further improved by Pabon, (1964) to get 80% yield of curcumin. Starting with other related aldehydes, curcumin derivatives and related compounds were also obtained. The synthesis by this method has recently been used in biosynthetic studies (Roughly and Whiting, 1973).

2.4.2. Chemical Properties

Curcumin obtained commercially is usually a mixture of the three curcuminoids, *viz.*, curcumin, demethoxycurcumin (*p*-hydroxy-cinnamoyl-feruloyl-methane) and bis-demethoxycurcumin (*p, p'*-dihydroxy-dicinnamoyl-methane). It is a yellow crystalline, odorless powder (mp 184-186°C), poorly soluble in water, petroleum ether, and benzene, soluble in methyl and ethyl alcohols, glacial acetic acid, and in propylene glycol and very soluble in acetone and ethyl ether. In a finely powdered form, curcumin can be dispersed in oil.

The most characteristic reaction of turmeric (and curcumin) is the formation of a characteristic red color when reacted with boric acid, due to the formation of the compounds named rubrocurcumin and rosocyanin. This

reaction is used as a test of identity of turmeric (and curcumin) and is believed to have structures isomeric with curcumin (Mayer, 1943).

2.4.3. Pharmacology and Biosynthesis of Curcumin

2.4.3.1. Medicinal and Pharmacological properties

Turmeric exemplifies a herb, for which clinical applications have evolved over time. Although the chemical structure of curcumin in turmeric was determined by Lampe in 1910, it was only in the seventies and eighties that the potential uses of curcuminoids in medicine have been studied. The ongoing clinical studies indicate that curcuminoids have unique anti inflammatory and anti oxidant properties and is potentially useful in the prevention of cancer and in the treatment of infection with human immuno deficiency virus (HIV) and also show promise in the prevention of Alzheimer's disease.

Anti oxidant properties

Curcuminoids are natural phenolic compounds with potent anti oxidant properties. Both turmeric and curcuminoids inhibited generation of potent free radicals like superoxide and hydroxyl radials (Reddy and Lokesh, 1992) and prevented lipid peroxidation, which also generates free radicals (Sreejayan and Rao, 1994). A potential use of this antioxidant effect is in the prevention of cardiovascular disease. Administration of 500mg curcuminoids daily to healthy humans for seven days could lower the lipid peroxides by 33% and blood cholesterol by 29% (Soni and Kuttan, 1992). Among the various curcuminoids, tetrahydrocurcumin had the strongest antioxidant activity (Osaka, 1995; Sugiyama, 1996), where both phenolic hydroxyl and β-diketone moiety are involved. As biological oxidation is a free radical mediated chain reaction, the efficiency of an anti oxidant is linked to this role

in preventing formation of free radicals and quenching the already existing free radicals. The C3-complex, "Curcuminoids" has been shown to be more effective as an anti oxidant than each of the components *viz.*, curcumin, demethoxycurcumin and bisdemethoxycurcumin used alone (Majid *et al.*, 1995). Demethylated forms of curcumin and trans- forms of ferulic acid and caffeic acid were found to be more potent, whereas complete methylation abolished anti oxidant activity.

In effect, curcumin acts as a scavenger for oxygen free radicals (Arora *et al.*, 1971). *In vitro*, curcumin can significantly inhibit the generation of Reactive Oxygen Species (ROS) like superoxide anions, hydrogen peroxide and nitrite radical generation, which play an important role in inflammation.

Chemopreventive Property

Curcuminoids possess anti carcinogenic property due to their oxygen radical scavenging ability (Soudamini and Kuttan, 1989). Among the various mechanisms, induction of apoptosis and inhibition of cell cycle progression plays an important role in its anticarcinogenic effect (Chen & Huang, 1998). Curcumin is a promising chemopreventive agent, by blocking the process of chemically induced toxicity due to the inhibition of the enzyme cytochrome P_{450} A1 (Commandeur and Vermeulen, 1996). Curcumin also stimulates the activity of caspase-8, which initiates signaling pathway of apoptosis and hence appears to exert its anti carcinogenic properties by inhibiting proliferation in gastric and colon cancer cells, as reported by Moragoda *et al.*, (2001). Oral administration of turmeric extract or curcumin could inhibit stomach tumors in mice (Nagabhushan and Bhide, 1992), skin tumor (Commandeur and Vermeulen, 1996).

Most carcinogens undergo metabolic changes in the body to 'activated' carcinogens, which binds to the cell DNA to form DNA adducts. During the

repair of damaged DNA, mutations may occur, which eventually give rise to cancer. The efficacy of curcumin in inhibiting the development of adenomas in the intestinal tract (Perkins, 2002) and in enhancing the cytotoxicity of chemotherapeutic agents in prostrate cancer (Hour *et al.*, 2002) is well established. Curcumin is also effective and works as an anti oxidant to inhibit the UV B radiation induced damages in cases of non-melanoma skin cancer (Afaq *et al.*, 2002). Shao (2002) reported the ability of curcumin to inhibit the proliferation of human breast cancer cells *in vitro*. The possible conversion of curcumin to tetrahydrocurcumin *in vivo* to act as a more promising chemopreventive agent was reported by Kawamori (1999) and Osawa (1995).

Anti microbial Property

Curcuminoids have also been shown to exhibit antimicrobial properties. Extracts from turmeric and the active principles were found to inhibit the growth of numerous gram positive and gram negative bacteria, fungi and the intestinal parasite *Entamoeba histolytica* (Ammon & Wahl, 1991). Curcumin also inhibits the *in vitro* production of aflatoxins - toxins produced by the mold *Aspergillus parasiticus*, which may grow and contaminate the poorly preserved foods (Soni *et al.*, 1992). Aflatoxin is a potent biological agent causing injury to liver, often resulting in liver cancer.

Curcuminoids are responsible for the antimicrobial activity against *M. pygenous* var.*aureus*, *Staphylococcus aureus* and *Mycobacterium tuberculosis*. One of the constituents of essential oil in turmeric- p-tolyl methyl carbenol - acts against *Bacillus coli*, *Paramecium caudautum*, *S. aureus* and *S. albus* (Apisariyakul *et al.*, 1995; Jayaprakasha *et al.*, 2001; Wuthi- Udomler *et al.*, 2000. and Koide *et al.*, 2002). An interesting property of curcuminoids is their anti-HIV effect, which has been demonstrated during *in vitro* and *in vivo* experiments, including a few human studies (Li, 1993; Copeland *et al.*, 1994).

Curcumin specifically inhibits HIV-protease and can be effectively used against AIDS.

Anti inflammatory activity

Turmeric is one of the oldest anti-inflammatory drugs used in ayurvedic medicines. Turmeric extract, volatile oil from turmeric and curcuminoids were reported to possess this property (Arora *et al.*, 1971; Ghatak and Basu, 1972; Chandra and Gupta, 1972). The inflammation associated with various forms of arthritis could be brought down by the administration of these compounds. Curcumin at dose of 1200 mg per day for 5-6 weeks could improve the rheumatoid arthritis in patients and the effect was comparable with phenyl butazone (Deodhar *et al.*, 1980). Curcumin is also effective against chronic respiratory diseases and the presence of olefinic with double bonds and hydroxyl groups are important for its anti-inflammatory activity. Curcumin exerts its action by inhibiting cyclo oxygenase and lipoxygenase enzymes, resulting in diminished production of inflammatory compounds *viz.*, arachidonine, prostaglandin and leukotrienes (Ammon *et al.*, 1993). Most of the anti inflammatory mechanisms of curcumin are comparable with the NSAID drugs phenyl butazone, which structurally resembles curcumin with two aromatic and two ketonic groups.

Anti diabetic property

Dietary curcumin is beneficial in ameliorating the diabetic nephropathy, which is probably mediated through its hyper lipidaemic effects (Suresh Babu and Srinivasan, 1998). Curcumin prevents galactose- induced cataract formation at very low doses (Suryanarayanan *et al.*, 2003). Both turmeric and curcumin decrease blood sugar level in alloxan-induced diabetes in rats (Arun and Nalini, 2002). Curcumin also decreases advanced

glycation end products, which induced complication in diabetes mellitus (Sajith lal *et al.*, 1998).

Thus curcumin is a non-toxic, highly promising natural antioxidant compound with a wide range of biological functions.

Other Pharmacological properties

Curcumin is reported to be useful in preventing the development of radio resistance following radiotherapy (Varadkar *et al.*, 2001; Inano Onada, 2002). Dietary curcuminoids have lipid-lowering effect in *vivo* (Asai and Miyasawa, 2001). The anti hepatotoxic property as well as the anti HIV effect of curcuminoids has been investigated by several workers (Kiso *et al.*, 1983; Li 1993; Copeland *et al.*, 1994; Reddy, 1996).

Curcuminoids thus represents a class of valuable phytonutrients with unique bio-protective properties. If regularly administered as nutritional supplement, these natural compounds would potentially help in maintaining good health and in slowing down the progression of various disease conditions. Topical application will help in neutralizing damaging free radicals at the surface of the skin, thereby retarding aging and damage due to UV radiation.

2.4.3.2. Biosynthesis

Two distinct pathways for the biosynthesis of curcumin had been suggested as early as in 1969 (Geissman) and in 1973 (Roughley and Whiting). Biosynthesis of flavour compounds of spices had been studied earlier, such as incorporation of phenylalanine into the aromatic ring in capsaicin (Bennet and Kirhy, 1968) and gingerol (Denniff and Whiting, 1976), the pungent compounds of capsaicin and ginger respectively. It was suggested that this

mechanism also would be active in the biosynthesis of curcumin, in the formation of the 3-methoxy-4—hydroxy phenyl moiety of curcuminoids. The structure established for curcumin suggested the reasonable biosynthetic mechanism involving two cinnamoyl units completely a control unit for malonate (Geissman and Crout, 1969). The proposed scheme was tested (Roughly and Whiting, 1971; 1973) by incorporation of labeled precursors. They concluded that an alternate scheme might exist for curcumin biosynthesis, which involves a cinnamate starter extending by five acetate or malonate units' and cyclization of the chain would give the second aromatic ring. Biosynthesis would be completed by hydroxylation and methylation. The effective symmetry of the tautomeric curcumin molecule and the degree of isotopic scrambling however prevents clear-cut interpretation in favour of either of the two alternate schemes discussed.

Using labelled phenolic acids and phenylalanine, the role of cinnamic acids in curcumin biosynthesis was investigated by Roughly and Whiting, (1973). They found that none of the cinnamic acids was quite as well incorporated, as phenylalanine into curcumin and caffeic acid was relatively unacceptable as a precursor.

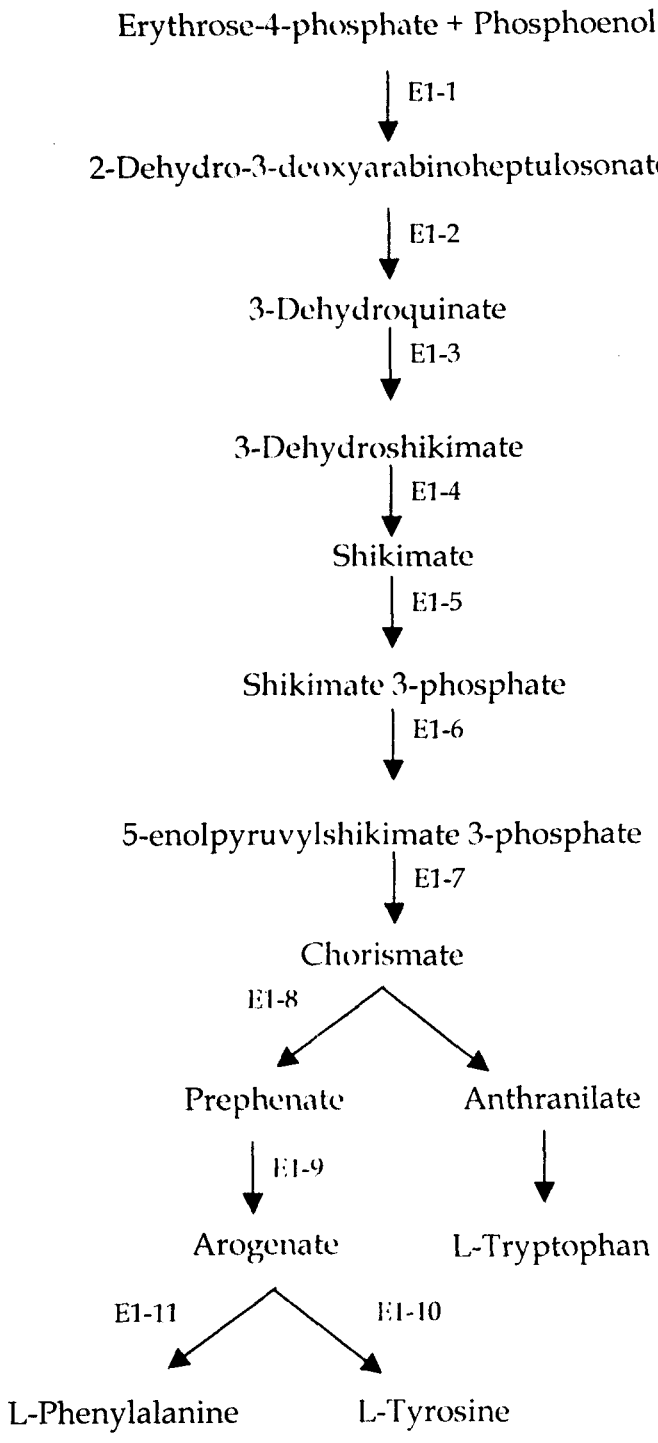
Apart from these two postulations, no recent information has been cited in literature, except for a study on the chemical synthesis of curcumin (Sastry, 1970). According to Sastry, synthetic curcumin on analysis contained all the three naturally occurring curcuminoids, which indicated the origin of these products from natural sources. Hence it is obvious that further work on the biosynthesis of curcumin is essential to draw conclusions.

PART B: Phenylalanine Ammonia Lyase

1. Phenyl propanoid pathway

Three different biogenetic routes namely shikkimate/ arogenate, acetate/ malonate, and acetate/ mevalonate pathway lead to plant phenolics. Of this shikkimate/ arogenate pathway leads, through phenylalanine, to the majority of plant phenolics, the phenyl propane (C_6 - C_3) derivatives (phenylpropanoids). It is the most important one in biosyntheses of plant phenolics.

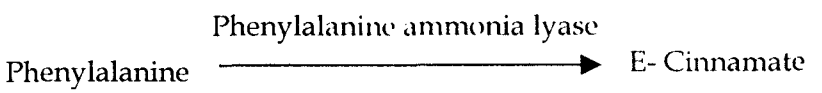
Phenylalanine, as well as the other two aromatic amino acids, tyrosine and tryptophan, is synthesized by the shikimate pathway. The first seven steps of the shikimate pathway (the prechorismate pathway) are common for the biosynthesis of all three aromatic amino acids (Dey and Harborne, 1997).



E1-1 : 2-Dehydro-3-deoxyarabinoheptulosonate aldolase
E1-2 : 3-Dehydroquinate synthase
E1-3 : 3-Dehydroquinate dehydratase
E1-4 : Shikimate 3-dehydrogenase
E1-5 : Shikimate kinase
E1-6 : 3-Phosphoshikimate 1-carboxyvinyltransferase
E1-7 : Chorismate synthase
E1-8 : Chorismate mutase
E1-9 : Prephenate aminotransferase
E1-10 : Arogenate dehydrogenase
E1-11 : Arogenate dehydratase

The bifurcation of arogenate to phenylalanine and tyrosine is an important point of regulation (feed back). Here the partition of the carbon flow between the two amino acids is controlled by two enzymes namely dehydratase which is inhibited by phenylalanine and the dehydrogenase by tyrosine. Both enzymes also inhibit the chorismate mutase activity (enzyme involved in the conversion of chorismate to prephenate). Further control possibly results from the fact that phenylalanine fluxes into protein and phenyl propanoid pathway, which is coordinated by parallel pathways that are located in different compartments and include several isozymes. One of the shikkimate pathway is thought to be located in chloroplasts, in which the aromatic amino acids are produced mainly for protein biosynthesis, whereas the second is probably membrane associated in cytosol, in which phenyalanine is also produced for the formation of phenylpropanoids.

The phenylalanine/ hydroxycinnamate pathway is defined as 'general phenylpropanoid metabolism'. It includes reaction leading from L-phenylalanine to the hydroxycinnamates and their activated forms, the coenzyme A (CoA) thioesters and the 1-O-acylglucosides. The latter accumulates in most plants as typical hydroxycinnamate conjugates. The enzyme Phenyalanine Ammonia Lyase (PAL) controls the interphase between the phenylalanine and secondary phenylpropanoid metabolism. PAL belongs to the class of carbon- nitrogen lyases (C-N cleavage) that forms a double bond. The active site of the enzyme contains a dehydroalanine residue whose methylene group binds to the amino group of phenylalanine (β- addition). The product elimination process of PAL activity generates, after a prototropic shift, *E*-cinnamic acid and the 'amino enzyme'. The enzyme is finally regenerated by release of ammonia.



A series of hydroxylation and methylation reactions, catalyzed by the enzymes cinnamic acid hydroxylase, 4- coumarate- 3- hydroxylase, ferulate-5-hydroxylase and caffeate/ 5-hydroxyferulate methyltransferase, respectively lead to the sequential formation of the common hydroxycinnamates, *viz.*, 4-coumarate, caffeate, ferulate and sinapate. All these phenolic acids exist as activated CoA form. The hydroxycinnamates are utilized in various pathways leading to flavonoids, stilbenes, coumarins and hydroxycinnamate compounds.

E- Cinnamate

↓ E2-2

4-Coumarate

↓ E2-3

Caffeate

↓ E2-5

Ferulate

↓ E2-4

5- Hydroxyferulate

↓ E2-5

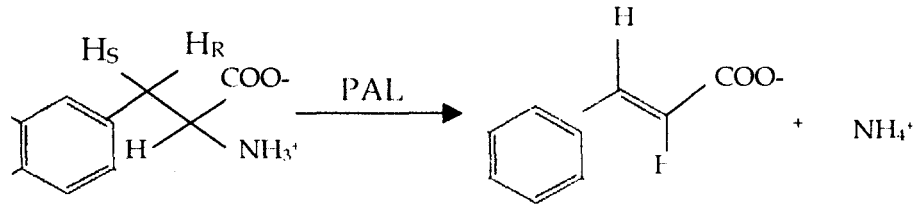
Sinapate

E2-2	:	Cinnamate 4-hydroxylase
E2-3	:	4-Coumarate 3-hydroxylase
E2-4	:	Ferulate 5-hydroxylase
E2-5	:	Caffeate/5-hydroxyferulate methyltransferase

2. Phenylalanine ammonia Lyase

PAL (E.C.4.3.1.5) catalyses the first reaction in the biosynthesis from phenylalanine of wide variety of phenyl propanoid compounds and fluctuations in the PAL levels are thought to be a key element in the control of

phenylpropanoid biosynthesis. It is currently the most studied enzyme concerned with secondary metabolism in plants. The enzyme was discovered in 1961 by Koukol and Conn. PAL has been found in all green plants including the higher cryptograms (Young, *et al.*, 1966) and the basidiomycetes (Bandoni, *et al.*, 1968). In algae, the only reported occurrence of the enzyme has been in Porphyridium, which has been confirmed (Landymore, & Towers, unpublished document). Phenylalanine ammonia lyase forms trans-cinnamate from L-phenylalanine, with β- elimination of ammonia and thus stands as a gateway to secondary metabolism in higher plants (Alunni *et al*, 2003; da Cunha, 1988).



L-Phenylalanine E-Cinnamate

This part of the review summarizes the chemistry, active site, localization, purification, regulation and kinetics of the PAL in various crops.

2.1. Mechanism of action of PAL

In the first step of the phenylpropanoid metabolic pathway, L-phenylalanine (L-Phe) is deaminated to form E-cinnamate, in a conversion catalyzed by phenylalanine ammonia lyase. The metabolic fate of the ammonium ion (NH_4^+) produced in this reaction was investigated in sweet potato (*Ipomoea batatas*) tuber discs. [^{15}N]-labeled substrates including L-Phe, in the presence or absence of specific enzyme inhibitors, were administered to sweet potato discs in light under aseptic conditions. ^{15}N -Nuclear magnetic resonance spectroscopic analyses revealed that the $^{15}NH_4^+$ liberated during the PAL reaction is first incorporated into the amide nitrogen of L-glutamine

(L-Gln) and then into L-glutamate (L-Glu). PAL-generated NH_4^+ after assimilation by the glutamine synthetase (GS; EC 6.3.1.2)/glutamate synthase (GOGAT; EC 1.4.1.13) pathway, recycled back to L-Phe via L-Glu as amino receptor and donor (Singh *et al.*, 1998).

Several fluoro and chlorophenylalanines were found to be good substrates of PAL from parsley. The kinetic constants for nine different fluoro- and chlorophenylalanines do not provide a rigorous proof for but are consistent with the proposed mechanism comprising an electrophilic attack of the methylidene-imidazolone cofactor of PAL at the aromatic nucleus as a first chemical step. Subsequent elimination of ammonia, concomitant with restoration of both the aromatic ring and the prosthetic group, completes the catalytic cycle (Schuster & Retey, 1995). A chemical model for the PAL reaction is described by Retey in 2003, based on the proposed mechanism of action of phenylalanine ammonia lyase (Skolaut & Retey, 2001).

Phenylalanine spectrospecifically labeled with isotopic hydrogen at C_3 has been used to establish the elimination of the pro-S proton from C_3 of L-phenylalanine, together with ammonia, in an antiperiplanar fashion from trans- cinnamic acid (Hanson and Havir, 1972, Ife and Haslam, 1971). Reversibility of the enzyme reaction has been demonstrated and the equilibrium constant for potato PAL has been estimated as 4.7 at 30°C, pH 6.8 and zero ionic states by Havir and Hanson, in 1968. There are no indications from tracer studies, however, of the *in vivo* synthesis of phenyalanine from cinnamate.

2.2. Active Site

Histidine Ammonia Lyase and Phenylalanine ammonia lyases (HAL and PAL) possess a catalytically essential electrophilic group, which has been

found to be methylidene imidazolone. Speculations on the active site of PAL through inhibition studies using cyanide and sodium borohydride showed dehydroalanyl residue at the active site (Hanson and Havir, 1970), resembling HAL. They are the only enzymes to have modified amino acid dehydroalanine (DHA) in their active site, for which the precursor has shown to be a serine residue. The region around this active site is highly conserved. Langer *et al.*, 1994, in his studies, replaced serine (202) residue by alanine, with a loss in the enzyme activity, indicating the importance of serine for the formation of the active enzyme. In addition to the active site, it was shown that the enzyme contained an allosteric binding site, based on the kinetic studies of PAL from potato and corn (Marsh *et al.*, 1968).

2.3. Localization

Differential centrifugation studies have indicated that the enzyme is localized in the soluble fraction (Kalghatgi *et al.*, 1976). In earlier studies, (Hrazdina *et al.*, 1984), evidence was obtained suggesting that the endoplasmic reticulum was a site for phenylpropanoid and flavonoid metabolism in petal tissue, and that (a) multienzyme complex (es) might be involved in this metabolism. Now, the possible role of membrane-bound multienzyme complexes in phenylpropanoid and flavonoid metabolism in three tissues has been investigated by (1) correlating enzyme induction kinetics and rates, (2) examining the molecular weight of putative complexes, (3) channeling of substrates, (4) determining the susceptibility of bound activities to trypsin digestion, and (5) investigating the structurally linked latency of bound activities. Results suggest that atleast a part and possibly the entire pathway--from phenylalanine to flavonoids is membrane (endoplasmic reticulum) associated, and that a multienzyme complex facilitates this metabolism. Phenylalanine ammonia lyase, the first enzyme of the biosynthetic sequence, and a flavonoid glucosyltransferase, the last, appear to be located in the lumen of the membranes. Cinnamate 4-

hydroxylase is membrane embedded, while other enzyme activities appear to be weakly associated with the cytoplasmic face of endoplasmic reticulum membranes (Hrazdina & Wagner, 1984). *p*-Hydroxycinnamic acid was found to be located within the plastids of the green alga *Dunaliella marina*. Thylakoid fractions disintegrated by ultrasonic treatment were capable of converting L-phenylalanine into *o*- and *p*-hydroxycinnamic acids; the hydroxylation reaction was increased by addition of NADPH. Hydroxycinnamic acids produced when [3-^{14}C] cinnamate was incubated with varying amounts of [4'-3H] L-phenylalanine exhibited a ^{3}H/^{14}C ratio 10-150 times higher than that of the cinnamic acid reisolated from the incubation mixture. The lack of equilibration between cinnamate formed from L-phenylalanine and cinnamate added to the solution supports the hypothesis that cinnamate, as an intermediate in hydroxycinnamate formation remains bound to the membrane enzyme complex. Protein gel blot analysis showed that tobacco PAL1 and bean PAL were localized in both soluble and microsomal fractions, whereas tobacco PAL2 was found only in the soluble fraction. Rasmussen & Dixon (1999) proposed that metabolic channeling of trans-cinnamic acid requires the close association of specific forms of PAL with C4H on microsomal membranes.

2.4. Purification of PAL

PAL has been purified from several different crops for the past 30-40 years. Dubery and Smith (1994) purified PAL from cotton hypocotyls (*Gossypium hirsutum*) by differential ammonium sulfate fractionation and hydrophobic interaction chromatography, with a yield of 52%. The enzyme was a tetramer with a molecular weight of 3,32,000 to 3,37,000. The isoelectric point was 4.6 and no isoforms were observed. The subunits of enzyme are unstable and break down to fragments with a molecular weight of 69,000 and 49,500 and the optimum pH of the reaction catalyzed by PAL was 8.9.

Negative cooperative interactions occurred between the substrate binding sites with a Hill coefficient of 0.87.

L-Phenylalanine ammonia lyase was purified from cotyledons of etiolated and far-red illuminated seedlings of radish by Fourcroy (1980) using absorption of the enzyme on L-Phenylalanine - Sepharose 4B and found that molecular weight of enzyme fluctuated according to pH and ionic strength. A mean value of 2,90,000 was determined for native form. By purifying L-phenylalanine through ammonium sulfate fractionation, DEAE-cellulose chromatography, Sephadex G-200 chromatography, and Q-Sepharose chromatography from the cytosolic fraction of leaf mustard (*Brassica juncea* var.*integrifolia*), it was found to consist of 4 subunits, each having an estimated molecular weight of about 40,000 on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with an optimal pH and temperature of 9.0 and 45°C, respectively (Lim *et al.*, 1997). PAL purified from far-red light-irradiated mustard cotyledons by Gupta and Acton (1979) consisted of a single protein and showed molecular weight of 2,40,000 +/- 9000. The enzyme constituted 0.01% of total cellular protein, did not catalyse the deamination of L-tyrosine, had a pH optimum of pH 8.6 and an isoelectric point of pH 5.6. Electrophoresis on denaturing polyacrylamide gels gave a single stained protein band corresponding to a subunit molecular weight of 55,000 indicating a tetrameric structure of equal (or near equal) size subunits and maximum activity at temperature 25°C.

Another purification study conducted by Lim *et al.*, (1998) in mustard leaf identified a second form of PAL (PAL II) by a combination of ion exchange chromatography and gel filtration. They found that PAL I and PAL II migrate at a different rate in native polyacrylamide gel electrophoresis and consists of 4 subunits, each having the molecular mass of about 37,000 Da with optimum pH and temperature of 8 and 45°C respectively.

Da Cunha (1988) purified PAL from leaves of *Phaseolus vulgaris* by Sephacryl S-200 gel filtration and Sepharose-4-B- succinyl- aminoethyl-L-Phenylalanine affinity chromatography possessing a molecular weight of 3,20,000 + /- 9000 and 3,30,000 + /-4000 respectively. After SDS electrophoresis only one band of molecular weight 83,000 + /-4000 was detected, indicating that the enzyme is an oligomer containing 4 subunits with an optimum pH between 8.8-9.2. The enzyme showed Km 1.25 mM for L-phenylalanine. By purification of differentially induced isoforms of PAL by elicitor - treated cell suspension, Bolwell *et al.*, (1985) observed four forms of the enzyme with identical molecular weight but differing apparent PI values of 5.4, 5.2, 5.05 + 4.85. This newly synthesized PAL (both *in vivo* and *in vitro*) gave a subunit with molecular weight of 77,000; a 70,000 molecular weight form is readily generated as a partial degradation product during purification. A preparation (purified 43-fold by ammonium sulphate precipitation, gel-filtration and ion-exchange chromatography) containing all four forms exhibited apparent negative rate cooperativity with respect to substrates. However, the individual forms displayed normal Michaelis-Menten kinetics, with Km values of 0.077 mM, 0.122 mM, 0.256 mM and 0.302 mM in order of decreasing apparent pI value. Microbial L-PAL, from *Rhizobacteria solani* a pathogenic fungus, was purified from the acetone dried powders and molecular weight was determined to be around 3,30,000 by Sephadex G-200 chromatography followed by density-gradient centrifugation. Enzyme was observed to be made up of two pairs of unidentified subunits, with a molecular weight of 70,000 (alpha) and 9000 (beta) respectively (Kalghatgi & Subba Rao, 1975). In *Phaseolus vulgaris*, purified enzyme preparations exhibited subunit molecular values of 77,000, 70,000 and 53,000 (Bolwell *et al.*, 1985). In tomato leaves, excision and light treatments increased PAL activity, which is contributed by three PAL isoforms, with similar native and subunit MW, but differing in pI, Km for Phenylalanine and optimal pH for activity.

2.5. Isozymes

The Molecular weights reported for PAL are 306 000 for maize (Havir and Hanson, 1968), 300 000 for mustard (Acton and Schopfer, 1975), 37,000 for mustard leaf (Lim *et al.*, 1998), 226 000 for *Streptomyces verticillatus* (Emes and Vining, 1970) and 330 000 for potato (Havir, and Hanson, 1968). The enzyme from mustard is unstable in dilute Tris Buffer and aggregates to a form with a MW greater than 600 000 (Acton and Schopfer, 1975). The potato enzyme has been reported to exist in two forms, one species being twice the MW of the other, but these forms are not interconvertible (Havir and Hanson, 1968).

On the other hand, two forms of PAL with different catalytic properties have been found in a number of tissues. In sweet potato the two forms are separable from TAL (Tyrosine ammonia lyase) and show, moreover, differing sensitivities to phenolic inhibitors (Minamikawa and Uritani, 1965). The two forms of PAL from *Quercus pedunculata*, separable on DEAE- cellulose, also differ in their sensitivities to phenolic compounds (Boudet *et al.*, 1971).

2.6. Regulation

There is a considerable interest in the regulation of phenylpropanoid biosynthesis, which is initiated by the deamination of phenylalanine by PAL. PAL, as the bridge between primary metabolism and natural product biosynthesis is a potential site for pathway regulation (Jones and Northcote, 1984). Mechanisms of regulation of PAL in response to a wide range of external stimuli have been studied in many systems. Inactivation (irreversible loss of enzyme activity) as a regulatory mechanism has been reported previously for many systems, while inhibition (reversible loss of enzyme activity) has been reported for some systems (French and Smith, 1975; Billette *et al.*, 1978). The PAL inactivating system described in sweet potato tubers

(Tanaka and Uritani, 1977) is very similar to that in sunflower leaf plastids. Trans- cinnamic acid, the product of PAL reaction, had been shown to play an important role in the modulation of PAL turnover (Shields, *et al.*, 1982; Bolwell *et al.*, 1986), by acting as an *in vivo* modulator of the synthesis of phenyl propanoid pathway enzymes.

Increased activity of PAL following excision of hypocotyls segments of dark-grown gherkin seedlings was prevented by the reaction product cinnamic acid. Density-labeling experiments show that cinnamic acid affects the rate of enzyme synthesis. By contrast, the regulation of phenylalanine ammonia-lyase activity by light has been shown to involve activation of existing inactive enzyme. It is proposed that regulation of synthesis by reaction products represents a mechanism for controlling the size of the pool of phenylalanine ammonia lyase, and that the activity of this pool is regulated by light (Johnson *et al.*, 1975). PAL is regulated through phosphorylation by a calcium dependent protein kinase (Allwood *et al.*, 2002).

The role of PAL in pathway regulation was investigated by measurement of product accumulation as a function of enzyme activity in a collection of near-isogenic transgenic tobacco plants exhibiting a range of PAL levels from wild type to 0.2%. The data indicated that PAL is a key step in the regulation of overall flux into the pathway and, hence, accumulation of major phenylpropanoid products, with the regulatory architecture of the pathway poised so that downstream steps control partitioning into different branch pathways (Bate *et al.*, 1994). The induction of L-phenylalanine ammonia-lyase activity during phaseollin biosynthesis in the *Phaseolus vulgaris--Colletotrichum lindemuthianum* interaction was regulated by an increase in enzyme concentration resulting from an increase in *de novo* synthesis of L-phenylalanine ammonia-lyase protein (da Cunha, 1988).

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A family of three genes encodes PAL. Liang *et al* (1989) had shown that the RNase protection with gene-specific probes that these genes are expressed differentially during development and in response to different environmental cues. While all three genes are expressed at high levels in roots, only PAL1 and PAL2 are expressed in shoots and only PAL1 is expressed in leaves. Mechanical wounding of hypocotyls induces all three genes, but fungal infection only activates PAL1 and PAL3. Illumination of etiolated hypocotyls activates PAL1 and PAL2 but not PAL3. Thus selective expression of PAL genes encoding functional variants is governed by a complex set of regulatory networks for developmental and environmental control of phenylpropanoid biosynthesis (Liang *et al.*, 1989).

To understand the regulation of phenylalanine ammonia-lyase (PAL) activity in the corn smut fungus, *Ustilago maydis*, Kim *et al* examined the effects of different media, metabolic effectors (including aromatic amino acids), and environmental factors on induction and repression of PAL activity. PAL was detected only in cell extracts and not in the culture medium. *U. maydis* PAL is constitutively produced at a low level in all media tested but its regulation can be influenced by aromatic amino acids. L-Tryptophan (0.3 mM) induces PAL activity 3- to 5-fold but tryptophan analogs and tryptophan-related metabolites do not. The enzyme is most readily induced during the early stationary phase of growth and the induced activity remains relatively constant during stationary stage. PAL induction was repressed by glucose but not by its reaction product, t-cinnamic acid. Induction did not require *de novo* protein synthesis, suggesting that some form of post-translational protein modification or a metabolic effect may be involved. This study shows that the regulation of *U. maydis* PAL is very different from the patterns known for plants and other fungi (Kim *et al.*, 2001).

According to Berglund (1996), vanadylsulfate increased PAL activity. PAL and phenolic compounds were studied in five potato cultivars (Agria, Cara, Liseta, Monalisa, and Spunta). PAL showed an induction pattern characterized by the presence of a maximum peak of activity after 4 days of minimal processing for all of the cultivars (Cantos *et al.*, 2002). Total phenolic content of soybean leaves increased following chitosan and chitin oligomer treatments, showing a positive correlation between enzyme activity and total phenolic content (Khan *et al.*, 2003). The effects of exogenously added L-phenylalanine (L-Phe) on the activities of PAL and 3, 4-dimethoxybenzyl alcohol (veratryl alcohol, VA) biosynthesis in ligninolytic cultures of *Phanerochaete chrysosporium* were investigated. Increasing PAL activity was detected in low nitrogen (LN) culture but not in high nitrogen (HN) culture. The addition of L-Phe into the LN culture caused a 25-fold increase in enzyme activity, which clearly shows that L-Phe, a substrate of the enzyme, served as an inducer of PAL. The increase in activity of PAL triggered by nitrogen starvation was correlated with biosynthesis of VA. However, PAL induced by the added L-Phe did not promote VA biosynthesis but suppressed the biosynthesis probably due to NH (4) (+) released from L-Phe (Hattori *et al.*, 1999).

Phenylalanine formed by two metabolic origins is subjected to phenolic biosynthesis, initiated by PAL, via the Shikkimate pathway. Primary origin, which refer to phenylalanine molecules, which remains unused in proteins, and secondary origin, which refers to those molecules of amino acid, released from protein in course of their catabolism. Margna *et al* (1989) attempted to differentiate between the L- phenylalanine of primary and secondary origin, by a combined treatment of L-AOPP, a potent inhibitor of PAL and glyphosphate, which specifically blocks the synthesis of aromatic amino acids. It was concluded that precursor pool available for phenolic synthesis consisted mainly of phenylalanine of primary origin in buckwheat

hypocotyls, while majority of phenolics were synthesized from phenylalanine of secondary origin.

2.7. Kinetics

Studies on initial velocity versus substrate concentration of PAL purified to homogeneity from the acetone-dried powder of the mycelial felts of the plant pathogenic fungus *Rhizoctonia solani* have shown significant deviations from Michaelis-Menten kinetics. The apparent Km value increases from 0.18 mM to as high as 5.0 mM with the increase in the concentration of the substrate and during this process the V_{max}, increases by 2-2.5-fold (Kalghatgi & Subba Rao, 1975). Margna (1977) had proved that substrate supply (Phe) rather than enzymic activity (PAL) is the most likely limiting factor in controlling phenylpropanoid accumulation. Thus administration of phenylalanine into seedling tissues can bring about a considerable increase in the accumulation of anthocyanins, with a low PAL activity (Ahmed & Swain, 1970). Mustard leaf PAL showed a K_m of 0.18 mM (Lim *et al.*, 1998), where as in *Phaseolus vulgaris* four values ranging from 0.07-0.302 mM which are indicative of isoforms of PAL were reported (Bolwell *et al.*, 1985) reported. Negative cooperativity (Hill coefficient, n = 1.08) was not detected over the substrate concentration range tested (Gupta & Acton, 1979). Most reported K_m fall midway in the range 0.3×10^{-4} to 1.5×10^{-2} M (Neish, 1961; Havir, and Hanson, 1968; Attridge *et al* 1971; Havir, *et al.*, 1971). In a detailed study of the kinetics of highly purified PAL from potato, Havir and Hanson (1968) have shown that the anomalous kinetics is replaced by Michaelis-Menton Kinetics in the presence of D- phenyalanine, a competitive inhibitor. Since V_{max} is not reduced under these conditions, the behavior of PAL is best explained in terms of allosteric interactions. While the enzyme may exist in two or more conformations with characteristic kinetic properties, the binding of D-phenylalanine causes a single form to predominate which displays simplified kinetics.

2.8. Inhibitors

The strongest inhibitors found so far for PAL are either cinnamic acid itself or hydroxy cinnamic acids (Camm & Towers, 1973). The hydroxylamine analogue of phenyalanine, L-alpha-Amino-oxy-ß-phenylpropionic acid, (AOPP) was found to be an extremely powerful inhibitor of PAL of higher plant and fungal origin (Amerhein & Godoke, 1977).

L (-) -, and D (+)-enantiomers of 1-amino-2-phenylethylphosphonic acid (PheP), a phosphoric analogue of phenylalanine, also inhibit the activity of potato tuber tissue *in vitro*. The apparent type of inhibition depends on concentration of PheP; as the concentration of D-PheP is raised from 10(-5) M to 2.5 X 10(-3) M, the type of inhibition shifts from competitive through mixed and non-competitive to uncompetitive. It specifically blocked light-induced phenylpropanoid synthesis in excised buckwheat hypocotyls and produced an up to 40-fold increase in the endogenous phenylalanine concentration, while the level of all other amino acids was hardly affected (Hollander *et al.*, 1979). Treatment of intact hypocotyls of etiolated gherkin seedlings with the PAL inhibitors alpha-aminooxyacetic acid and L-alpha-aminooxy-beta-phenylpropionic acid during illumination caused enhanced formation of PAL and reduced the accumulation of hydroxycinnamic acids. PAL formation in the segments is inhibited by cinnamic acid and, to a lesser extent, *p*-coumaric acid, while it is slightly enhanced by caffeic acid and is not affected by ferulic acid (Amrhein & Gerhardt, 1979).

The conformationally restricted phenylalanine analogue 2-aminoindan-2-phosphonic acid (AIP) inhibits PAL from parsley competitively in a time-dependent manner. A non-dialysable inhibitor of PAL has been partially purified from dark-grown gherkin hypocotyls (MW less than 20 000), thermolabile, sensitive to proteolysis digestion, and apparently hydrophobic.

Kinetic experiments show that the inhibitor is competitive with phenylalanine for the lyase and that its association with the lyase is reversible. The activities of phenylalanine ammonia-lyase and cinnamic acid 4-hydroxylase are often concurrently regulated and both have regulatory roles in phenol metabolism; it is suggested that the inhibitor may be specifically involved in controlling their activities *in vivo* (Billett, *et al.*, 1978). PAL activity in radish seedlings was inhibited by the competitive inhibitor AIP (Chen & Clure, 2000).

The influence of the allelochemicals, ferulic and vanillic acids, on PAL, activity and their relationships with phenolic acid content and root growth of soybean (*Glycine max* (L.) Merr.) were examined by Herrig and coworkers (2002). Both compounds (at 0.5 and 1mM) decreased root length, fresh weight and dry weight and increased phenolic acid contents. At 1mM, ferulic acid increased (82%) while VA reduced (32%) PAL activities.

2.9. Light

The normal development of higher plants occurs only in light (photomorphogenesis). The effect of light is due to intracellular development of a morphogenetically active effecter molecule (Pfr, a chromoprotein). The photomorphogenesis (= development through the presence of Pfr of the pattern due to the primary differentiation) is to be attributed to differential enzymatic induction and repression. This opinion is confirmed by examples. The correlation between the enzymatic activity and the structure (form, shape) still remains an unsolved problem. However, the elimination of other control mechanisms leads us to the conclusion that phytochrome most probably does control synthesis of this enzyme in mustard, which agrees with the previous findings for parsley cells (Acton & Schopfer, 1975).

Blue light and UVB light induction experiments using red light and dark grown seedlings showed that the flavonoid biosynthetic genes are

induced most effectively by UVB light and that blue light induction is mediated by a specific blue light receptor (Kubasek *et al.*, 1992). Kubasek *et al* show that seedlings exhibit a transient potential for induction of four genes namely PAL, chalcone synthase, chalcone isomerase and dihydroxyflavanol reductase which is distinct from that observed for chlorophyll a/b-binding protein (CAB). The potential for flavonoid gene induction was similar in seedlings grown in darkness and red light, indicating that induction potential is not linked to cotyledon expansion or the development of photosynthetic capacity. Evidence for metabolic regulation of these enzymes was also proposed by them (Kubasek *et al.*, 1998).

In dark-grown *Raphanus sativus* seedlings the level of PAL was higher in cotyledons than in root and hypocotyls (Amrhein & Gerhardt, 1979). Only PAL was significantly increased by light (Tome *et al.*, 1975). Enzyme activity in excised hypocotyl segments floating on buffer increased in the dark as well as in the light, while hydroxycinnamic acids accumulated only in the light. The extractable activity of PAL and the concentration of sugar esters of p-coumaric and ferulic acids in the hypocotyls of etiolated gherkin seedlings increase upon irradiation with white light. L-alpha-Aminooxy-beta-phenylpropionic acid (AOPP), a potent competitive inhibitor of phenylalanine ammonia-lyase (PAL), blocked light-induced phenylpropanoid synthesis in excised buckwheat hypocotyls. In the presence of AOPP, illuminated hypocotyls accumulated nearly 3 times more phenylalanine than hypocotyls kept in the dark, indicating an enhancing effect of light on the flow of carbon through the shikimate pathway (Hollander *et al.*, 1979). The activity of PAL during the life cycle of barley plants (*Hordeum distichon* L.) exposed to UV-A radiation (355nm) during 15, 30 and 60 min day^{-1} was studied. In comparison with the control plants, a stimulatory effect on PAL activity was observed. This effect was directly related to the exposure time to UV-A radiation (Baztan, and Torres, 1988).

MATERIALS AND METHODS

Neema Antony "Investigations on the Biosynthesis of Curcumin in Turmeric (Curcuma longa L.)" Thesis. Indian Institute of Spices Research , University of Calicut, 2005

MATERIALS AND METHODS

CONTENTS

MATERIALS AND METHODS

3.1. General

3.1.1. Propagation of experimental plants

For the major experiments, the released varieties, viz., Alleppey, Prabha, Prathibha, Suguna and Sudarsana from Indian Institute of Spices Research, Calicut were used. In addition, fifteen more accessions were also taken for further experiments on Phenylalanine Ammonia Lyase (PAL).

Rhizomes from the above varieties /accessions were planted in 3m x 1m beds @ 1kg per bed during June - July of each year. The plants were grown under normal cultural and management practices. For radiotracer studies, which were carried out at CWRDM, Calicut, potted plants were used. For light studies on PAL, rhizomes were sown in polybags and were kept in dark for germination.

3.1.2. Selection and mode of sampling

Plant tissues were sampled specifically for each experiment. For primary and secondary metabolites, all tissues except psuedostem were used, while for PAL assay, localization, purification and for RNA isolation, only turmeric leaves were sampled. Incorporation studies using labelled compounds were done using all the vegetative parts of the plant.

For primary and secondary metabolites, samples were collected at 120 Days after sowing (DAS). As for PAL assay, leaf samples were taken at monthly intervals starting from 30DAS upto 180DAS. Since PAL activity was found to be maximum in the early germination phase, leaves were sampled at

60DAS for localization and purification studies. In all the three tracer experiments, two months old plants were taken.

Dried and powdered samples were used for the analysis of oleoresin and curcuminoids. Data were compiled from three consecutive planting seasons, for the majority of the experiments.

3.2. Primary metabolites

Starch

For the estimation, 100mg sample was homogenized in hot 80% ethanol to remove sugars and centrifuged. The residue was washed repeatedly with hot 80% ethanol till the washings did not give colour with anthrone reagent. To the dried residue, 5.0ml of water and 6.5ml of 52% perchloric acid were added and kept at 0°C for 20min to complete hydrolysis. Centrifuged and the supernatant was collected. The extraction was repeated using fresh perchloric acid and the supernatants were pooled and made up to 100ml. 0.1ml aliquots were used for starch estimation using anthrone reagent by the method of Hodge and Hofreiter (1962).

Dry recovery

Accurately weighed samples from five varieties of turmeric rhizomes during developmental stages were dried to constant weight in hot air oven at 50°C (ASTA, 1978). The percentage dry recovery was calculated gravimetrically.

3.3. Secondary metabolites

3.3.1. Extraction, TLC and HPLC of phenolic acids

3.3.1.1. Extraction

5g of turmeric samples (leaf, pseudostem, rhizome and root) was extracted with 20ml of 2N Hydrochloric acid and digested in a boiling water bath for 20 min. The filtrate was extracted thrice with 10ml diethyl ether in a separating funnel. The ether extracts were pooled and extracted twice with 10ml 5% sodium carbonate. Sodium carbonate fractions were pooled and acidified to pH 3 with 5% sulphuric acid, which was then extracted thrice with 10ml diethyl ether. The ether layer was evaporated to dryness and then dissolved in 0.5ml alcohol (Bate & Smith, 1954) and stored for further analysis, by TLC and HPLC.

3.3.1.2. Separation and identification using TLC

TLC plates of thickness 1mm were casted with Silica gel G. Phenolic acid samples were spotted on the plates along with standards viz., coumaric acid, caffeic acid and ferulic acid. Toluene: Acetic acid in the ratio 4: 1, served as the mobile phase and the plates were sprayed with a mixture of 20% sodium carbonate and Folin's reagent in the ratio 1:2 respectively. Blue coloured spots appeared indicating the presence of phenolic acids, which were compared with values of authentic standards.

3.3.1.3. Separation of phenolic acids using HPLC

Preparation of Standard solution

Standards of each of the phenolic acids (1mg/ml) (Sigma), viz. p-coumaric acid, ferulic acid, caffeic acid, sinapic acid and chlorogenic acid were prepared. 20 µl of each standard was injected.

Chromatographic conditions

Shimadzu LC- 10At vp High Performance Liquid Chromatograph was used. The UV detector was Shimadzu SPD-SAV. The stationary phase was Octa Di Silane (ODS), pre-packed in a 250x45mm ID column. The mobile phase was 0.1% phosphoric acid (A) and 100% acetonitrile (B). With a concentration gradient as given below. Flow rate was 1 ml/min.

Time (min)	Mobile phase
0.00-0.01	5 % B
0.01-5.00	5 % B
5.00-10.00	10% B
10.00-25.00	15% B
25.00-45.00	40% B
45.00-55.00	95% B
55.00-65.00	5 % B

20µl of the sample prepared as above was injected into HPLC (Shipkova *et al.*, 1998).

3.3.2. Oleoresin

Dried and powdered samples of rhizome and root were used. About 10g sample was weighed and transferred to the column. The column was filled with 3 times the weight of powdered sample of acetone and kept for overnight percolation. Acetone was drained into a pre weighed beaker and column was washed twice with 15ml acetone. The extracts were pooled and evaporated at 80°C over a water bath. Oleoresin was determined gravimetrically (ASTA, 1978).

3.3.3. Essential oil (Modified Clevenger Method)

To determine the amount of water insoluble steam volatile oil, 30g sample was weighed and transferred to a short neck, 1-liter round-bottom flask with 500ml water. The apparatus was assembled using the proper Clevenger trap (ASTA, 1978) and was heated with stirring for 3hrs. Oil was collected in the trap until two consecutive readings taken at one-hour intervals showed no change of oil volume in the trap. The oil collected was noted estimating to the nearest 0.02ml.

3.3.4. Extraction and estimation of curcumin

3.3.4.1. Separation by thin layer chromatography

Weighed amount of oleoresin fraction extracted as above was dissolved in 0.5ml of alcohol and was spotted on silica gel G plate (1mm thickness), along with standard curcumin. The solvent system was methanol: water in the ratio 95: 5. Three yellow fluorescent spots were identified on drying. The R_f values of each spot from the origin was calculated and compared with those of standards (Srinivasan, 1953).

3.3.4.2. Quantification by HPLC

Preparation of standard graph

1mg of pure curcumin - (Sigma, USA) was dissolved in 50ml alcohol. Curcumin, demethoxycurcumin and bismethoxycurcumin were analyzed by HPLC. Standard graphs were drawn for the three curcuminoids with concentrations ranging from 10, 20, 40, 80, 100, 200, 300, and 400ng/ml.

Sample Extraction

To 100mg each of dried powder from leaf, psuedostem, root and rhizome. 30ml of alcohol was added and subjected to steam distillation for $2^1/_2$hrs. This was made up to 100ml with alcohol and 1ml of this was diluted 12.5 times with alcohol. 20µl samples of standard curcumin and above test sample were injected into the column for analysis.

Chromatographic conditions

Shimadzu LC- 10 AT VP high performance liquid chromatograph was used. The fluorescence detector was RF- 10A-XL. The stationary phase was Nucleosil NH2 (Shimpak -CLC), particle size 5µm, pre-packed in a 250 x 4.6mm. I.D. column. The mobile phase was ethanol, with a flow-rate of 0.6 ml/min. A rheodyne injector with a 20µl loop was used. The analyses were carried out at ambient temperature. Detection conditions: Fluorimetric, excitation wavelength 420nm, and emission wavelength 470nm (Tonnessen & Karlsen, 1983).

3.4. Tracer Studies

3.4.1. Exposure of turmeric plants to ^{14}C- labelled carbon dioxide

Turmeric rhizomes (var. Prathibha) were sown in polybags. Two months old turmeric plants of uniform size (30 Nos.) were selected for the exposure studies. Seedlings were placed in a persplex glass chamber (1m x 0.5m x 0.5m) designed for the purpose. ^{14}C- labelled sodium carbonate ($Na_2{}^{14}CO_3$), 0.5mCi, purchased from BRIT (Board of Radiation and Isotope Technology), BARC, Mumbai was placed in a vial in the center of the chamber and concentrated hydrochloric acid was added drop by drop through a slit provided at the top. Addition of 1ml of conc. hydrochloric acid can release 967

x 10 ^{17}, ^{14}C- atoms as carbon dioxide, which will be available for incorporation (Dixit & Srivastava, 2000).

$$Na_2{}^{14}CO_3 + 2HCl \rightarrow 2\ NaCl + {}^{14}CO_2 + H_2O$$

The plants were allowed to assimilate the ^{14}C labelled carbon dioxide for 24hrs. At the end of this period, a saturated solution of KOH was added into the central vial containing the radio labelled isotope and left for 15min. to absorb excess $^{14}CO_2$. The chamber was then opened.

Leaf, pseudo stem, rhizome and root samples were collected at 0hr, 24hrs, 48 hrs, 96hrs, 1week and then every one month interval, till 6th month for phenolic acid and curcumin analysis. Fresh samples were used for phenolic acid extraction while dry and powdered samples were used for curcumin analysis.

3.4.2. Exposure of turmeric plants to 1-^{14}C-phenylalanine

Two months old turmeric plants as above were selected for the studies. The roots were immersed for 48 hrs in a trough containing L- (1-^{14}C) labelled phenylalanine (50µCi) (Amersham Pharmacia) dissolved in 100ml-distilled water for absorption. Leaf, pseudostem, rhizome and root samples were collected at 0hr, 48hrs, 1week and then every one-month interval till 5th month for detecting the incorporation of the label in phenolic acids. Only rhizomes and roots were sampled for detecting the incorporation in curcumin.

3.4.3. Exposure of turmeric plants to 2-^{14}C-malonyl CoA

Two months old turmeric plants raised in poly bags as above were selected for the studies. The roots were immersed for 48hrs in a trough containing (2-^{14}C) labelled malonyl coenzyme A (5µCi) (Amersham

Pharmacia) dissolved in 100ml water for absorption. Incorporation was detected in curcumin, extracted from the rhizome and root at specific intervals.

3.4.4. Measurement of incorporation using Liquid Scintillation Counter-Wallac Model-1410

Phenolic acids

0.2ml of the phenolic acid extract was made up to 4ml with distilled water and to this 6ml of scintillation cocktail consisting of naphthalene and primary and secondary flours namely 2,5-diphenyloxazole and 2,2'-p-phenylene-bis-5-phenyloxazole in Dioxane were added (Michael, 1979). The activity was measured using liquid scintillation counter (Wallac, Finland Model- 1410).

Curcumin

4ml of the curcumin extract and 6ml of scintillation cocktail of the same composition described above was added and the activity was measured.

3.5. Phenylalanine Ammonia Lyase in turmeric leaves

3.5.1. Assay of phenylalanine ammonia lyase activity

Sample Preparation

Leaf tissue (2g) was homogenized with 5ml of 0.01 M borate buffer pH 8.8 in a chilled mortar at 4°C and centrifuged at 10,000g for 20min. The supernatant served as the enzyme source.

Procedure

The reaction mixture consisted of 1ml enzyme, 1ml borate buffer and 1ml of 0.15M -L- phenylalanine. The mixture was incubated at 38°C for 60 min. The reaction was stopped by the adding 0.5ml of 1M- trichloro acetic acid and incubated at 37°C for 5min. The amount of trans-cinnamic acid produced was determined in an UV-160A Shimadzu spectrophotometer at 270nm (Brueske, 1980). In control, phenylalanine was added after arresting the enzyme activity by adding TCA and kept for 1-hour incubation along with test. A standard graph of different concentrations ranging from 10 - 60µmoles of trans-cinnamic acid was prepared from the working standard with a concentration of (100µmoles/ml). The specific activity of PAL was expressed as µg of trans-cinnamic acid released per minute per mg protein.

3.5.1.1. Estimation of protein

During the studies on PAL, the determination of protein formed a routine procedure. This was necessary for calculating the specific activity of the enzyme at every stage of the study and for calculating the fold enrichment during enzyme purification. In all studies, total protein was estimated in the tissue by the colorimetric method of Lowry *et al.*, (1951).

Bovine serum albumin (BSA) was used as standard. A working standard containing 100µg/ml BSA was used for this purpose.

3.5.2. Localization of phenylalanine ammonia lyase

3.5.2.1. Isolation of microsomal fractions

Turmeric leaves (30g) were homogenized using an ultra turra blender (4 x 15 s) and grinding medium containing 100mM tris-hydrochloric acid (pH 7.6) in the cold room (4°C). Homogenate was centrifuged at 12,000g for 15min. From the above-prepared sample, supernatant (approx. 15ml per tube) was immediately layered onto a 30% (w/w) sucrose solution (10ml) in a 38ml centrifuge tube. After centrifugation in a Beckman rotor (SW-27) for 35min at 27,000 rpm, microsomes were removed from the 6-30% interphase and were carefully brought to a final concentration of 18% sucrose. This suspension was then placed at the 15-20% sucrose interphase of a gradient consisting of 39% sucrose (4ml), 37.5% sucrose (4ml), 20% sucrose (6ml) and 15% sucrose (12ml). Centrifugation was performed at 5000 rpm (10min), then at 10, 000 rpm (15min), and at 27,000 rpm (55min) (Czichi and Kindl, 1977). Subsequently, microsomes were recovered from the 20-37.5% sucrose interphase. Phenylalanine ammonia lyase activity in the microsomal fraction was estimated along with the microsomal marker enzymes viz.; fumarase, catalase and glucose-6- phosphatase.

3.5.2.2. Sub fractionation of microsomes

Turmeric leaves (25g) were homogenized in a grinding medium containing 50mM tris-hydrochloric acid (pH-7.9) and centrifuged at 12,000g for 15min. Extract was layered on a gradient consisting of 28ml 15-45% (w/w) linear sucrose gradient and a 5ml top layer of 8% sucrose. All sucrose solutions were contained in 10mM tris -hydrochloric acid, pH - 7.5, 1mM potassium chloride, 1mM EDTA, 0.1mM magnesium chloride. Gradients were centrifuged at 27,000 rpm for 4 hrs (Czichi and Kindl, 1977). All gradients used for the isolation of microsomal subfractions were fractionated by

collecting successive 1.2ml samples using micropipette. PAL activity was measured in each fraction and also the presence/ absence of activity of the marker enzymes, namely fumarase, catalase and glucose-6-phosphatase was also measured.

3.5.2.3. Assay of fumarase (Mitochondrial marker)

Reaction mixture contained 3.6ml buffer pH 8.4, 10µl extract and 10µl 1M malate (15mM final molarity). Reaction started with the addition of malate and activity was measured as the increase of OD at 240nm, based on fumarate formation (Beeckmans and Kanarek 1982).

3.5.2.4. Assay of catalase (Cytosolic marker)

Reaction mixture contained 2ml of buffer (phosphate buffer pH 7.2), 1ml of distilled water, 0.5ml of 0.1% H_2O_2 and 25µl of sample (enzyme extract). The rate of reaction was measured at 15sec. intervals for 90 sec., with a lag time of 10 sec. at 230nm (Luck, 1974).

3.5.2.5. Assay of glucose- 6-Phosphatase activity (Microsomal marker)

Reaction mixture contained 0.6ml 0.1M tri acetate of sodium cacodylate, pH 6.5, 0.2ml 0.15M glucose-6-phosphate pH 6.5, 0.6ml distilled water and 0.1ml of the fraction, which was incubated at room temperature for 30 minutes. Reaction was stopped by adding 1ml of 10% TCA (w/v). All tubes were centrifuged at 8000g for 5min. Inorganic phosphate liberated in the reaction was measured using Fiske & Subbrao method (1925).

3.5.3. Purification of phenylalanine ammonia lyase

In order to study the properties and kinetics of PAL, an enzyme fraction, which was relatively pure, was necessary. Attempts were made to purify the enzyme as detailed below.

Leaf homogenate was filtered through a muslin cloth, followed by centrifugal clarification at 20,000g for 20min. and was subjected to purification procedures *viz.* ammonium sulphate fractionation, dialysis, ion exchange chromatography and sephacryl S-300 gel filtration chromatography. The enrichment achieved, together with the quantitative recovery of the enzyme activity in these steps was calculated. All steps were carried out at 4°C (Whetten and Sederoff, 1992).

Sample Preparation

All purification steps were carried out in a cold room at 4°C. Approximately 50g of frozen leaf was ground to a powder in a waring blender cooled with liquid nitrogen. The powder was then transferred to a glass beaker and a two - fold (v/w) excess of buffer containing 0.1M potassium phosphate (pH 7.5) with 5mM dithiothreitol was added. The frozen mixture was stirred gently with a spatula until the buffer thawed, then squeezed through 10 layers of cheesecloth (premoistened with the same buffer) and filtered through one layer of miracloth. The filtrate was centrifuged at 20,000g for 30min.

3.5.3.1. Ammonium sulphate fractionation

One of the most versatile steps in the purification of enzymes is the salting out using ammonium sulphate. Ammonium sulphate is a particularly useful salt for the fractional precipitation of proteins. From the above supernatant, a small portion (2ml) was set aside for the estimation of protein

for basal enzyme activity measurements, and the bulk (98ml) of the supernatant was taken in a beaker. Solid ammonium sulphate (enzyme grade) for 20% ammonium sulphate saturation was weighed and added to the supernatant slowly with continuous mixing by means of a magnetic stirrer. The suspension was kept in the cold to allow flocculation of proteins. It was then centrifuged at 15,000 g for 15 min in a refrigerated centrifuge. The supernatant was collected and precipitate (F_1) was suspended in a medium, which contained 20mM tris-hydrochloric acid (pH 7.5) buffer with 5mM DTT and 10% (v/v) ethylene glycol dissolved in it (Buffer B). The supernatant was brought to 30% ammonium sulphate saturation by further addition of solid ammonium sulphate followed by centrifugation and collection of the supernatant. The precipitate F_2 was dissolved in the same buffer as F_1. Same steps were repeated for 40%, 50%, 60%, 70% and 90% saturation conducted as above and the precipitates (F_3, F_4, F5, F_6 and F_7 respectively) were resuspended as earlier, after each step. The final supernatant was discarded. All the suspensions were dialyzed against two changes of buffer B, centrifuged and the supernatants were collected and used for enzyme studies. The enzyme activity in the six residual fractions and in the original supernatant was determined after appropriate dilutions.

From the preliminary experiments on ammonium sulphate fractionation, seven saturation levels were tested for enzyme activities, namely from 0-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70% and 70-90%. Of these, 4 fractions precipitating between 40 and 70% of saturation was taken for further purification, as these fractions possessed PAL activity. In order to recover most of the enzyme in a single fraction, but at the same time to exclude unwanted proteins, the following procedure was adopted. The ammonium sulphate saturation was brought initially to 30% and the precipitate was discarded. The saturation was then raised to 40, 50, 60 and 70% and precipitate was collected in each step. Precipitate from 40% to 70%

fraction was resuspended in a minimum volume of buffer B and dialyzed against two changes of buffer B and was used in the subsequent experiments.

3.5.3.2. DEAE-Sephacel column chromatography

Column preparation

200g of the preswollen DEAE Sephacel (Sigma) was suspended in 500ml water in a beaker and washed with 500ml water in a funnel to remove the methanol. This was suspended in 500ml water, sonicated for 2min. and allowed the slurry to settle for 30min. The sediment was again resuspended in 500ml of 0.4M sodium chloride in 20mM tris-hydrochloric acid (pH 7.5), (strongest eluent used during chromatography), sonicated for 2min. and kept aside for 30min. Decanted off the fines and washed with 500ml 20mM tris-hydrochloric acid (pH 7.5) buffer containing 10 (v/v) ethylene glycol and 5mM dithiothreitol which is the starting buffer and a slurry (10% w/v) was made. The slurry was poured into the column using blunt end of the glass rod by opening the stopcock upto a total height of 20cm. The liquid level was decreased to just above the adsorbent.

Enzyme purification

Insoluble material after dialysis was removed by centrifugation at 14,000g for 20min. The supernatant was diluted to a protein concentration of less than 15mg/ml and loaded onto a 2.5 x 20cm column of DEAE-Sephacel. The column was washed with buffer containing 20mM tris-hydrochloric acid (pH 7.5) until the protein content of the effluent returned to baseline level, determined by an on-line UV monitor. A linear gradient elution with sodium chloride concentration ranging from 0.1 to 0.4M in 250ml each of buffer containing 20mM Tris-Hydrochloric acid (pH 7.5) was performed and finally by buffer containing 400mM tris-hydrochloric acid (pH 7.5). Both buffers

contained 10v/v ethylene glycol and 5mM DTT and 5ml fractions were collected. Fractions containing PAL activity were pooled and concentrated to 1ml using centrifugal ultra filtration devices (Centriprep 30; Amicon).

3.5.3.3. Gel filtration using Sephacryl S-300

Column Packing

This step was rendered to purify the PAL fractions obtained after DEAE-Cellulose column chromatography, as the SDS-PAGE showed few light bands, which might be due to other proteins present in the fraction apart from PAL.

Sephacryl S-300 (pre swollen) in 20% ethanol was diluted with buffer D containing 20mM tris-hydrochloric acid, 5mM sodium bisulfate (pH 7.5) and 5mM DTT and was mixed to uniform suspension. The gel was packed into glass column, 50cm in length and 1.5cm in diameter, to a height of 40cm (total bed volume was about 80ml) using a blunt end glass rod, by continuously pouring without gap. The column was fixed vertically and the top layer of the gel was made perfectly horizontal. A filter paper disc was placed above the gel surface. All the steps were performed in the cold room at 4^0C. The stopcock was opened to drain off the equilibration medium above the filter paper. The column was washed with five times the column volume with the elution buffer.

Enzyme purification

1.0 ml of the sample was carefully layered above the filter paper using a pipette and an equal volume of the equilibration medium was drained off. The top of the column was carefully rinsed with a small quantity of equilibration medium and the same was drained off as before. Protein was eluted from the column using excess equilibration medium. A total effluent

volume equal to the void volume was drained off at a flow rate of 0.5ml/minute. Proteins were collected as single fraction of 5ml following the exclusion of the void volume with one -fold dilution. The fractions thus obtained which is almost colourless, served as the source of enzyme for preliminary studies. Fractions containing PAL activity were pooled and concentrated as before, and the purified enzyme preparation was stored at -70°C.

3.5.3.4. SDS-polyacrylamide gel electrophoresis of proteins

Electrophoresis was carried out to determine the molecular weight of the purified PAL protein (Laemmli, 1970)

Reagents

* *Acrylamide and N, N'-methylenebisacrylamide*: A stock solution containing 30g of acrylamide and 0.8g N, N'-methylenebisacrylamide was prepared in deionized, warm water (to assist the dissolution of the bisacrylamide) and made upto 100ml.
* *Sodium dodecyl sulfate (SDS)*: A 10% (w/v) stock solution was prepared in deionized water and stored at room temperature.
* *Tris buffer for the preparation of resolving gels*: 3M tris Hydrochloric acid, pH 8.8
* *Tris buffer for stacking gel- 0.*5M Tris hydrochloric acid, pH 6.8
* *TEMED (N, N, N', N'-tetramethylethylenediamine)*:
* *Ammonium persulfate*: 1.5% (w/v) stock solution was prepared in deionized water and stored at 4°C.
* *SDS Reservoir buffer*: 0.25M Tris, 1.92M Gly, 1% SDS, pH 8.3

Stock Solution	Separating Gel -10%	Stacking Gel -2.5%
Acrylamide: bisacrylamide	10ml	2.5 ml
Separating Gel Buffer Stock	3.75ml	-
Stacking Gel Buffer Stock	-	5.0ml
10% SDS	0.3 ml	0.2ml
1.5% APS	1.5ml	1.0ml
Water	14.45ml	11.3ml
Degas Mixture in Vacuum Pump		
TEMED *(N, N, N', N'-tetramethylethylenediamine)*	0.015ml	0.015ml
Total	30ml	20ml
Reservoir Buffer Stock- Diluted 20ml to 200ml before use		

Preparation of SDS-Polyacrylamide Gels

In an Erlenmeyer flask, appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel, using the values given in the above table was prepared. The mixture was swirled rapidly and poured into the gap between the glass plates, leaving sufficient space for the stacking gel. Using a pasteur pipette, 0.1% SDS (for gels containing $\leq$ 8% acrylamide) was carefully layered on the top. The gel was placed in a vertical position at room temperature. The overlay prevents oxygen from diffusing into the gel and inhibiting polymerization. After polymerization was complete (30 min), the overlay was poured off and the top of the gel was washed several times with demonized water to remove any unpolymerized acrylamide.

The stacking gel was prepared using the values given in table. The stacking gel solution was poured directly onto the surface of the polymerized resolving gel, without delay. Immediately insert clean Teflon comb was inserted into the stacking gel solution, care being taken to avoid trapping air bubbles. More stacking gel solution was added to fill the spaces of the comb completely. Place the gel in a vertical position at room temperature.

Sample preparation

The samples were prepared by heating them to 100°C for 3 min. in 1 x SDS gel-loading buffer to denature the proteins.

1 x SDS gel-loading buffer containing

- 50mM Tris.HCl (pH 6.8)
- 100mM Dithiothreitol
- 2% SDS (electrophoresis grade)
- 0.1% Bromophenol blue
- 10% Glycerol

(1 x SDS gel-loading buffer lacking dithiothereitol can be stored at room temperature. Dithiothreitol should then be added, just before the buffer is used, from a 1M stock.)

Sample containing marker proteins (Biogene, USA) of known molecular weights (Phosphprylase B- MW 97,400, Bovine serum albumin- MW 66,200, Glutamate dehydrogenase- MW 55,000, Ovalbumin- MW 42,700, Aldolase- MW 40,000, Carbonic anhydrase MW 31,000, Soyabean trypsin inhibitor- MW 21,500 and Lysozyme- MW 14,400) also were denatured as above.

Electrophoresis

After polymerization was complete (30min.), the samples (15µl) were loaded in a predetermined order into the bottom of the wells. An equal volume of 1 x SDS gel-loading buffer was loaded into any wells that are unused. Electrophoresis was run with a voltage of 8 V/cm to the gel. After the dye front had moved into the resolving gel, the voltage was increased to 15 V/cm and the gel was run until the bromophenol blue reached the bottom of the resolving gel (about 4 hours) (Hames, and Rickwood, 1994).

The glass plates were removed from the electrophoresis apparatus and one set of gels was stained with Coomassie Brilliant Blue and another set using silver staining.

Staining

Coomassie Brilliant Blue

0.25g of Coomassie Brilliant Blue R250 was dissolved in 90ml of methanol: H_2O (1:1 v/v) and 10ml of glacial acetic acid. The solution was filtered through a Whatman No. 1 filter to remove any particulate matter.

The gel was immersed in at least 5 volumes of staining solution and placed on a slowly rotating platform for a minimum of 4 hours at room temperature. Destaining of the gel was done by soaking it in the methanol/acetic acid solution.

After destaining, gels were stored indefinitely in water containing 20% glycerol in a sealed plastic bag without any diminution in the intensity of staining.

Silver Staining

50% methanol was added to gel @ 100ml/gel. Staining solution [Solution A - 0.4g $AgNO_3$ in 8ml water, Solution B - 42 ml 0.36 % NaOH and 2.8ml NH_4OH (1.8ml NH_4OH + 1ml H_2O] was added after keeping for one hr to fix the proteins (Solution A (8ml) and B (44.8ml) was mixed well and made upto 200ml) and shaken for 15min. Gel was washed twice with DDW for 5min. Developing solution (1.25ml 1% citric acid plus 0.125ml 37% HCHO 250ml) was then added and kept for 1-2min. or until bands appeared. Gel

was removed and washed with DDW and stored in 10% methanol (Hames, and Rickwood, (1994).

3.5.4. Kinetics and effect of external factors on PAL activity from turmeric leaves

For all studies on the properties of the purified phenylalanine ammonia lyase, the Sephacryl S-300 filtrate was used as the source of enzyme.

Determination of Michaelis-Menten constant (K_m Value)

The activity of purified PAL at varying concentrations of phenylalanine in the assay system ranging from 5mM to 100mM (0.1, 0.15,0. 25, 0.50, 0.75 & 1.00mM) was measured. The standardized assay system was used. The K_m value for phenylalanine was determined using the double - reciprocal method of Line Weaver-Burk (Dixon and Webb, 1979).

Effect of temperature

This was tested over a temperature range of 20°C to 50°C, while keeping the other assay conditions constant. The pooled active fractions of sephacryl S-300 gel filtrate were used as the source of enzyme. The system was incubated for 60 minutes at varying temperatures viz; 20, 25, 30, 35, 38, 40, 45 and 50°C and PAL activity was assayed using the method of Bruske, (1980).

Effect of pH

The reaction was conducted over a range of pH 2- 10 while keeping the other assay conditions constant. The pooled active fractions of Sephacryl S-300 gel filtrate were used as the source of enzyme. The system was incubated

for 60 minutes at different pH *viz.* 2, 4, 6, 7, 8, 8.8, and 10. PAL activity was assayed as above

Effect of inhibitors

PAL assay was conducted with the above purified sample with inhibitors at different concentrations *viz.* Cinnamic acid at concentration 0.03mM, 0.01mM, 0.3mM and 1mM and ferulic acid, coumaric acid and chlorogenic acid concentrations of 0.01M, 0.5M and 1M. These inhibitors were added along with the assay system before incubation

Effect of light on PAL Activity

Turmeric rhizomes var. Alleppey was germinated in dark (etiolated) for 21 days in polybags. From these, three sets were taken and were exposed to three sources of light viz. red, blue and white for 72hrs. The samples from each set were collected and PAL activity was estimated at intervals of 6hrs. A control was kept in dark and a control germinated in daylight was used as the dark and light control.

3.6. Amplification of PAL specific gene from turmeric plants

Plant materials

Turmeric plants were maintained in a green house at 26°C. Before RNA isolation, the leaves were thoroughly washed with diethyl pyro carbonate (DEPC) treated water, and were transferred to sterile plastic bags, immediately freezed using liquid nitrogen and stored at -80°C.

RNA isolation

2g of the frozen leaves were pulverized in the presence of liquid nitrogen in a pre-cooled mortar and pestle with 250mg PVPP (polyvinyl poly pyrollidone), transferred to a polypropylene tube containing 5ml of denaturing buffer. Added 0.5ml, 2M Sodium acetate (pH4.0) and mixed by inverting the tube. 5ml of water-saturated phenol was added and mixed the tubes gently by inverting. 1ml of chloroform: isoamyl alcohol (24:1) was then added. The tubes were incubated on ice for 20min., and centrifuged at 10,000g for 20min at 4°C. The supernatant was carefully transferred to a fresh polypropylene tube. An equal amount (5ml) of cold isopropanol was added, mixed well and incubated at -20°C for 1 hour to precipitate RNA. Centrifugation was done at 10,000g for 20min at 4°C. The pellet containing total RNA was dissolved in 1.5 ml of denaturing buffer and distributed equally in three microfuge tubes (0.5ml). Equal volumes of cold isopropanol was added to each tube, mixed well and incubated at -20°C for 1 hour, centrifuged at 10,000g for 15min at 4°C and supernatant was discarded. The RNA pellet was resuspended in 75% ethanol, incubated at room temperature for 15min, centrifuged for 10min at 10,000g at 4°C, dried under vacuums for 15min and dissolved in 50µl of nuclease free water or 100% formamide (for long time storage). Aliquots in 1.5ml tubes were stored at -80°C until use.

Purity of the RNA samples was estimated spectrophotometrically at the wavelengths of 260 and 280nm. (Sambrook *et al.* 1989).

Denaturing gel electrophoresis

To confirm the integrity of RNA samples from *Curcuma longa* L. extracted by the above protocol, they were resolved in 1% denaturing agarose gel and visualized by staining with ethidium bromide following the protocol by Sambrook *et al.* (1989).

Primer Designing

Primer designing for amplification of PAL sequence was done using Primer designing software (Premier).

The amino acid sequence used to derive primer: ^{338}HGGNFOG^{344}.

Nucleic acid sequence corresponding to the above conserved motif in rice chitinase gene ^{163}RGGNGGNAARTTRCA^{1177}

Forward Primer sequence used in amplification from mRNA 5'RGGNGGNAARTTRCA 3'

Reverse Primer used in amplification; oligo $(dT)_{15}$

The total length of the chitinase cDNA sequence is approximately 2150 for PAL and the region chosen for primer was at position 1163. Therefore the fragment of approximately 900bp was expected to be amplified using the above primers.

Reverse Transcription and PCR amplification

Primers were designed based on PAL gene sequences obtained from database. Reverse transcription was performed using oligo dT $_{(18)}$ primer. After annealing, the RNA strand was reverse transcribed with reverse transcriptase enzyme. The synthesized cDNA was used for subsequent PCR following the conventional reaction procedures.

First strand cDNA synthesis

In a 0.2ml tube, 1.5µL primer (50mM), Oligo dT $_{(18)}$ and 1µg of RNA were combined and adjusted the volume to 10µL with DEPC treated water. RNA and primer were denatured by incubating at 65°C for 2min and then kept on ice. To the above tube, 4µL of 5x cDNA synthesis buffer, 1.0µL of dNTPmix (10mM) (Finnzymes, Finland), 1µL of RNase inhibitor (40U/µL) (Bangalore Genie, India), 0.75µL of $MgCl_2$ (50mM) (Finnzymes, Finland) were added and the volume was made up to 19µL using DEPC treated water. The thermocycler was then programmed at 37°C for 60min. After incubating the tubes at 37°C for 10min, the thermocycler was paused and 1µL of Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase (100U/µL) (GenHunter Corporation, USA) was added and mixed before continuing incubation. At the end of reverse transcription, incubating the tubes at 75°C for 5min did the enzyme denaturation. The tubes were then spun briefly and stored at -20°C for later use.

PCR amplification

Only 10% of the cDNA synthesized (2µL), was used for subsequent PCR amplification. To the tube containing 2µL of the first strand reaction mixture, 2.0 µL of 10x DNA polymerase buffer, 1.0µL of dNTP mix (10mM), 0.75µL of $MgCl_2$ (50mM), 1.25µL Oligo dT $_{(18)}$ (250ng/reaction), 2.0µL of 2µM PAL specific primer (Bangalore Genei, Bangalore) and 1.5µL of DNAzyme EXT DNA Polymerase (1U/µL) (Finnzymes, Finland) were added and the volume was made up to 20 µL using nuclease free water. PCR amplification was carried out using the following thermocycling conditions: 94°C for 2 min, followed by 40 cycles of denaturing at 94°C for 30sec, annealing at 40°C for 2min and 68°C for 3min. The 40th cycle was followed by a final extension step at 68°C for 10min.

3.7. Statistical significance

Analysis of variance for all the parameters studied was done. The level of significance was determined using Duncan Multiple Range Test (DMRT). Values taken for statistical analysis are the mean of three observations.

RESULTS

Neema Antony "Investigations on the Biosynthesis of Curcumin in Turmeric (Curcuma longa L.)" Thesis. Indian Institute of Spices Research , University of Calicut, 2005

RESULTS

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RESULTS

4.1. Primary and Secondary Metabolites

Since the prime components for the biosynthesis of curcumin originate from the carbon source and starch being the major storage form of carbohydrate, it was felt necessary to carryout the analysis of the same and correlate the results with formation of secondary metabolites. As such, the levels of starch, along with essential oil, oleoresin and curcuminoids have been determined and observations recorded below.

4.1.1. Starch

Sampling was done from fourth month onwards (120 DAS). At this stage, the transformation of bulbs to rhizomes will be complete. The percentages of starch during rhizome development among the five varieties *viz.*, Prabha, Prathibha, Alleppey, Suguna and Sudarsana are shown in Table 1 and Fig.1. Statistical analysis indicated highly significant variation at all levels of comparison between the varieties studied and the respective stages. In general, there was a gradual increase in the starch content in all the varieties. However the rate of increase was higher between 180 & 210 DAS, while at full maturity the pattern varied between varieties. During rhizome development, Prabha and Prathibha showed a similar pattern with higher content of starch at full maturity (56-59%) and Suguna and Sudarsana showed lower levels (50-51.7%), with Alleppey showing an intermediate value. Lower values of starch at the initial stages indicated the diversion of carbon source to metabolites other than starch.

Table 1. Percentage of starch in turmeric rhizomes during plant growth

Varieties	120 DAS	150 DAS	180 DAS	210 DAS	240 DAS
Prabha	39.6	42.3	47.8	54.2	56.1
Prathibha	38.5	39.9	42.9	60.1	59.4
Alleppey	39.7	40.8	43.3	46.2	54.9
Suguna	39.6	42.7	44.6	52.1	51.7
Sudarsana	39.9	41.2	45.5	54.1	50.2

Values given are mean of three observations. DAS - Days after sowing

Table 1a. ANOVA

Dependent Variable: STARCH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
VARIETY	110.194	4	27.549	15.766	0.000 (HS)
STAGES	2829.482	4	707.371	404.829	0.000 (HS)
VARIETY * STAGES	412.621	16	25.789	14.759	0.000 (HS)
Error	87.367	50	1.747		
Total	166941.040	75			
Corrected Total	3439.663	74			

a R Squared =0 .975 (Adjusted R Squared =0 .962). HS= Highly Significant

4.1.2. Dry recovery

Along with starch, the pattern of dry recovery was also noted (Table 2 and Fig 2). Statistical analysis showed highly significant variation among the stages and varieties (Table 2a). The dry recovery showed an increasing trend during rhizome development, with varieties Suguna and Sudarsana showing minimum percentage (15.9% & 13.8% respectively). Prabha showed highest dry recovery (26.2%), followed by Alleppey (23.9%) and Prathibha (20.9%).

Table 2. Percentage of dry recovery of turmeric rhizomes during development

Varieties	120 DAS	150 DAS	180 DAS	210 DAS	240 DAS
Prabha	9.30	12.6	15.3	25.7	26.2
Prathibha	8.98	11.6	14.6	20.2	20.9
Alleppey	8.10	9.3	14.1	19.5	23.9
Suguna	8.60	9.8	11.9	13.5	15.9
Sudarsana	8.40	9.1	10.5	13.2	13.8

Values given are mean of three observations. DAS - Days after sowing

Table 2a. ANOVA

Dependent Variable: DRYRECOVERY

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
VARIETY	451.827	4	112.957	1391.482	0.000 (HS)
STAGES	1471.615	4	367.904	4532.100	0.000 (HS)
VARIETY * STAGES	283.384	16	17.711	218.183	0.000 (HS)
Error	4.059	50	0.081		
Total	17346.383	75			
Corrected Total	2210.885	74			

a R Squared =0.998 (Adjusted R Squared =0.997). HS - Highly Significant

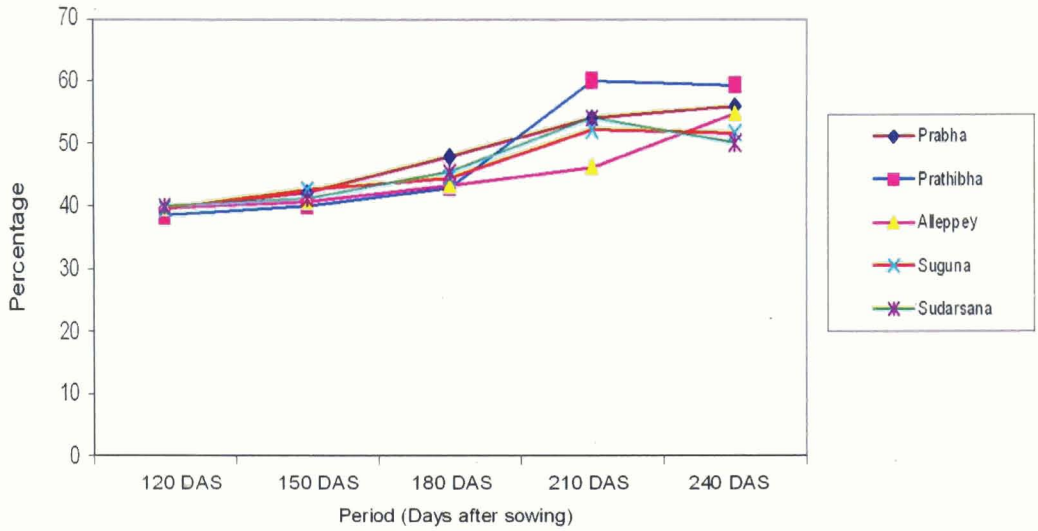


Fig. 1. Percentage of starch in rhizomes of released varieties of turmeric during development

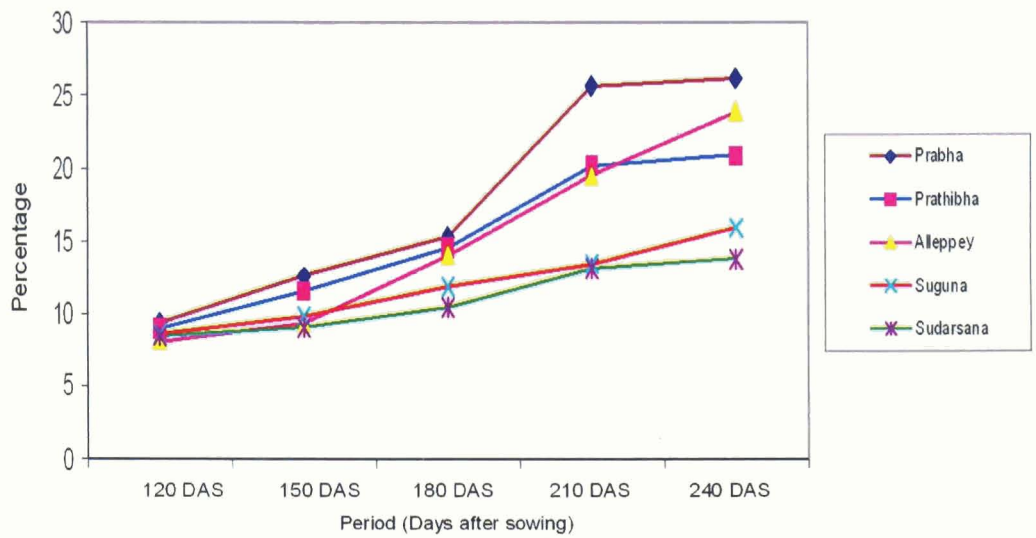


Fig. 2. Percentage of dry recovery in rhizomes of released varieties of turmeric during development

4.1.3. Phenolic acids

Separation and identification of phenolic acids in turmeric leaves, rhizomes and roots by TLC

Phenolic acids were extracted from the leaves, roots and rhizomes (var. Suguna) during the early germination phase (120 DAS), as described in Materials and Methods. TLC on silica gel showed the presence of caffeic, *p*-coumaric and ferulic acids to be the major phenolic acids in leaves. Slight traces of few other phenolic compounds were noted (Plate 5) whereas roots and rhizomes had similar pattern showing only caffeic acid and ferulic acid. Complete absence of higher phenolic acids viz. sinapic acid and chlorogenic acid were noted in all the three cases.

HPLC of phenolic acids in turmeric leaves, rhizomes and roots

Separation of phenolic acids in leaves, roots and rhizomes (var. Suguna) during early germination phase (120 DAS) showed specific peaks for caffeic, *p*-coumaric and ferulic acids. Leaves contained 0.70, 7.3 and 15% of *p*-coumaric, caffeic and ferulic acids. Rhizomes and roots contained 0.44 and 0.054% of *p*-coumaric acid, 9.44 and 0.373% caffeic acid and 0.158 and 0.077% ferulic acids respectively (Table 3. and Fig. 3). No significant peaks corresponding to sinapic acid was observed in the case of rhizomes and roots, whereas leaf sample showed a minor but non-significant peak.

Table 3. Percentage of phenolic acids in turmeric plant parts

Sample	*p*-Coumaric acid	Caffeic acid	Ferulic acid
Leaves	0.700	7.300	15.00
Rhizomes	0.440	9.440	0.158
Roots	0.054	0.373	0.077

Values given are mean of three observations

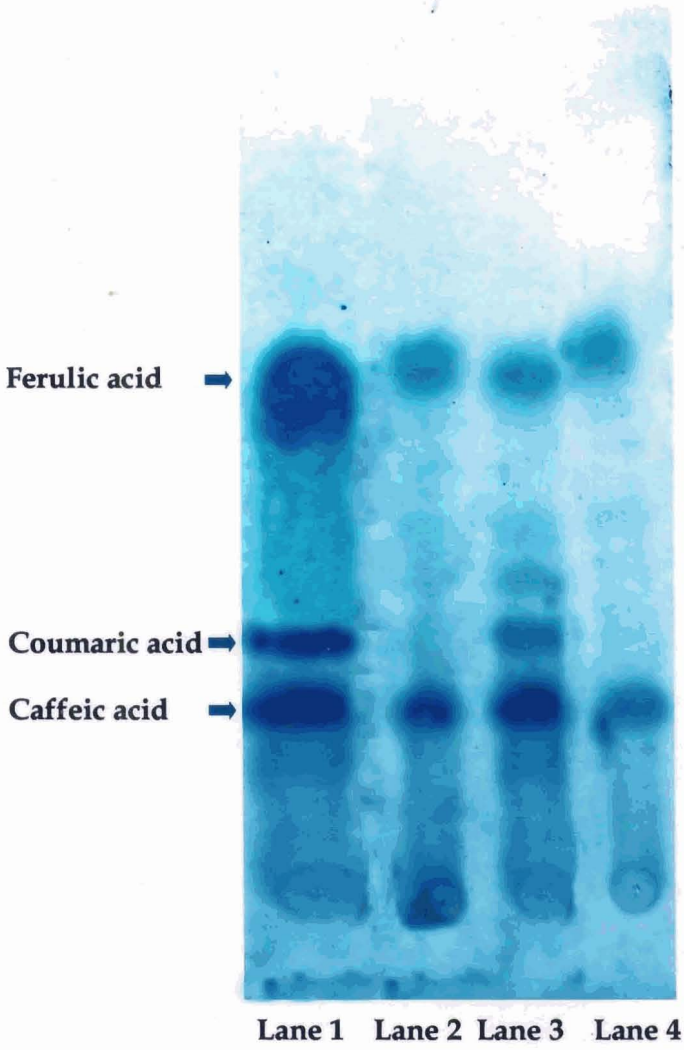


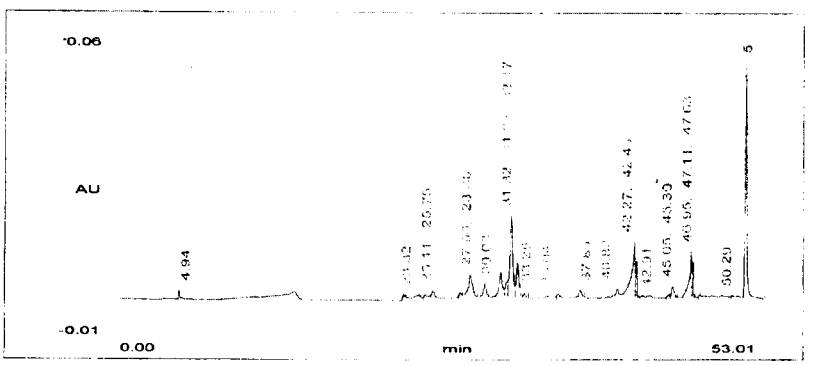
Plate 5. TLC pattern of phenolic acids in turmeric leaves

Lane 1 - Phenolic acid standards
Lane 2 - Rhizome
Lane 3 - Leaves
Lane 4 - Root

REPORT

Sample Name: sug-root-30.11.02-2ml **Data File: ...F2\sample06.DAT**
Method File: C:\Program Files\Indtech Instruments\Winchrom99\UV\UV DETECTOR.Met
Detector: UV-VIS. **System: HPLC**
Date: 9 Jul 2003 **Time: 12:59:12**
Run: ch1: 5
Type of Analysis : Percent On Area and Height
Type of Analysis : Percent On Area and Height

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
4	30	5	4	20.0



No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Area/ Ht
1	4.94	626	71484	1.5302	0.6759	BB	0.078
2	23.32	306	77211	0.7480	0.7300	BB	0.172
3	25.11	0	5714	0.0000	0.0540	BB	0.177
4	25.75	308	103985	0.7529	0.9831	BB	0.230
5	27.96	221	49331	0.5402	0.4664	BB	0.152
6	28.80	1159	578772	2.8330	5.4721	BB	0.341
7	30.03	695	218908	1.6988	2.0697	BB	0.215
8	31.32	1453	608389	3.5516	5.7521	BV	0.286
9	31.82	866	280055	2.1168	2.6478	VV	0.221
10	32.17	4640	1822946	11.3417	17.2354	VV	0.268
11	32.69	2004	750671	4.8984	7.0974	VB	0.256
12	33.25	159	41344	0.3886	0.3909	TTT	0.177
13	36.04	175	52949	0.4278	0.5006	BB	0.207
14	37.86	387	114342	0.9460	1.0811	BB	0.202
15	40.88	398	107082	0.9728	1.0124	BB	0.184
16	42.27	4009	1250023	9.7993	11.8186	BV	0.213
17	42.48	2829	432880	6.9150	4.0928	VB	0.104
18	42.91	0	3714	0.0000	0.0351	BB	0.141
19	45.05	0	9007	0.0000	0.0852	BB	0.154
20	45.39	582	191418	1.4226	1.8098	BB	0.225
21	46.95	3597	773240	8.7923	7.3108	BV	0.147
22	47.11	2683	305693	6.5581	2.8902	VB	0.078
23	47.63	0	7287	0.0000	0.0689	BB	0.111
24	50.29	0	3506	0.0000	0.0331	BB	0.126
25	51.40	13814	2716777	33.7660	25.6864	BB	0.134
		4e+04	10576728				

Fig. 3. HPLC pattern of phenolic acids from turmeric roots

4.1.4. Oleoresin

Table4. and Fig.4 show the percentage of oleoresin in turmeric rhizomes during the different developmental stages. Oleoresin showed a gradual decline as the rhizomes mature. Significant variations were observed in all cases (Table 4a). DMRT analysis of the results showed Suguna and Sudarsana having lower levels among the varieties, while similar pattern was observed in varieties Prabha and Prathibha. Highest content of oleoresin was obtained for var. Alleppey.

Table 4. Percentage of oleoresin in turmeric rhizomes during plant growth

Varieties	120 DAS	150 DAS	180 DAS	210 DAS	240 DAS
Prabha	24.01	17.95	14.00	9.36	6.69
Prathibha	20.88	17.95	14.90	10.72	9.48
Alleppey	23.96	19.38	18.90	11.96	10.72
Suguna	22.52	11.49	11.40	8.87	8.83
Sudarsana	17.55	15.42	12.60	9.09	9.64

Values given are mean of three observations. DAS - Days after sowing

Table 4a. ANOVA

Dependent Variable: Oleoresin rhizome

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
VARIETY	183.741	4	45.935	182.316	0.000 (HS)
STAGES	1591.307	4	397.827	1578.970	0.000 (HS)
VARIETY * STAGES	166.283	16	10.393	41.249	0.000 (HS)
Error	12.598	50	0.252		
Total	17369.430	75			
Corrected Total	1953.929	74			

a R Squared =0.994 (Adjusted R Squared =0.990), HS= Highly Significant

Table 5 and Fig. 5 show the percentage of oleoresin in turmeric roots, during development. Table 5a represents the ANOVA for the oleoresin values in roots for the different stages. It is seen that the percentage at 120 DAS varied from 8 to 10% while a steep increase was seen at 150 DAS. Thereafter the values were found to decline, which varied from 7.3 to 9.8% at 240 DAS. Here, higher values were shown by var. Suguna and Sudarsana as per DMRT.

Table 5. Percentage of oleoresin in turmeric roots during rhizome development

Variety	120 DAS	150 DAS	180 DAS	210 DAS	240 DAS
Prabha	8.0	12	9.6	10.0	7.3
Prathibha	8.6	14	10.0	11.0	7.8
Alleppey	9.0	15	12.0	8.5	8.8
Suguna	10.0	17	13.0	11.0	8.5
Sudarsana	9.6	14	15.0	9.6	9.8

Values given are mean of three observations. DAS - Days after sowing

Table 5a. ANOVA

Dependent Variable: Oleoresin ROOT

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
VARIETY	62.249	4	15.562	268.184	0.000 (HS)
STAGES	352.253	4	88.063	1517.598	0.000 (HS)
VARIETY * STAGES	68.371	16	4.273	73.640	0.000 (HS)
Error	2.901	50	0.058		
Total	9175.551	75			
Corrected Total	485.774	74			

a R Squared = 0.994 (Adjusted R Squared = 0.991), HS= Highly Significant

4.1.5. Essential oil

Essential oil percentage in rhizomes during plant growth among the five released varieties is shown in Table 6 and Fig. 6. Statistical analysis (ANOVA) is given in Table 6a. Higher levels of essential oil were seen at 180 and 210 DAS, which then showed a decline. All varieties showed a decrease at 120 DAS and 150 DAS, which then showed significant, increase, with a further decline at full maturity (240 DAS).

Table 6. Essential oil percentage in turmeric rhizomes during plant growth

Varieties	120 DAS	150 DAS	180 DAS	210 DAS	240 DAS
Prabha	3.6	3.0	4.3	5.3	3.0
Prathibha	4.0	3.6	4.3	5.3	3.0
Alleppey	4.6	4.0	5.0	5.4	3.3
Suguna	3.6	2.6	4.0	4.0	3.0
Sudarsana	4.0	3.8	5.0	5.0	3.3

Values given are mean of three observations. DAS - Days after sowing.

Table 6a. ANOVA
Dependent Variable: ESSENTIAL OIL RHIZOME

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
VARIETY	8.830	4	2.208	93.672	0.000 (HS)
STAGES	35.905	4	8.976	380.885	0.000 (HS)
VARIETY * STAGES	3.891	16	0.243	10.320	0.000 (HS)
Error	1.178	50	0.024		
Total	1248.205	75			
Corrected Total	49.804	74			

a R Squared = 0.976 (Adjusted R Squared =0.965), HS = Highly significant

Table 7 and Fig. 7 show the essential oil percentage in leaves during rhizome development. ANOVA for the values are given in Table 7a.
In short, highest oil content was visible in var. Suguna, followed by var. Sudarsana. Other three varieties, showing lower levels, always formed a group at all stages.

In the final stage, at 240 DAS, leaves got dried up and samples were not available for analysis. In general, essential oil values were found to decline towards maturity in the leaves. However, Suguna and Sudarsana were found to contain higher percentage of essential oil at all stages. A 3-fold increase was seen at 210 DAS in these varieties as compared to others. Turmeric leaves from these varieties can be a good source of essential oil.

Table 7. Percentage of essential oil in turmeric leaves during plant growth

Variety	120 DAS	180 DAS	210 DAS
Prabha	2.20	1.6	1.12
Prathibha	2.60	1.9	0.99
Alleppey	3.00	1.7	0.99
Suguna	4.40	3.3	2.92
Sudarsana	3.11	2.9	2.82

Values given are mean of three observations. DAS - Days after sowing

Table 7a. ANOVA

Dependent Variable: ESSENTIAL OIL LEAVES

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
VARIETY	27.318	4	6.829	179.366	0.000 (HS)
STAGES	15.745	2	7.872	206.758	0.000 (HS)
VARIETY * STAGES	4.548	8	0.569	14.932	0.000 (HS)
Error	1.142	30	0.038		
Total	289.340	45			
Corrected Total	48.753	44			

a R Squared =0.977 (Adjusted R Squared = 0.966), HS = Highly significant

Tables 8 and Fig. 8 show the essential oil percentage in turmeric roots. Statistical analysis of the values is given in Table 8a. It is interesting to note that varieties Suguna and Sudarsana are good sources of volatile oil, with 11-12% in the initial stages of rhizome development. During the later stages of maturity, no specific pattern was noticed, except for a good decline as compared to earlier stages.

Table 8. Essential oil percentage in turmeric roots during plant growth

Variety	120 DAS	150 DAS	180 DAS	210 DAS	240 DAS
Prabha	4.5	4.3	3.5	4.6	4.9
Prathibha	5.3	5.6	4.4	3.0	4.3
Alleppey	4.0	3.7	4.6	3.8	3.7
Suguna	8.4	12	5.8	8.1	5.0
Sudarsana	11	6.7	5.2	5.1	3.4

Values given are mean of three observations. DAS - Days after sowing

Table 8a. ANOVA table

Dependent Variable: ESSENTIAL OIL ROOT

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
VARIETY	161.364	4	40.341	2030.587	0.000 (HS)
STAGES	69.756	4	17.439	877.802	0.000 (HS)
VARIETY * STAGES	137.293	16	8.581	431.921	0.000 (HS)
Error	0.993	50	0.020		
Total	2551.010	75			
Corrected Total	369.407	74			

a R Squared =0.997 (Adjusted R Squared =0.996), HS = Highly significant

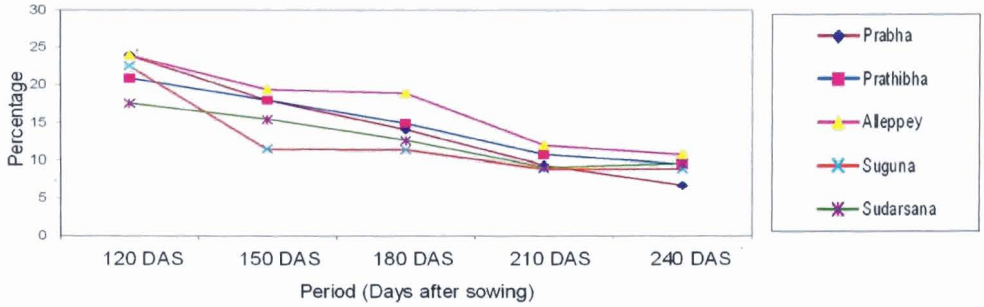


Fig. 4. Percentage of oleoresin in rhizomes of released varieties of turmeric during development

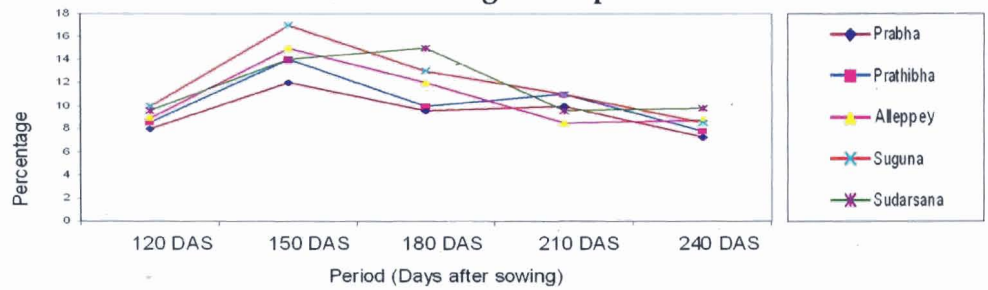


Fig. 5. Percentage of oleoresin in roots of released varieties of turmeric during rhizome development

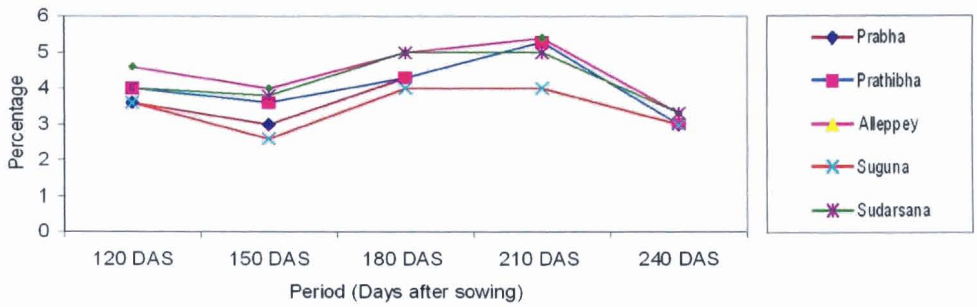


Fig. 6. Percentage of essential oil in rhizomes of released varieties of turmeric during development

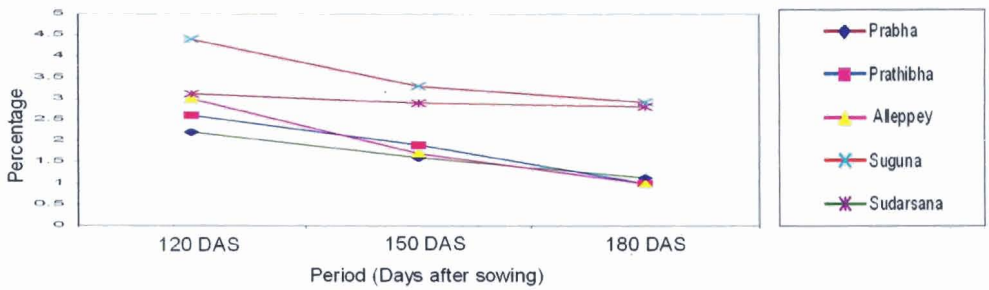


Fig. 7. Percentage of essential oil in leaves of released varieties of turmeric during rhizome development

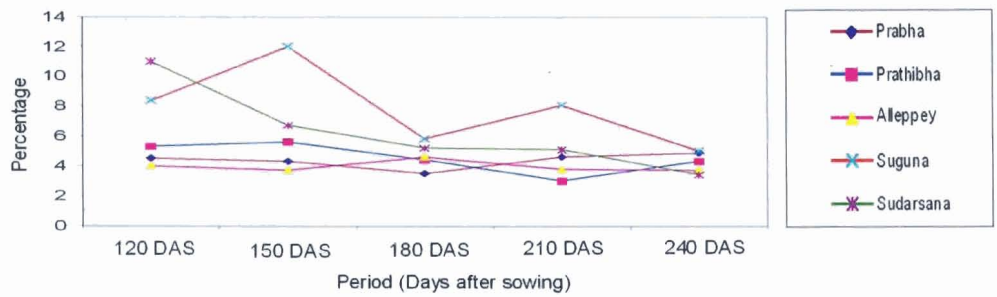


Fig. 8. Percentage of essential oil in roots of released varieties of turmeric during rhizome development

4.1.6. Curcuminoids

Quantification of curcuminoids by HPLC in rhizomes and roots of turmeric varieties /accessions

Curcuminoids in rhizomes and roots during the developmental changes were quantified using HPLC to observe the order and rate of biosynthesis of the three forms. Since fluorescence detection is the most sensitive HPLC detection method for curcuminoids, the detection of three curcuminoids were done using fluorescence detector, the proportion of the three forms being in the ratio 10.4: 2.2:1. The results could also be correlated with the levels of rate determining biosynthetic enzymes.

4.1.6.1. Distribution of Curcuminoids

Tables 9 to 17 and Figs. 9-27 show the percentage of curcumin in rhizomes and roots at one month intervals during rhizome development among the released varieties *viz.*, Prabha, Prathibha, Alleppey, Suguna, Sudarsana and acc. nos. 109, 88 and 64 during developmental stages. The separation was carried out using HPLC, into curcumin (C), demethoxy curcumin (DMC) and bis-demethoxy curcumin (BDMC), which are subsequently referred to as curcumin I, II and II respectively.

var. Alleppey

Table 9 shows the levels of three curcuminoids in rhizomes and roots for the var. Alleppey. Regarding curcumin I, all the stages contained higher levels except at 30 DAS. The highest value was observed at 150 DAS. As for curcumin II, the values ranged between 0.17% (30 DAS) to 0.98% (180 DAS). Curcumin III (BDMC) also showed lowest levels in the initial stage (0.12%) with two peak levels at 90 DAS (1.19%) and at 150 DAS (1.21%).

Figs. 9-14 show the HPLC pattern of the levels of the three curcuminoids in Alleppey rhizomes. Fig. 15 shows their percentage distribution in rhizomes. Curcumin I, (curcumin), which is the major pigment, contributes about 66 to 72%, the highest being observed at 90 DAS and 150 DAS (70.8 and 71.8% respectively). Curcumin II showed a wider variation, ranging from 8.3 to 19.8%. with a decline in the levels of curcumin II was seen at 90 and 150 DAS. BDMC (curcumin III) was not altered during the developmental stages, except at 90 and 150 DAS.

The variations in the three forms in roots are given in Table 9 and Fig. 16. In roots, curcumin I at higher levels was seen at 60 DAS and curcumin II was found to be higher at 30 and 120 DAS and curcumin III showed high value at 90 DAS.

Table 9. Curcuminoids in turmeric rhizomes and roots (var. Alleppey)

Growth Stages (DAS)	Curcumin I		Curcumin II		Curcumin III	
	Rhizome	Root	Rhizome	Root	Rhizome	Root
30	0.56 ± 0.014 (66.1)	0.032 ± 0.0 (66.1)	0.168±0.003 (19.7)	0.01±0.001 (20.8)	0.121±0.004 (14.2)	0.006±0.00 (12.5)
60	2.65 ± 0.08 (67.5)	0.300±0.002 (82.4)	0.733±0.036 (18.6)	0.06±0.001 (16.5)	0.551±0.008 (14.1)	0.004±0 (1.1)
90	4.13 ± 0.04 (70. 9)	0.360±0.035 (69.23)	0.511±0.008 (8.7)	0.09±0.005 (17.3)	1.190±0.036 (20.6)	0.070±0.005 (13.5)
120	3.09 ± 0.095 (66.2)	0.210±0.052 (72.4)	0.879±0.003 (18.82)	0.06±0.01 (20.7)	0.700±0.02 (14.97)	0.030±0.0 (10.4)
150	4.40 ± 0.12 (71. 8)	1.200±0.12 (71.4)	0.513±0.003 (8.37)	0.30±0.027 (17.9)	1.210±0.02 (19.7)	0.180±0.01 (10.7)
180	3.29 ± 0.096 (65.9)	0.880±0.014 (76.5)	0.978±0.005 (19.8)	0.18±0.021 (15.7)	0.723±0.06 (14.5)	0.100±0.007 (8.7)

(Values expressed as percentage, Mean of three observations), DAS -Days after sowing
Values in parentheses denote the percentage distribution among the three curcuminoids in each stage.

REPORT

Sample Name: Cur ALLEPPEY Rhi 1ST month Data File: ...s\Cur B 135.DAT
Method File: C:\Program Files\Indtech Instrumeats\Winchrom99\DEMOCur.Met
Detector: Flur. System: HPLC
Date: 6 Mar 2003 Time: 12:26:52
Run: ch1: 4
Type of Analysis : Percent On Area and Height
Type of Analysis : Percent On Area and Height

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
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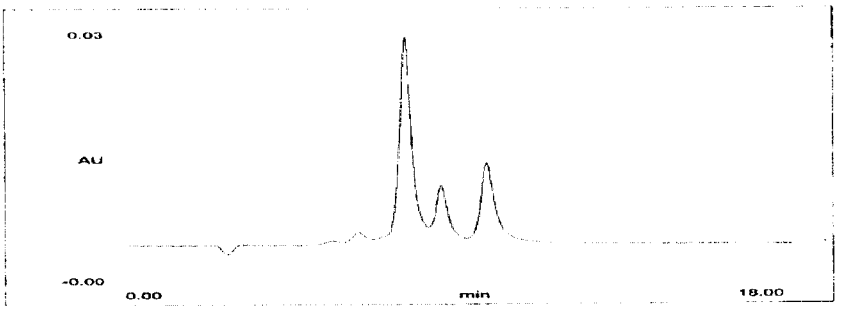

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Area/ Ht
1	3.20	275	173143	1.1329	1.1155	BB	0.430
2	5.62	241	170649	0.9928	1.0994	BV	0.483
3	6.31	791	566766	3.2586	3.6514	VV	0.489
4	7.58	13890	8381235	57.2217	53.9956	VV	0.412
5	8.55	3813	2464750	15.7082	15.8790	VV	0.441
6	9.77	5264	3765536	21.6858	24.2592	VB	0.488
		2e+04	15522080				

Fig. 9. HPLC pattern of curcuminoids in turmeric rhizomes at 30 DAS (var. Alleppey)

REPORT

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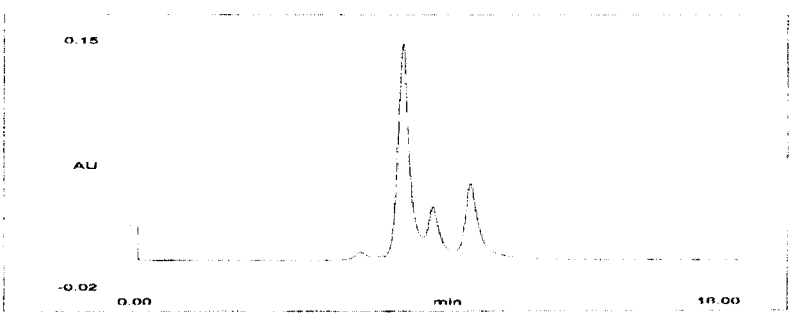
Method File: C:\Program Files\Indtech Instrumeats\Winchrom99\DEMOCur.Met
Detector: Flur. System: HPLC
Date: 22 Feb 2003 Time: 10:3:20
Run: ch1: 1
Type of Analysis : Percent On Area and Height
Type of Analysis : Percent On Area and Height

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
16	30	5	4	20.0



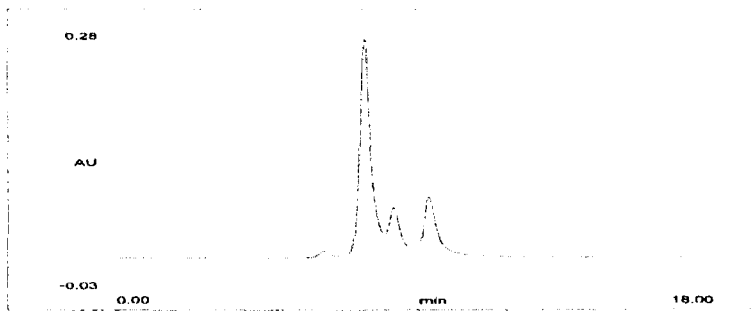
No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Area/ Ht
1	3.55	0	14149	0.0000	0.0206	BB	0.164
2	6.25	156	95396	0.1396	0.1388	S	0.417
3	6.95	2547	1553023	2.2795	2.2589	BV	0.416
4	7.49	1145	433640	1.0248	0.6307	S	0.259
5	8.15	68040	38280985	60.8952	55.6793	BV	0.384
6	9.01	16345	10712264	14.6286	15.5809	VV	0.447
7	10.08	23500	17663141	21.0323	25.6909	VB	0.513
		1e+05	68752598				

Fig. 10. HPLC pattern of curcuminoids in turmeric rhizomes at 60 DAS (var. Alleppey)

REPORT

Sample Name: Rhi Alleppey 3rd month Data File: ...s\Cur B 107.DAT
Method File: C:\Program Files\Indtech Instruments\Winchrom99\DEMOCur.Met
Detector: Flur. System: HPLC
Date: 22 Feb 2003 Time: 12:24:34
Run: ch1: 7
Type of Analysis : Percent On Area and Height
Type of Analysis : Percent On Area and Height

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
16	30	5	4	20.0



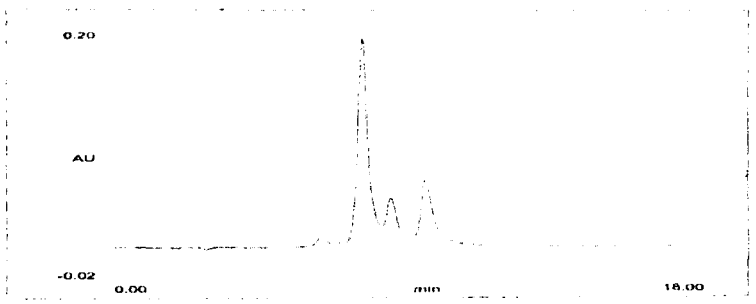
No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Area/ Ht
1	2.75	0	5885	0.0000	0.0049	BB	0.149
2	5.45	123	71911	0.0683	0.0600	S	0.399
3	6.19	3611	2207792	2.0051	1.8417	BV	0.417
4	7.40	124536	82561864	69.1517	68.8726	VV	0.453
5	8.26	17732	7499962	9.8461	6.2564	TTT	0.289
6	9.32	34089	27528875	18.9288	22.9644	VB	0.551
		2e+05	119876289				

Fig. 11. HPLC pattern of curcuminoids in turmeric rhizomes at 90 DAS (var. Alleppey)

REPORT

Sample Name: Cur ALLEP Rhi 4TH month Data File: ...s\Cur B 128.DAT
Method File: .MET
Detector: Flur. System: HPLC
Date: 5 Mar 2003 Time: 12:49:34
Run: ch1: 0
Type of Analysis : Percent On Area and Height
Report printed on : 5/3/2003 at : 14:27:27

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
16	30	5	4	20.0



No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Area/ Ht
1	3.17	284	145409	0.2044	0.1703	BB	0.349
2	5.60	172	97659	0.1238	0.1144	BV	0.388
3	6.25	4133	3081984	2.9752	3.6096	VV	0.509
4	6.83	198	52813	0.1425	0.0619	TTT	0.182
5	7.49	86142	48474554	62.0101	56.7726	VV	0.384
6	8.35	20058	12898585	14.4389	15.1066	VV	0.439
7	9.40	27929	20632705	20.1050	24.1647	VB	0.504
		1e+05	85383708				

Fig. 12. HPLC pattern of curcuminoids in turmeric rhizomes at 120 DAS (var. Alleppey)

REPORT

Sample Name: Cur Alleppey Rhi5th month Data File: ...es\Cur B119.DAT
Method File: .MET
Detector: Flur. System: HPLC
Date: 25 Feb 2003 Time: 12:20:22
Run: ch1: 0
Type of Analysis : Percent On Area and Height
Report printed on : 5/3/2003 at : 14:30:39

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
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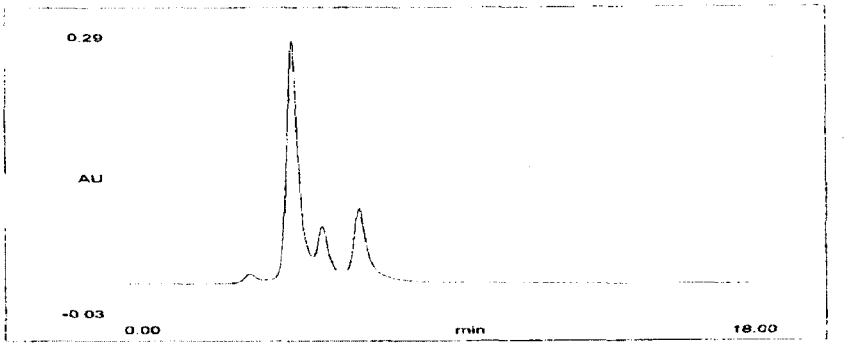

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Area/ Ht
1	2.78	151	58359	0.0799	0.0471	BV	0.264
2	3.44	4938	2999494	2.6136	2.4229	VV	0.415
3	4.59	126853	83822738	67.1414	67.7081	VV	0.451
4	5.41	18525	7534633	9.8050	6.0861	TTT	0.278
5	6.41	38467	29384911	20.3600	23.7358	VB	0.521
		2e+05	123800134				

Fig. 13. HPLC pattern of curcuminoids in turmeric rhizomes at 150 DAS (var. Alleppey)

REPORT

Sample Name: Cur Alleppey Rhi 6th month Data File: ...es\Cur B120.DAT
Method File: .MET
Detector: Flur. System: HPLC
Date: 25 Feb 2003 Time: 12:41:36
Run: ch1: 0
Type of Analysis : Percent On Area and Height
Report printed on : 5/3/2003 at : 14:34:41

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
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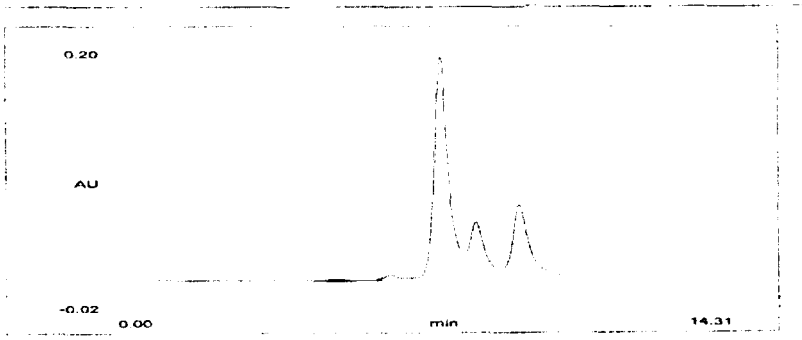

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Area/ Ht
1	6.26	2071	1213054	1.4460	1.3833	BV	0.400
2	7.44	89291	50151192	62.3453	57.1891	VV	0.383
3	8.23	22686	14352250	15.8400	16.3664	VV	0.432
4	9.23	29172	21977091	20.3687	25.0612	VB	0.514
		1e+05	87693588				

Fig. 14. HPLC pattern of curcuminoids in turmeric rhizomes at 180 DAS (var. Alleppey)

var. Suguna

The values of the three curcuminoids in rhizomes and roots in Suguna are given in Table 10 and Fig. 17. Highest levels of curcumin I was seen at 90 DAS, with a minimum value at 150 DAS. The same pattern was seen for curcuminoids II and III. All the three forms exhibited a sudden decline at 150 DAS.

The distribution between curcumin I, II and III in rhizomes is given in Fig.17. Curcumin I contributed to the major portion of curcuminoids, with the percentage distribution ranging from 65-71.1%. Curcumin II showed a steady percentage up to 120 DAS and then increased at 150 DAS and again declined at 180 DAS Curcumin III also showed a mixed trend with higher accumulation at initial stages.

Table 11 and Fig. 18 show the variations in the three forms in roots. Curcumin I was found to be at higher levels at 150 DAS, curcumin II at 90 and 120 DAS and Curcumin III between 30 and 120 DAS.

Table 10. Curcuminoids in turmeric rhizomes and roots (var. Suguna)

Growth Stages (DAS)	Curcumin I		Curcumin II		Curcumin III	
	Rhizome	Root	Rhizome	Root	Rhizome	Root
30	3.66 ± 0.28 (65.2)	0.04±0.01 (83.3)	0.47± 0.03 (8.4)	0.005±0.0001 (10.4)	1.48±0.036 (26.4)	0.003±0.001 (6.3)
60	3.31 ± 0.07 (65.2)	0.07±0.001 (63.6)	0.41±0.052 (8.1)	0.02±0.0 (18.2)	1.3±0.035 (26.9)	0.02±0.001 (18.2)
90	5.05 ± 0.087 (71.03)	0.21±0.01 (63.6)	0.59±0.044 (8.3)	0.07±0.027 (21.2)	1.48±0.046 (20.8)	0.05±0.003 (15.2)
120	4.63 ± 0.1 (69.8)	0.32±0.027 (62.8)	0.59±0.046 (8.9)	0.11±0.01 (21.6)	1.41±0.027 21.27	0.08±0.001 (15.7)
150	2.47 ± 0.076 (66.9)	2.6±0.0 (63.4)	0.34±0.01 (9.21)	0.80±0.02 (19.5)	0.88±0.036 (23.9)	0.72±0.001 (17.6)
180	3.37± 0.027 (69.3)	0.410.02 (70.7)	0.39± 0.036 (8.03)	0.11±0.02 18.9)	1.10± 0.096 (22.6)	0.06±0.00 10.4

Values expressed as percentage (Mean of three observations), DAS -Days after sowing
Values in parentheses denote the percentage distribution among the three curcuminoids in each stage.

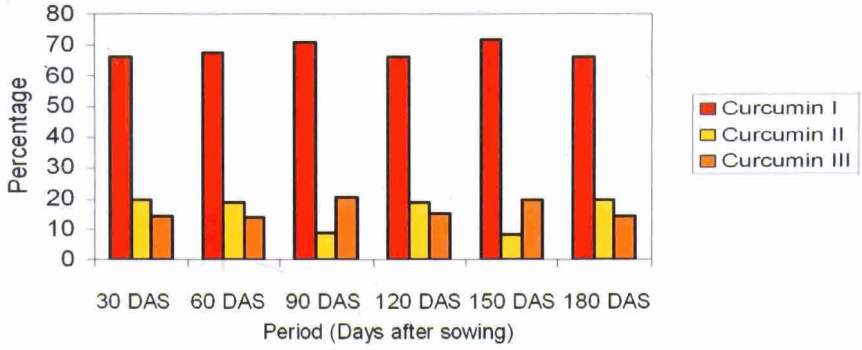

Fig. 15. Pattern of distribution of curcuminoids in turmeric rhizomes (var. Alleppey) during development

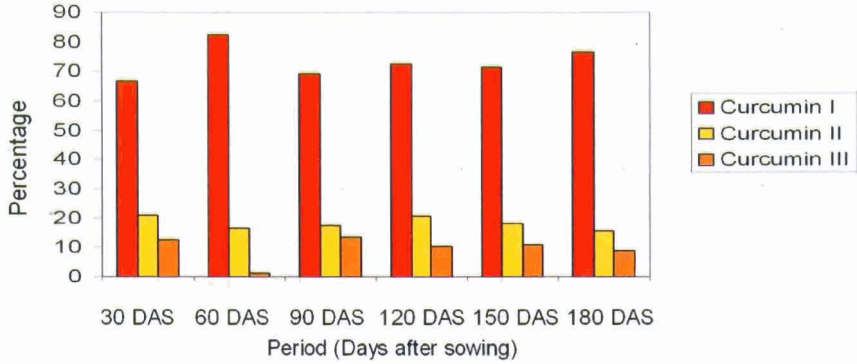

Fig. 16. Pattern of distribution of curcuminoids in turmeric roots (var. Alleppey) during rhizome development

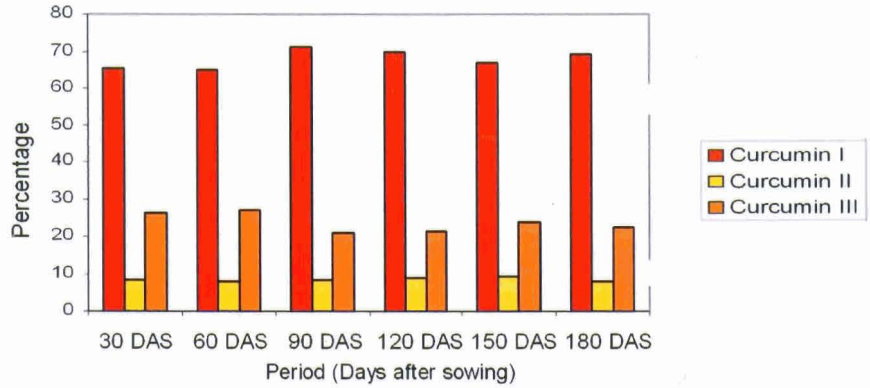

Fig. 17. Pattern of distribution of curcuminoids in turmeric rhizomes (var. Suguna) during development

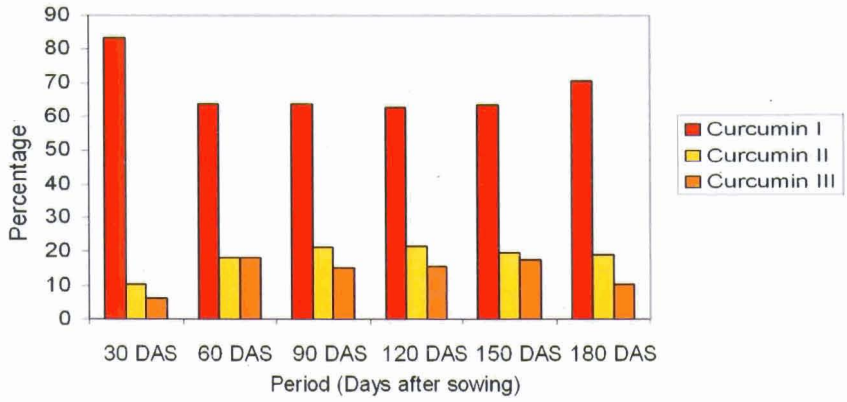

Fig. 18. Pattern of distribution of curcuminoids in turmeric roots (var. Suguna) during rhizome development

var. Sudarsana

In Sudarsana, curcumin I was found to increase up to 120 DAS with a sudden decrease followed by a slight increase (180 DAS). Curcumin II levels remained steady up to 90 DAS with a 4- fold increase at 120 DAS. In the case of curcumin III almost a steady level (range 1.4 -1.6) was noticed up to 120 DAS followed 3-fold decrease at subsequent stages (Table 11). Curcumin I, II, and III were present at highest levels 90,120 and 30 DAS respectively. In roots, (Table 11 & Fig. 20), all three forms were seen at higher levels at 120 DAS.

Table 11. Curcuminoids in turmeric rhizomes and roots (var. Sudarsana)

Growth Stages	Curcumin I		Curcumin II		Curcumin III	
	Rhizome	Root	Rhizome	Root	Rhizome	Root
30 DAS	3.80 ±0.22 (66.8)	0.005 ±0.003 (33.3)	0.49 ± 0.036 (8.6)	0.007± 0.001 (46.7)	1.40 ±0.04 (24.6)	0.003 ±0.0001 (20.0)
60 DAS	3.46 ± .06 (67.5)	0.029 ±0.001 (67.4)	0.42 ± 0.027 (8.2)	0.008 ±0.001 (18.6)	1.26 ±0.027 (24.6)	0.006 ±0.001 (13.9)
90 DAS	4.43±0.027 (69.1)	0.260 ±0.027 (60.5)	0.48 ± 0.03 (7.5)	0.090 ±0.01 (20.9)	1.51 ±0.1 (23.6)	0.080 ±0.01 (18.6)
120 DAS	5.50 ±0.092 (60.9)	0.400 ±0.02 (61.5)	1.90 ±0.1 (21.1)	0.140 ±0.02 (21.5)	1.62 ±0.044 (17.9)	0.110 ±0.017 (16.9)
150 DAS	2.00 ± 0.052 (66.5)	0.340 ±0.017 (65.4)	0.57 ± 0.02 (18.9)	0.100 ±0.021 (65.4)	0.45 ±0.04 (14.9)	0.080 ±0.003 (15.4)
180 DAS	3.39 ± 0.12 (62.4)	0.360 ±0.02 (69.2)	1.08 ± 0.027 (19.9)	0.100 ±0.01 (19.2)	0.96 ±0.027 (17.7)	0.06 ±0.017 (11.5)

(Values expressed as percentage, Mean of three observations), DAS -Days after sowing
Values in parentheses denote the percentage distribution among the three curcuminoids in each stage.

var. Prabha

Table 12 shows the levels of curcuminoids in rhizomes and roots in var. Prabha. In rhizomes, curcumin I, II and III were found to be very low at 30 DAS (0.33, 0.098 and 0.077 respectively). All three were found to increase upto 90 DAS (4.41, 1.41 and 0.99 respectively) followed by a decrease (2-fold) at 150 DAS.

Percentage distribution of curcumin I, II and III in rhizomes were found to range between 51.5 - 66.2%, 19.1 - 27.4% and 14.6 - 22.0%, respectively (Fig. 21). Regarding curcumin II, similar percentage distribution was seen during 30, 120 and 150 DAS. Curcumin III was found to be lower at 30 and 120 DAS while similar values were shown at 60 and 90 DAS.

Roots showed a different type of distribution, with highest values at 90 DAS for curcumin I, II and III. However, at 180 DAS, curcumin II showed higher levels, which was reflected in a lower percentage of curcumin III at this stage (Table 12 and Fig. 22).

Table 12. Curcuminoids in turmeric rhizomes and roots (var. Prabha)

Growth Stages	Curcumin I		Curcumin II		Curcumin III	
	Rhizome	Root	Rhizome	Root	Rhizome	Root
30 DAS	0.33 ± 0.042 (66.0)	0.015 ±0.004 (78.9)	0.098± 0.003 (19.6)	0.002 ±0.0 (10.5)	0.077 ±0.007 (15.4)	0.002 ±0.0 (10.5)
60 DAS	1.39 ± 0.017 (66.19)	0.023 ±0.003 (44.1)	0.400 ± 0.0 (19.1)	0.006 ±0.0 (17.7)	0.309 ±0.02 (14.7)	0.005 ±0.0006 (14.7)
90 DAS	4.41 ± 0.044 (64.9)	0.720 ±0.017 (77.4)	1.410 ± 0.06 (20.7)	0.140 ±0.04 (15.1)	0.990 ±0.046 (14.6)	0.070 ±0.0036 (7.53)
120 DAS	2.29 ± 0.01 (64.9)	0.360± 0.015 (76.6)	0.690 ±0.027 (19.6)	0.080 ±0.01 (17.0)	0.550 ±0.027 (15.6)	0.040 ±0.0 (8.5)
150 DAS	2.49 ± 0.063 (63.9)	2.600 ±0.17 (63.4)	0.773 ± 0.09 (19.8)	0.800 ±0.01 (19.5)	0.640 ±0.062 (16.4)	0.720 ±0.056 (17.6)
180 DAS	1.66 ± 0.058 (51.4)	0.570 ±0.025 (68.7)	0.8 ± 0.013 (27.7)	0.180 ±0.008 (21.7)	0.710 ±0.016 (21.9)	0.076 ±0.002 (9.2)

(Values expressed as percentage, Mean of three observations), DAS -Days after sowing
Values in parentheses denote the percentage distribution among the three curcuminoids in each stage.

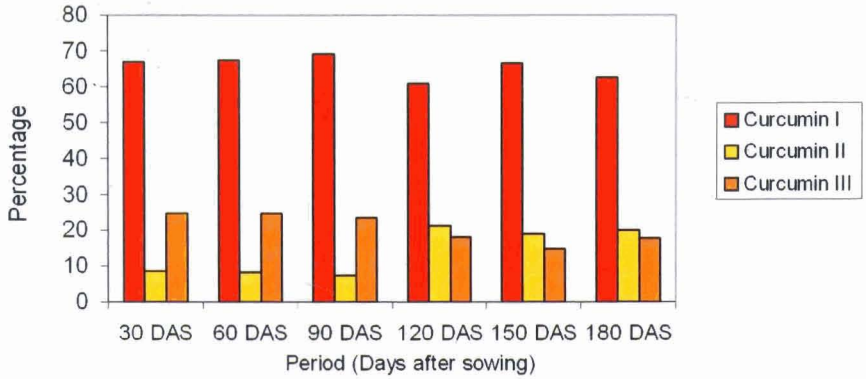

Fig. 19. Pattern of distribution of curcuminoids in turmeric rhizomes (var. Sudarsana) during development

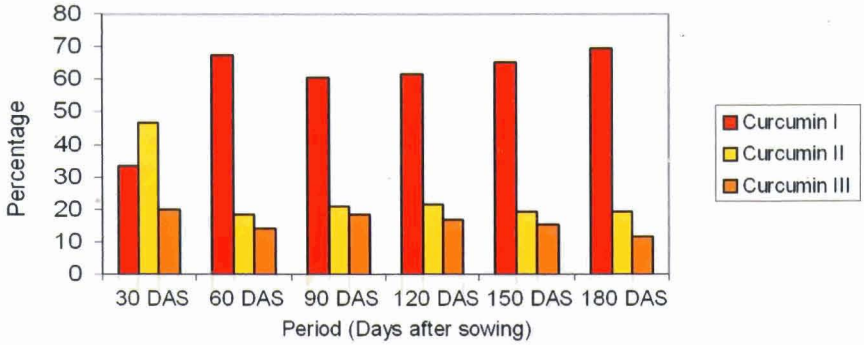

Fig. 20. Pattern of distribution of curcuminoids in turmeric roots (var. Sudarsana) during rhizome development

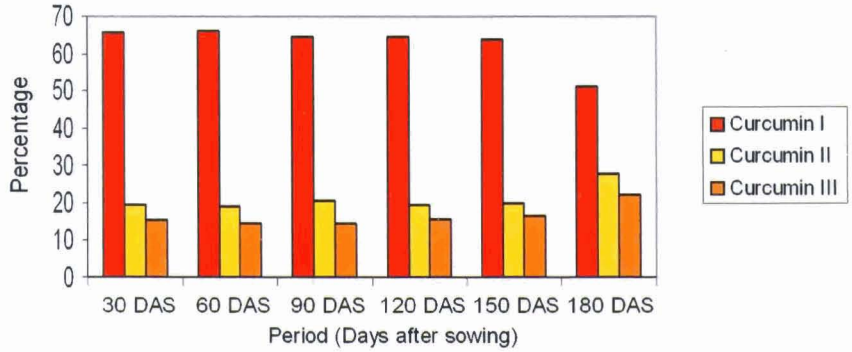

Fig. 21. Pattern of distribution of curcuminoids in turmeric rhizomes (var. Prabha) during development

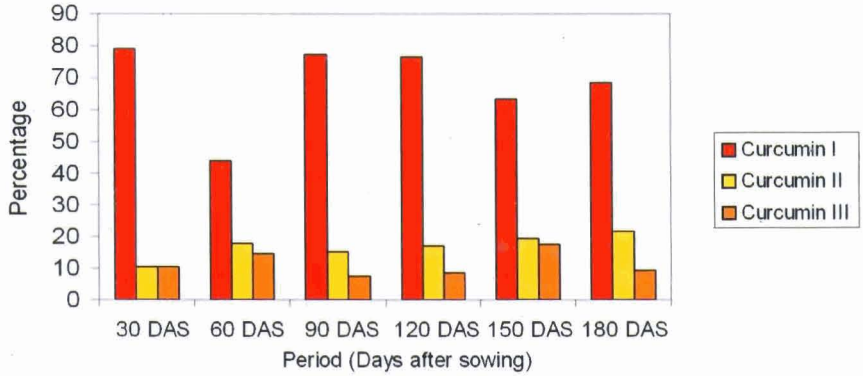

Fig. 22. Pattern of distribution of curcuminoids in turmeric roots (var. Prabha) during rhizome development

var. Prathibha

In this variety, curcuminoids during different stages were found to increase up to 90 DAS followed by a decline (Table 13). Curcumin II showed fluctuations in the values throughout the stages. Curcumin III in the initial stages increased up to 90 DAS, which then showed a declining trend.

The percentage distribution ranged from 66.6 to 78.9%, 8.31 to 21.03% and 13.0 to 20.2% for curcumin I, II and III respectively (Fig.23). Highest value for curcumin I and II was found at 120 DAS and for curcumin III, low values were seen at 90 DAS.

The distribution of curcuminoids, in roots exhibited a similar pattern (Fig. 24) in all the developmental stages. Higher values are seen for curcumin II and III (Table 13) at 90 DAS, and at 150 DAS, for curcumin I.

Table 13. Curcuminoids in turmeric rhizomes and roots (var. Prathibha)

Growth Stages	Curcumin I		Curcumin II		Curcumin III	
	Rhizome	Root	Rhizome	Root	Rhizome	Root
30 DAS	0.63 ± 0.036 (68.5)	0.047 ±0.001 (74.6)	0.17 ± 0.01 (18.5)	0.01 ±0.0 (15.9)	0.120 ± 0.02 (13.0)	0.006 ±0.0 (9.5)
60 DAS	2.60 ± 0.046 (66.7)	0.300 ±0.017 (69.8)	0.73 ± 0.027 (18.7)	0.08 ±0.001 (18.6)	0.580 ±0.027 (14.9)	0.050 ±0.01 (11.6)
90 DAS	5.16 ± 0.036 (71.5)	0.410 ±0.0 (70.7)	0.60 ± 0.017 (8.3)	0.10 ±0.0 (17.2)	1.460 ±0.053 (20.2)	0.070 ±0.01 (12.1)
120 DAS	3.14 ± 0.053 (78.9)	0.250 ±0.01 (65.8)	1.10 ±0.01 (21.0)	0.08 ±0.01 (21.1)	0.990 ±0.0 (18.9)	0.050 ±0.01 (13.2)
150 DAS	4.13 ±0.02 (66.6)	0.900 ±0.027 (73.8)	1.14 ± 0.15 (18.4)	0.20 ±10.85 (16.4)	0.930 ±0.063 (15.0)	0.120 ±0.0 (9.8)
180 DAS	2.60 ±0.078 (68.0)	0.130 ±0.01 (74.7)	0.74 ±0.12 (19.3)	0.03 ±0.0 (17.2)	0.483 ±0.024 (12.6)	0.014±0.001 (8.1)

(Values expressed as percentage, Mean of three observations), DAS -Days after sowing
Values in parentheses denote the percentage distribution among the three curcuminoids in each stage.

Accession no. 109

Curcumin I, II and III increased from 30 DAS to 120 DAS and decreased from 150 DAS onwards, with highest value at 120 DAS (Table 14). Curcumin II increased up to 150 DAS showing highest value at 150 DAS (0.97).

Fig. 25 shows the percentage distribution of curcuminoids present in Acc.109. Curcumin I and III were found to exhibit high levels at 120 DAS whereas curcumin II was highest at 150 DAS.

Table 14. Curcuminoids in turmeric rhizomes (Acc. 109)

Growth Stages (DAS)	Curcumin I	Curcumin II	Curcumin III
30	0.18 ± 0.01 (64.3)	0.07 ± 0.005 (25.0)	0.03 ± 0.004 (10.7)
60	0.97 ± 0.12 (59.9)	0.36 ± 0.027 (22.2)	0.29 ±0.03 (17.9)
90	1.50 ± 0.13 (60.9)	0.56 ± 0.022 (22.8)	0.41 ±0. 036 (16.7)
120	3.35 ± 0.07 (65.6)	0.96 ± 0.044 (18.8)	0.80 ±0.044 (15.7)
150	2.26 ± 0.036 (58.7)	0.97 ± 0.12 (25.2)	0.61 ±0.051 (15.8)
180	2.10 ± 0.1 (59.3)	0.85 ± 0.03 (24.0)	0.59 ±0.027 (16.7)

(Values expressed as percentage, Mean of three observations), DAS -Days after sowing
Values in parentheses denote the percentage distribution among the three curcuminoids in each stage.

Accession no. 88

Acc. 88 belongs to a low curcumin type. Table15 shows the levels of curcuminoids at different stages. A gradual increase upto 90 DAS followed by a decrease was seen for all the three curcuminoids.

Fig. 26 shows the percentage distribution of curcuminoids at different growth stages. The accession showed higher percentage at 60 and 180 DAS for curcumin I, while Curcumin II exhibited higher values at 120 and 150 DAS. Curcumin III was highest at 90 DAS.

Table 15. Curcuminoids in turmeric rhizomes (Acc. 88)

Growth Stages (DAS)	Curcumin I	Curcumin II	Curcumin III
30	0.45 ± 0.035 (55.6)	0.21 ± 0.017 (25.9)	0.130 ±0.017 (16.1)
60	0.76 ± 0.044 (60.8)	0.28 ± 0.027 (22.4)	0.210 ±0.01 (16.8)
90	1.01 ± 0.085 (55.8)	0.45 ± 0.036 (24.9)	0.350 ±0.036 (19.3)
120	0.74 ± 0.027 (56.1)	0.37 ± 0.072 (28.0)	0.210 ±0.027 (15.9)
150	0.57 ± 0.02 (57.6)	0.27 ± 0.044 (27.3)	0.160 ±0.01 (16.2)
180	0.43 ± 0.027 (59.7)	0.19 ± 0.018 (26.4)	0.097 ±0.003 (13.5)

(Values expressed as percentage, Mean of three observations), DAS -Days after sowing
Values in parentheses denote the percentage distribution among the three curcuminoids in each stage.

Accession no. 64

This accession also belongs to low curcumin type. Curcumin I showed a 3-fold increase at 60 DAS as compared to initial value, which remained stationary during the preceding stages (Table 16). Here, curcumin II showed least value at 30 DAS, which increased up to 150 DAS and then declined, whereas curcumin III showed highest value at 90 and 120 DAS with a declining trend upto 180 DAS.

Fig. 27 shows the percentage distribution of curcumin I, II and III in Acc.64. Highest percentage of curcumin I was seen at 180 DAS and for curcumin II and III at 120 DAS and 90 DAS respectively.

Table 16. Curcuminoids in turmeric rhizomes (Acc. 64)

Growth Stages (DAS)	Curcumin I	Curcumin II	Curcumin III
30	0.24 ± 0.004 (64.9)	0.09 ± 0.002 (24.3)	0.04 ±0.004 (10.8)
60	0.92 ± 0.07 (60.9)	0.40 ±0.01 (26.5)	0.19 ±0.01 (12.6)
90	0.88 ± 0.01 (53.7)	0.42 ±0.003 (25.6)	0.34 ±0.01 (20.7)
120	0.94 ± 0.06 (20.7)	0.56 ±0.002 (31.3)	0.29 ±0.004 (16.2)
150	0.89 ± 0.01 (60.1)	0.34 ± 0.02 (22.9)	0.25 ±0.027 (16.9)
180	0.91 ± 0.039 (83.5)	0.13 ± 0.039 (11.9)	0.05 ±0.003 (4.6)

(Values expressed as percentage, Mean of three observations), DAS -Days after sowing
Values in parentheses denote the percentage distribution among the three curcuminoids in each stage.

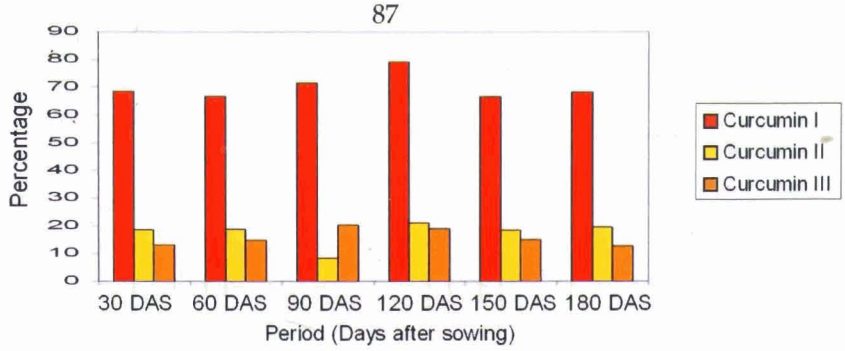

Fig. 23. Pattern of distribution of curcuminoids in turmeric rhizomes (var. Prathibha) during development

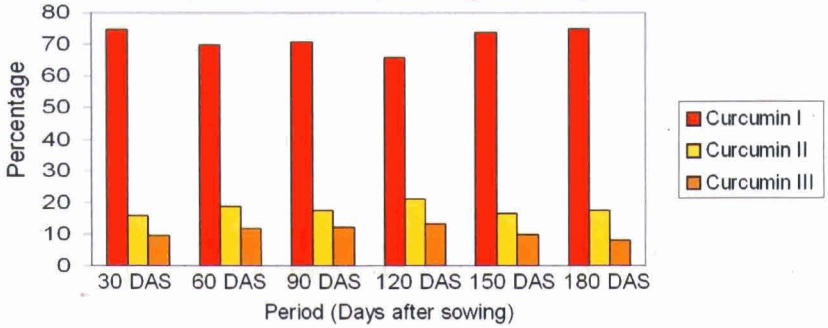

Fig. 24. Pattern of distribution of curcuminoids in turmeric roots (var. Prathibha) during rhizome development

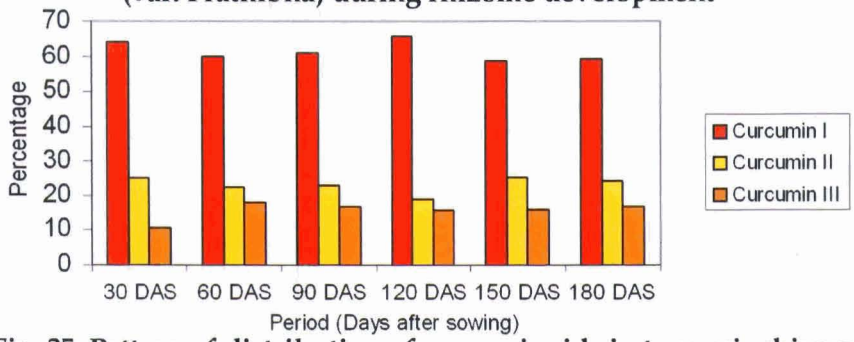

Fig. 25. Pattern of distribution of curcuminoids in turmeric rhizomes (Acc. 109) during development

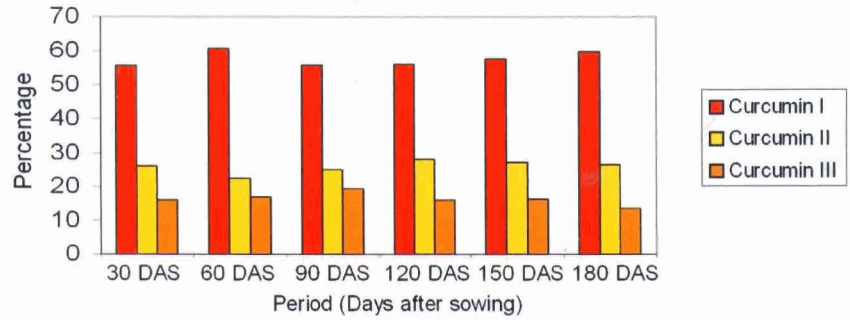

Fig. 26. Pattern of distribution of curcuminoids in turmeric rhizomes (Acc. 88) during development

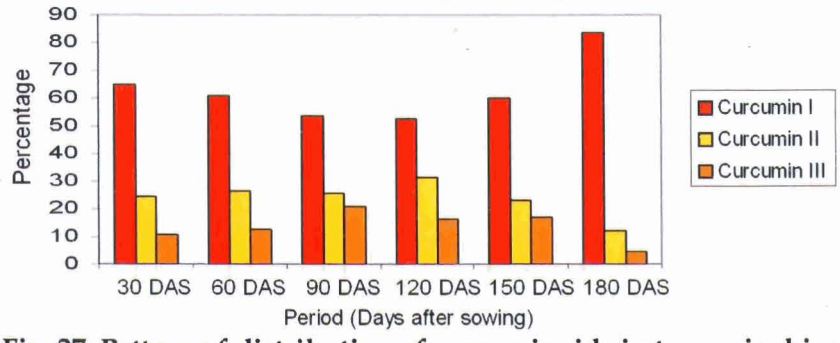

Fig. 27. Pattern of distribution of curcuminoids in turmeric rhizomes (Acc. 64) during development

4.1.6.2. Summary of results for Curcumin I, II & III

The results explained above for individual variety / accession gave the following indications. This included a total comparison of the curcuminoids between stages and between var./acc. The respective ANOVA are given as Tables 17a, 17b and 17c for rhizomes and 18 a, 18b and 18c for roots.

Rhizomes

During early germination phase (30 DAS), higher levels of curcumin I are seen in varieties Suguna and Sudarsana, as compared to Alleppey, Prabha, Prathibha and the high curcumin accession (Acc. 109). The same trend was exhibited by curcumin II and III also. During the next stage (60 DAS), the pattern remained same for curcumin I and III even though a 5-fold increase were seen among the varieties except varieties Suguna and Sudarsana. A different pattern was observed in the case of curcumin II for varieties Alleppey and Prathibha, were a 2-fold rise was observed, compared to other accessions.

Three months after sowing, i.e., at 90 DAS, all varieties / accessions exhibited higher curcumin I and III, with var. Suguna showing highest levels for curcumin I. Varieties Sudarsana, Prathibha and Suguna showed higher levels of curcumin III. The values ranged from 1.5 to 5.16%.

At 120 DAS, values for curcumin I showed 5.5% for var. Sudarsana while var. Prabha showed the least (2.29%). Same pattern was observed for curcumin III, with varieties Suguna and Sudarsana showing maximum values and var. Prabha exhibiting the minimum.

At 150 DAS, varieties Prabha and Prathibha showed higher value for curcumin I. The values for all the varieties ranged from 2.0 - 4.4%. As for

curcumin II, var. Prathibha showed highest levels, with values ranging from 0.34 - 1.14%. Regarding curcumin III, var. Alleppey had highest levels, of curcumin III with values ranging from 0.45 - 1.2%. Interestingly, varieties Suguna and Sudarsana exhibited low values in all forms of curcuminoids.

At 180 DAS, curcumin I showed a stabilized value, ranging from 2.20 - 3.3%, for all the varieties studied. Curcumin II showed a varied pattern, with highest values showed by var. Sudarsana. Here the values ranged from 0.39 - 1.1%. Regarding curcumin III, highest content was seen in varieties Sudarsana and Suguna, with values ranging from 0.6 - 1.14%.

In the low curcumin accessions (Acc. 88 and 64), curcumin I did not show much variation from the high curcumin varieties during early germination phase. But from 60 DAS to 180 DAS, the levels were found to be drastically reduced. Regarding curcumin II and III, the same trend was noticed at all stages. In these two varieties, the level of curcuminoids was low in all stages, confirming the varieties as low curcumin accessions.

Table 17a. ANOVA

Dependent Variable: CUR1

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
VAR	170.873	7	24.410	3618.676	0.000 (HS)
STAGE	57.144	5	11.429	1694.224	0.000 (HS)
VAR * STAGE	78.875	35	2.254	334.074	0.000 (HS)
Error	0.648	96	0.007		
Total	1068.761	144			
Corrected Total	307.539	143			

a R Squared = 0.998 (Adjusted R Squared = 0.997), HS = Highly Significant.

Table 17b. ANOVA

Dependent Variable: CUR2

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
VAR	2.952	7	0.422	189.763	0.000 (HS)
STAGE	4.536	5	0.907	408.139	0.000 (HS)
VAR * STAGE	5.850	35	0.167	75.207	0.000 (HS)
Error	0.213	96	0.002		
Total	59.393	144			
Corrected Total	13.552	143			

a R Squared =0 .984 (Adjusted R Squared =0 .977), HS = Highly Significant

Table 17c. ANOVA

Dependent Variable: CUR3

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
VAR	17.780	7	2.540	1954.304	0.000 (HS)
STAGE	4.721	5	0.944	726.520	0.000 (HS)
VAR * STAGE	8.574	35	0.245	188.492	0.000 (HS)
Error	0.125	96	0.001		
Total	91.884	144			
Corrected Total	31.200	143			

a R Squared =0 .996 (Adjusted R Squared = 0.994), HS = Highly Significant

Roots

Generally, roots showed higher values at 150 DAS for all varieties, with lower values at 30 DAS. Among the varieties, highest curcumin I was seen in Prabha (1.6) followed by Alleppey (1.2) and Prathibha (0.9), with Suguna and Sudarsana showing lesser percentage.

Curcumin II was found to be low in all varieties, with highest values at 150 DAS for Alleppey, Prabha and Prathibha (0.3, 0.8, and 0.2 % respectively). For Suguna and Sudarsana, higher levels were seen at 120 DAS (0.11 and 0.14 % respectively). Highest percentage of curcumin II was seen in Prabha (0.8%).

Regarding curcumin III, similar pattern was shown by all varieties, with highest value at 150 DAS for Alleppey, Prabha and Prathibha (0.18, 0.72 and 0.12% respectively).

Table 18a. ANOVA

Dependent Variable: CUR1

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
VAR	3.385	4	0.846	494.098	0.000 (HS)
STAGE	8.916	5	1.783	1041.226	0.000 (HS)
VAR * STAGE	10.301	20	0.515	300.742	0.000 (HS)
Error	0.103	60	0.002		
Total	36.584	90			
Corrected Total	22.704	89			

a R Squared =0.995 (Adjusted R Squared =0.993), HS= Highly Significant

Table 18b. ANOVA

Dependent Variable: CUR2

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
VAR	14.683	4	3.671	0.935	0.450
STAGE	29.181	5	5.836	1.487	0.208
VAR * STAGE	75.275	20	3.764	0.959	0.521
Error	235.537	60	3.926		
Total	364.179	90			
Corrected Total	354.676	89			

a R Squared = 0.336 (Adjusted R Squared = 0.015).

Table 18c. ANOVA

Dependent Variable: CUR3

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
VAR	0.133	4	0.033	229.714	0.000 (HS)
STAGE	0.480	5	0.096	662.142	0.000 (HS)
VAR * STAGE	0.826	20	0.041	284.900	0.000 (HS)
Error	0.009	60	0.000		
Total	1.978	90			
Corrected Total	1.447	89			

a R Squared =0.994 (Adjusted R Squared =0.991), HS= Highly Significant

4.1.6.3. Total Curcuminoids (Rhizomes and Roots)

Table 19 shows a summarized pattern of curcuminoids in the varieties and accessions selected for this particular study. Varieties Alleppey, Prabha and Prathibha with higher vegetative growth and similar agronomic characters showed uniform pattern as compared to the early germinating ones *viz.*, Suguna and Sudarsana. In the former case, the curcuminoids ranged from 0.50 to 0.92% at 30 DAS, while a spurt (3-fold) in the activity was seen after 60 DAS. This higher rate of increase continued up to 90 DAS. A varietal variation was also observed, varieties Prabha and Prathibha showing a higher rate of increase. At 120 DAS, the values declined by 20-50% in these three varieties, which subsequently showed a uniform increase upto 150 DAS. The values declined at 180 DAS and remained stable upto maturity (Table 19).

An entirely different picture is observed in the early germinating varieties, Suguna and Sudarsana. Higher levels of total curcuminoids were seen at 30 DAS (5.6-5.69%). Ensuing growth stages also exhibited higher levels of curcumin upto 120 DAS. A decline to the tune of 33 to 44% was seen at 150 DAS in these varieties, which then showed an enhancement in the next stage. The levels then remained stationary up to maturity.

Generally roots contained lower levels of the pigment than rhizomes. It was interesting to note that in varieties Prabha, Prathibha and Alleppey, higher levels of the three forms of curcuminoids were seen at 150 DAS, while in the early germinating *viz.*, Suguna and Sudarsana a peak activity is observed at 120 DAS.

Table 19. Total curcuminoids in rhizomes and roots in selected turmeric varieties / accessions during plant growth

Growth Stages (DAS)	Alleppey		Suguna		Sudarsana		Prabha		Prathibha		Acc. 109	Acc. 88	Acc. 64
	Rhizomes	Roots	Rhizomes	Roots	Rhizomes	Roots	Rhizomes	Roots	Rhizomes	Roots	Rhizomes	Rhizomes	Rhizomes
30	0.853	0.048	5.61	0.048	5.69	0.015	0.50	0.019	0.92	0.063	0.28	0.81	0.37
60	3.930	0.364	5.08	0.11	5.13	0.043	2.10	0.034	3.90	0.430	1.62	1.25	1.51
90	5.830	0.520	7.11	0.33	6.41	0.430	6.80	0.930	7.22	0.580	2.46	1.81	1.64
120	4.670	0.290	6.63	0.51	9.02	0.650	3.53	0.470	5.23	0.380	5.11	1.32	1.79
150	6.130	1.680	3.69	4.10	3.01	0.520	3.90	4.100	6.20	1.220	3.85	0.99	1.48
180	4.990	1.150	4.86	0.58	5.43	0.520	3.23	0.830	3.83	0.174	3.50	0.72	1.09

(Values expressed as percentage, Mean of three observations), DAS -Days after sowing

4.2. Tracer Studies

4.2.1. Incorporation studies using ^{14}C- carbon dioxide

Incorporation of ^{14}C in phenolic acids

Tables 20 and 21 show the incorporation of $^{14}CO_2$ in the phenolic acids and the distribution of the label in the vegetative parts of turmeric during rhizome development (Plate 6). Since turmeric plants subjected to radiolabelling were of two months old, the observations were taken up to six months i.e., till full maturity of the rhizomes. But, no detectable incorporation of ^{14}C was observed after one month. Hence the results are explained only up to 1-month period.

In leaves, a higher level of incorporation was seen up to one week, with maximum value at 96hrs (Table 20). A gradual increase in DPM was seen from 7.478 x 10^4 DPM mg^{-1} F.W at 24hrs to 22.4 x 10^4 DPM mg^{-1} F.W at 96hrs. Afterwards a decline was observed up to one month. In pseudostem, maximum incorporation was seen at 24hrs, which then declined at 48hrs, and again showed a peak at one week, with a decline afterwards.

Rhizomes showed a steady incorporation up to 96hrs, the values ranging from 11.2 to 11.9 x 10^4 DPM mg^{-1} F.W, which further showed a decrease. In contrast to the above, negligible incorporation was observed in roots at 24hrs (63.0), with a spurt in the values at 48hrs (9.9 x 10^4 DPM mg^{-1} F.W). A decline was seen at 96hrs, followed by a 2-fold increase after one week. The values showed a diminishing trend afterwards.

Table 21 gives the percentage distribution of ^{14}C among the vegetative parts at definite intervals. The results indicated highest ^{14}C accumulation in the pseudostem at 24hrs and also after one week whereas in leaves, the distribution was higher at 48 and 96hrs. In general, higher accumulation of ^{14}C was observed in leaves, followed by rhizomes (Fig. 28 a, b, c, d and e).

Plate 6. Exposure of ^{14}C-Carbonate to turmeric seedlings

Plate 7. Root absorption of 1-^{14}C-Phenylalanine of turmeric seedlings

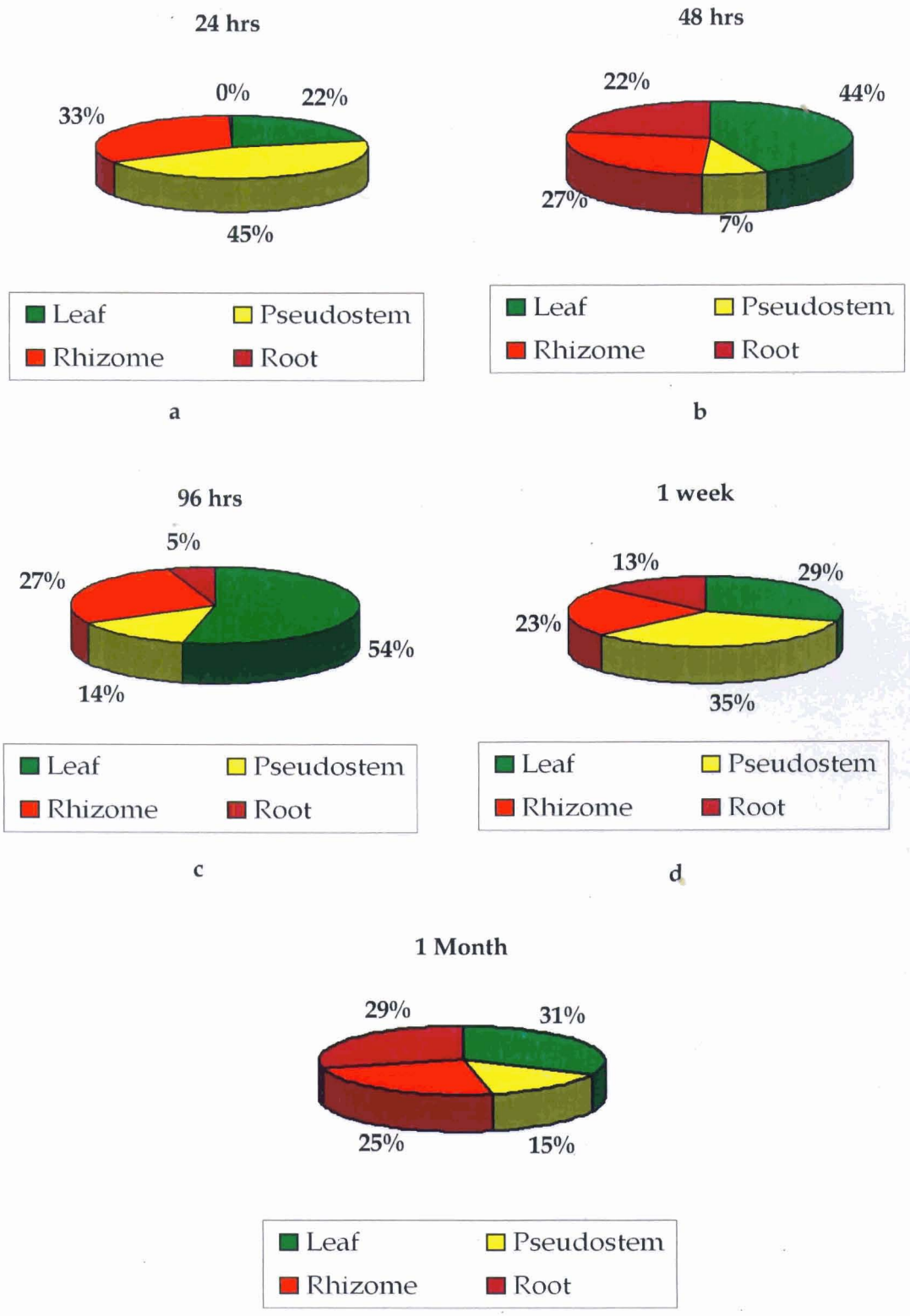

Fig. 28. Pattern of distribution of ^{14}C-CO_2 in phenolic acids among the vegetative parts of turmeric

a. 24 hrs b. 48 hrs c. 96 hrs d. 1 week e. 1 month

Table 20. Incorporation of ^{14}C-CO_2 in phenolic acids as DPM mg^{-1} F.W in various vegetative parts of turmeric during rhizome development

Period of exposure	Root	Rhizome	Pseudostem	Leaf
24 hrs	63	11207	15137	7478
48 hrs	9897	11927	3333	19297
96 hrs	2211	11417	5718	22420
1 W	4880	8500	12600	10843
1 M	1089	948	559	1205

Values are mean of three observations. DPM-Disintegrations per minute
hrs-Hours, W-Week, M-month.

Table 21. Percentage distribution of ^{14}C-CO_2 in phenolic acids among vegetative parts of turmeric

Period of exposure	Root	Rhizome	Pseudostem	Leaf
24 hrs	0.188	33.1	44.70	22.1
48 hrs	22.300	26.8	7.49	43.4
96 hrs	5.290	27.34	13.70	53.6
1 W	13.300	23.1	34.20	29.4
1 M	28.600	24.9	14.70	31.8

Values are mean of three observations. DPM-Disintegrations per minute
hrs-Hours, W-Week, M-month.

Incorporation of ^{14}C as curcumin

Incorporation of ^{14}C -CO_2 as curcumin in rhizomes and roots is shown in Table 22. Since, from our earlier observations, curcuminoids could not be detected either by TLC or by HPLC in leaves and pseudostem, they were not taken for the study. To confirm the presence of curcumin incorporation in rhizomes and roots, a TLC was run using Silica gel G and the separated curcumin fractions in alcohol was subjected to liquid scintillation counting (LSC) to check the presence of the label in curcumin. ^{14}C was found to be incorporated in these fractions, which confirmed the presence of curcumin in the alcohol extract of the tissues, used for the study.

The results indicated higher incorporation of ^{14}C label in roots and rhizomes at 96hrs. In roots, an increased level of incorporation was visible at

all stages as compared to rhizomes, whereas peak incorporation was observed at 96hrs, followed by a decline. The studies continued up to five months, after which negligible or near zero detection was seen.

Table 22. Incorporation of ^{14}C-CO_2 in curcumin as DPM mg^{-1} F.W in rhizomes and roots of turmeric during development

Period of exposure	Rhizome	Root
24hrs	77138	166094
48 hrs	78973	134596
96hrs	83822	181045
1 W	72679	55391
1 M	7143	34871
2 M	925	1188
3 M	1167	5148
4 M	189	3213
5 M	547	1246

Values are mean of three observations. DPM-Disintegrations per minute
hrs-Hours, W-Week, M-month.

4.2.2. Root absorption studies

In higher plants, feeding experiments have established that aromatic amino acids, phenylalanine and tyrosine are the primary precursors of some secondary plant products. Hence feeding the labeled compounds and measuring the subsequent isotope dilution can outline the biosynthetic pathways. Thus root incorporation studies were conducted (Plate 7) with 1-^{14}C -phenylalanine and 2-^{14}C- malonyl CoA and the results are presented below.

4.2.2.1. Studies using 1-^{14}C-phenylalanine

Incorporation of ^{14}C -phenylalanine into phenolic acids

^{14}C -phenylalanine was absorbed through the roots of two months old turmeric plants for a period of 48hrs. Extraction of phenolic acids from the

vegetative parts of the plant was done, as described under Materials and Methods and incorporation of ^{14}C - was determined using LSC. The results are presented in Table 23.

Since the absorption was mainly through roots in this study, higher levels of absorption were seen in roots at 24 and 48hrs, followed by rhizomes, which declined after one week. Leaves and pseudostem did not show any incorporation. Leaves and pseudostem exhibited lower rate of incorporation during this period as compared to roots and rhizomes. After one month, a decline in the incorporation was observed in these vegetative parts. Subsequently, only negligible incorporation was seen up to five months and hence data are not shown here.

Table 23. Incorporation of 1-^{14}C-phenylalanine as DPM mg^{-1} F.W in phenolic acids in vegetative parts of turmeric during rhizome development.

Period of exposure	Rhizome	Root	Pseudostem	Leaf
48 hrs	3730	82,78,66	0.00	0.00
1 W	895	183	228	336
1 M	300	5011	95	157

Values are mean of three observations. DPM-Disintegrations per minute
hrs-Hours, W-Week, M-month.

Table 24 shows the incorporation of ^{14}C label in curcumin during the absorption of 1-^{14}C phenylalanine. As in the case of phenolic acids, highest accumulation was noticed in the roots and rhizomes up to a period of one month. At two months, a slight increase in the incorporation was noticed in roots only. At 4th month, roots and rhizomes showed higher incorporation. During subsequent stages, negligible values were obtained and hence data are not shown.

Table 24. Incorporation of 1-^{14}C-phenylalanine as DPM mg^{-1} F.W in curcumin from turmeric during rhizomes and roots.

Period of exposure	Rhizome	Root
48 hrs	16,605	84,914
1 W	10,170	65,640
1 M	828	10,339
2 M	250	15,145
3 M	200	705
4 M	956	1006

Values are mean of three observations. DPM-Disintegrations per minute
hrs-Hours, W-Week, M-month.

4.2.2.2. Studies using 2-^{14}C-Malonyl CoA

Rhizomes and roots only were sampled for the extraction of curcumin. 2-^{14}C-Malonyl CoA was allowed to absorb through the roots, for 48hrs. In roots, significant incorporation was seen up to one week, after which it was poor and erratic (Table 25). In rhizomes also, negligible incorporation was seen, which showed a declining trend.

Table 25. Incorporation of 2-^{14}C-Malonyl CoA as DPM mg^{-1} F.W in curcumin

Period of exposure	Rhizome	Root
1W	53.00	1498.0
1M	34.00	227.6
2M	31.30	53.6
3M	26.10	34.3
4M	24.26	28.2
5M	23.90	24.5
6M	23.90	30.7

Values are mean of three observations. DPM-Disintegrations per minute
hrs-Hours, W-Week, M-month.

4.3. Phenylalanine Ammonia Lyase (PAL)

4.3.1. PAL activity during rhizome development

Table 26 shows the level of PAL in turmeric leaves during different stages of rhizome growth, starting from 30 DAS up to 150 DAS. The values are shown for five released varieties of turmeric and in a few turmeric accessions. Leaves were not available for sampling after this period and hence enzyme activity could not be assayed.

Table 26a gives the ANOVA for the PAL activity between varieties and stages. All the levels of correlation were found to be significant and the level of significance was tested using DMRT according to which the accessions could be classified into 8 groups based on the PAL activity.

It is seen that both at 60 DAS and 90 DAS, PAL activity showed highest value in all the released varieties, which then showed a decline during the ensuing period. Almost 2-3-fold increase was seen during this period.

Among the 15 more accessions assayed for PAL activity, higher levels were found at 60 DAS and 90 DAS in majority of the cases. A few accessions (Accession nos. 126, 290, 329, 339, 367 and 298) showed maximum activity at 60 DAS, while some accessions (Acc. no.672) did not cause any significant increase. Accessions 656, 585, 310, 288 and 328 showed a peak activity at 90 DAS. At 120 DAS and 150 DAS, more or less uniform pattern is shown by all accessions studied.

Table 26. Phenylalanine ammonia lyase activity in leaves of turmeric varieties / accessions during different growth stages

Varieties / accessions	Specific activity of Phenylalanine ammonia lyase activity in turmeric leaves (unitsx 10^{-2})				
	30 DAS	60 DAS	90 DAS	120 DAS	150 DAS
Alleppey	5.68	18.49	11.14	4.31	4.02
Suguna	4.78	10.10	13.35	3.23	3.15
Sudarsana	4.96	7.35	6.05	2.45	3.04
Prabha	2.69	5.64	2.95	2.05	1.87
Prathibha	2.57	7.33	9.34	3.77	3.85
126	3.52	7.39	5.02	3.61	3.09
656	4.02	7.98	22.79	5.82	4.83
290	2.07	7.74	4.18	3.11	3.24
585	4.86	7.22	8.65	4.05	3.92
329	2.65	12.98	7.07	5.56	5.83
310	1.42	2.30	9.86	3.08	2.99
288	1.89	7.10	14.58	5.99	4.75
298	2.53	13.30	10.31	3.06	3.28
335	2.69	9.75	8.59	3.15	3.88
285	1.69	3.91	1.21	2.02	2.45
339	2.91	8.73	6.19	2.54	2.86
672	1.58	2.71	2.22	1.76	1.94
303	2.42	4.88	3.64	2.79	2.14
367	4.26	8.46	4.28	3.80	3.61
328	3.35	2.84	7.11	3.07	3.15

Values are mean of three observations. DAS- Days after sowing

Table 26a. ANOVA

Dependent Variable: PAL ACTIVITY

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
VARIETY	1031.836	19	54.307	198.633	0.000 (HS)
STAGES	1545.263	4	386.316	1412.986	0.000 (HS)
VARIETY * STAGES	1445.622	76	19.021	69.572	0.000 (HS)
Error	54.681	200	0.273		
Total	12105.422	300			
Corrected Total	4077.402	299			

a R Squared =0.987 (Adjusted R Squared = 0.980), HS= Highly significant.

A correlation study conducted between PAL activity and total curcuminoids content in five released varieties showed high significance

4.3.2. Sub cellular localization of PAL in turmeric leaves

4.3.2.1. Cell fractionation of PAL

Cellular localization of PAL was carried out after extracting the enzyme using Tris HCl (pH 7.6) as described in Materials and Methods. Removal of microsomes from the extract was done after layering over a 30% concentration of sucrose. This was allowed to spin at 27,000 rpm for 35min. The layer formed between 6% and 30% contained major microsomal fractions with a PAL activity of 226.3 x 10^{-2} units. The remaining zones and interfaces were devoid of PAL activity.

The interface (6-30%) was again layered between 15 and 20% sucrose concentration to recover the microsomes in a gradient consisting of 15 to 39% and centrifuged at 27,000 rpm. The specific activity of PAL in this zone was determined and found to be 395.9 x 10^{-2}units.

The microsomal marker enzyme glucose-6-phosphatase was assayed in the microsomal fraction and showed a specific activity of 2.88 x 10^{-2}units (Table 27). The activities of catalase and fumarase which are cytosolic and mitochondrial markers showed negative values. The presence of both PAL and glucose-6-phosphatase in the microsomal fraction confirms the localisation of PAL in the microsomes.

Table 27. Activity of PAL and marker enzyme located in 6-30% sucrose density gradient

Sucrose concentration	Specific Activity of PAL (units x 10^{-2})	Specific activity of Glucose -6- phosphatase (microsomal marker enzyme) (units x 10^{-2})	Protein concentration (mg/ml)
6-30% interphase	395. 9	2.88	2.65

Catalase and fumarase showed negative activity.

4.3.2.2. Localization of PAL in the microsomal sub fractions

Another experiment using sucrose density gradient centrifugation could locate PAL in the different sub fractions of microsomal particles. In this case, the extracting buffer contained EDTA and $MgCl_2$, with 8% sucrose. The extract was layered over a linear gradient of sucrose starting from 15% to 45% and was centrifuged at 27,000 rpm for 4hrs. This experiment differed from the earlier one in that the sucrose gradient consisted of KCl, EDTA, and $MgCl_2$ to maintain the osmoticum of the cells and to provide a buffering action.

Tables 28 and 29 show PAL activity in the different sub fractions obtained after centrifugation, along with the marker enzyme specific for microsomes *viz.*, glucose-6-phosphatase. Fractions 15-17, 21, 24, 25 responded both to PAL and microsomal marker enzymes, out of a total of 32 fractions collected after density gradient centrifugation (Table 30). Total PAL activity was observed in the combined fraction (15-17) as 720.4 x 10^{-2} units. Fraction 21 had PAL activity of 285.2 x 10^{-2} units, while fractions 24 and 25 indicated PAL activity of 392.2 x 10^{-2} units. Thus it is seen that maximum activity for PAL was observed at portions with almost medium density of sucrose, followed by the two other peaks at slightly higher dense areas.

Catalase and fumarase, which are specific markers for cytosol and mitochondria showed negative values. This confirmed the absence of PAL in these organelles. Glucose-6-phosphatase, the specific marker for microsomal fractions, exhibited an activity ranging from 0.675 to 2.33 x 10^{-2} units in the fractions positive to PAL.

Based on the earlier studies on PAL localisation in other crops it can be presumed that the relative amount of PAL activity recovered in the microsomal fractions is highest at the portion of endoplasmic reticulum

possibly at 23-25% sucrose, while activities are also present in proplastids and plasma membrane (approximately at 29.5% and 34-35% sucrose).

Table 28. Distribution of PAL and Glucose-6-phosphatase activities in the sub fractions

Fraction Number (1.2 ml/tube)	Activity Phenylalanine ammonia lyase	Activity of glucose-6-phosphatase
1- 14	Nil	Nil
15-17	Present	Present
18-20	Nil	Nil
21	Present	Present
22-23	Nil	Nil
24-25	Present	Present
26-32	Nil	Nil

Table 29. Activity of PAL and marker enzyme in collected fractions

Fraction No.	Activity of PAL (μ g of cinnamic acid released)	Glucose-6-Phosphatase activity (Phosphoric acid released min^{-1} mg^{-1} protein x 10^{-2})	Protein μg / ml
1	Nil	Nil	170.87
2	Nil	Nil	254.11
3	Nil	Nil	250.43
4	Nil	Nil	211.05
5	Nil	Nil	296.97
6	Nil	Nil	313.74
7	Nil	Nil	345.25
8	Nil	Nil	346.81
9	Nil	Nil	408.32
10	Nil	Nil	398.92
11	Nil	Nil	447.44
12	Nil	Nil	394.53
13	Nil	Nil	469.74
14	Nil	Nil	419.28
15	6.920	0.8581	449.43
16	17.606	1.215	413.05
17	11.787	1.2013	414.59
18	Nil	Nil	366.60
19	Nil	Nil	412.06
20	Nil	Nil	403.12
21	15.311	0.675	447.44
22	Nil	Nil	247.69
23	Nil	Nil	165.49
24	1.818	0.967	175.83
25	2.472	2.334	67.117
26	Nil	Nil	54.834
27	Nil	Nil	43.069
28	Nil	Nil	30.739
29	Nil	Nil	26.534
30	Nil	Nil	28.849
31	Nil	Nil	27.951
32	Nil	Nil	21.431

Table 30. Specific activity of fractions positive to PAL and marker enzyme

Tube No. (each tube contain 5 ml sample)	Specific activity of PAL (units x 10^{-2})	Specific activity of glucose-6-phosphatase (units x 10^{-2})
15	1.283	0.8581
16	3.552	1.2150
17	2.369	1.2013
21	2.852	0.6750
24	0.862	0.9668
25	3.060	2.3340

Catalase and fumarase showed negative activity.

4.3.3. Purification of PAL in turmeric leaves

The purification scheme developed for *Curcuma longa* resulted in 157-fold purification from the crude extract after ammonium sulphate precipitation and two chromatographic steps, with a yield of about 15% of the activity present in the original homogenate (Table 35). Column chromatography of the enzyme over DEAE - cellulose could give almost 16-fold purification, with a specific activity of 870.4 x 10^{-2} units. When this protein was again subjected to gel filtration over sephacryl S- 300, it nearly gave way to another 10-fold purification. $(NH_4)_2SO_4$ precipitation could recover about 73.3% of the activity from the crude homogenate, while anion exchange chromatography could retain 30% of the original activity. After the second chromatographic step over Sephacryl S- 300, 15% of the yield recovery of PAL was observed, with a higher level of purification.

Ammonium sulphate fractionation was carried out at 40-70% saturation. The pellet was dissolved in buffer B and subjected to DEAE cellulose column chromatography. Table 31 shows the molarity of sodium chloride, used in buffer B, for elution of the enzyme. Linear gradient elution with 250 ml each of buffer B and C showed maximum concentration of protein between 0.1 and 0.2M, which corresponded with fraction numbers 21-30. The protein content of the 50 fractions were determined by monitoring the absorbance at 280 nm. Soluble protein applied to the column was distributed

in various fractions covering all molarity ranges (Fig. 29). The PAL activity was determined in the fractions (Table 32). It was seen that only fractions 21-30 contained PAL activity.

Table 31. Presence of PAL activity in different molar solutions of sodium chloride during DEAE-Cellulose chromatography

Tube No. (5ml/tube)	Molarity of sodium chloride	PAL activity
1-10	0.0 M	Nil
11-20	0.0- 0.1 M	Nil
21-30	0.1-0.2 M	Present
31- 40	0.2-0.3 M	Nil
41-50	0.3-0.4 M	Nil

Table 32. Phenylalanine Ammonia Lyase activity in collected fractions

Tube No. (5ml/sample)	Molarity of sodium chloride	Concentration of protein (mg/5ml)	Specific activity of PAL (units x 10^{-2})
21	0.1-0.2 M	0.15	22.0
22		0.207	59.1
24		0.326	109.3
25		0.728	275.0
26		0.620	218.1
27		0.453	124.4
28		0.304	112.8
29		0.220	68.3
30		0.185	31.5

Gel Filtration using sephacryl S- 300

The most active fractions of PAL (21-30) after DEAE- cellulose chromatography were pooled and concentrated, subjected to gel filtration over sephacryl S- 300. This gave one major peak (Fraction 14-19) with PAL activity and a few minor peaks, which were devoid of PAL activity (Tables 33 & 34). The protein values were determined by UV (280 nm) in these fractions and depicted in Fig. 34.

The fractions containing PAL activity were concentrated using centrifugal ultra filtration devices (Centre prep 30: Amicon). SDS-PAGE was carried out using the concentrated protein, as described in Materials and Methods. Plate 8 describes the banding pattern of the proteins separated during the various stages of purification. A thick fast moving band was

consistently seen in all the lanes, indicating the different stages of purification (Plate 9).

Table 33. Presence/absence of PAL in the collected fractions

Tube No. (each tube contain 5ml sample)	Phenylalanine ammonia lyase activity
1-13	Nil
14-19	Present
20-51	Nil

Table 34. PAL activity in the fractions

Tube No. (each tube contain 5ml sample)	Concentration of protein (mg/5ml)	Specific activity of PAL (units x 10^{-2})
14	0.009	270.5
15	0.015	488.3
16	0.055	4450.3
17	0.048	2561.5
18	0.021	1030.4
19	0.011	120.0

The reference proteins used in determining the molecular weight (MW) are Rabbit phosphoryalse b (MW 97,400), Bovine serum albumin (MW 66,200), Glutamate Dehydrogenase (MW 55,000), Ovalbumin (MW 42,700), Aldolase (MW 40,000), Carbonic anhydrase (MW 31,000), Soya bean trypsin inhibitor (MW 21,500) and Lysozyme (MW 14,400). Electrophoresis of the purified enzyme (Plate 9) gave a thick single stained protein band corresponding to a subunit molecular weight ranging from 38,000 to 40,000, indicating that the enzyme is an oligomer consisting of four subunits.

Table 35 summarises the various steps used for the purification of PAL and the levels of purification seen at every step. 157-fold purification was obtained for PAL with a specific activity of 8822.4 units.

Table 35. Summary table for purification of PAL

Sample	Protein (mg/ml)	PAL activity (μ g of cinnamic acid released)	Specific activity (units x 10^{-2})	Fold	Yield (%)
Crude	161.200	54.30	56.2	1.00	100.0
Ammonium sulphate precipitation	71.050	39.80	91.9	1.63	73.3
DEAE- Cellulose	3.150	16.45	870.4	15.50	30.3
Sephacryl S-300	0.153	5.09	8822.4	157.00	15.0

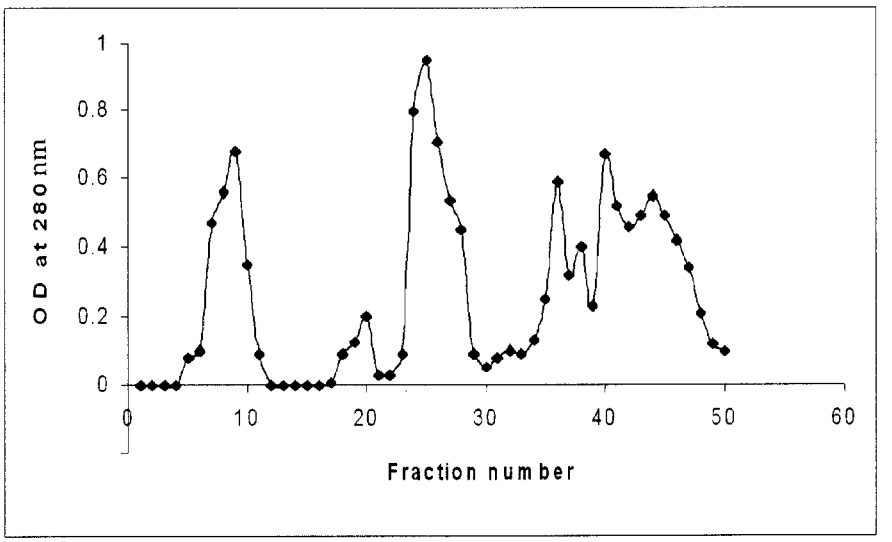

Fig. 29. Elution profile of DEAE Cellulose column chromatography (Details as described in Materials and methods)

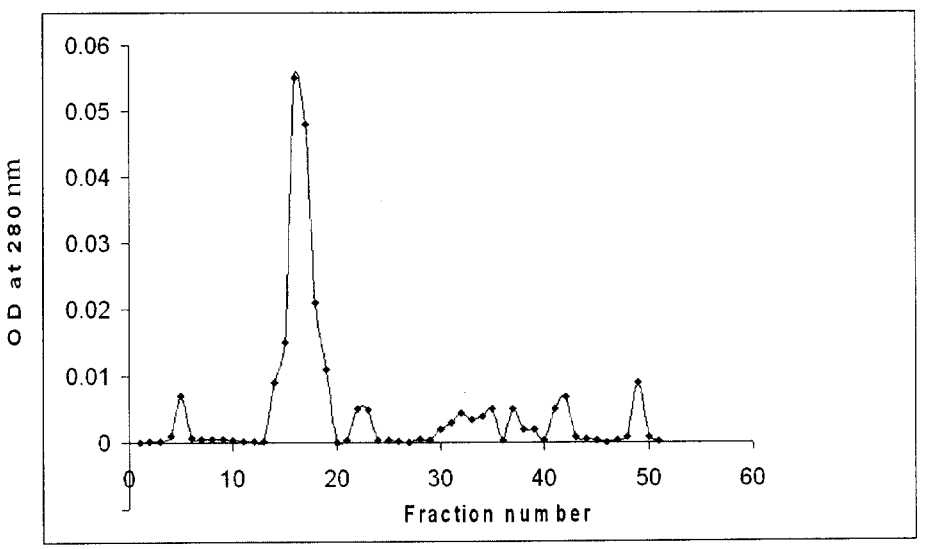

Fig. 30. Elution profile of Sephacryl S- 300 gel filtration (Details as described in Materials and methods)

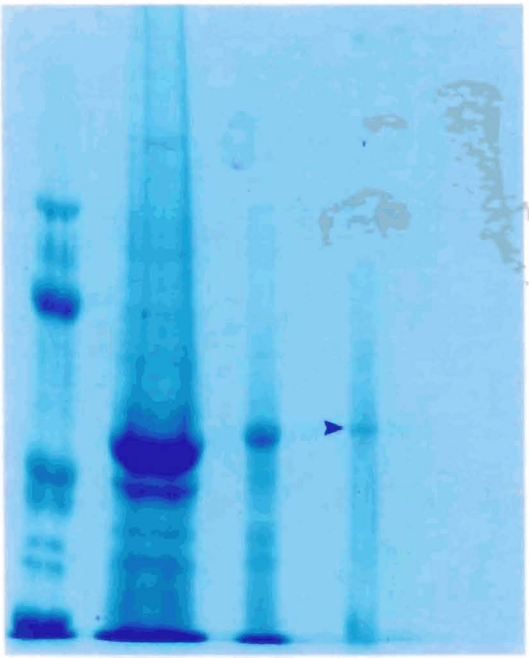

Plate 8. Protein profile at different levels of purification of phenylalanine ammonia lyase in turmeric leaves

Lane 1 - Sample after centrifugation
Lane 2 - After Ammonium Sulphate precipitation
Lane 3 - After DEAE Sephacel chromatography
Lane 4 - After Sephacryl - S 300 gel chromatography

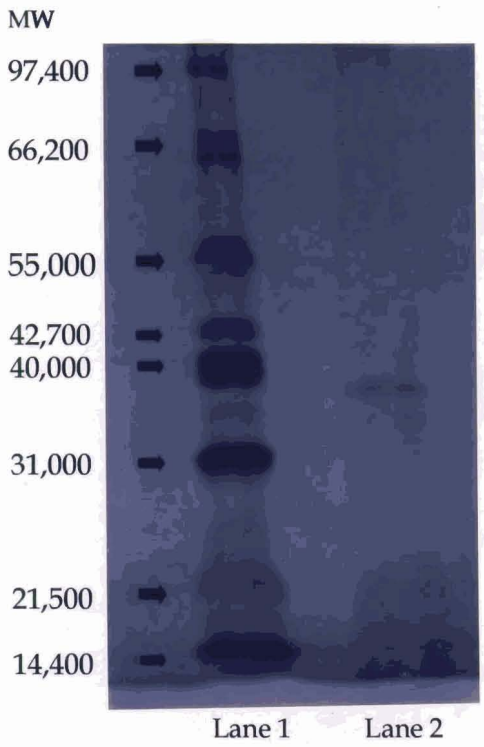

Plate 9. SDS-PAGE of purified phenylalanine ammonia lyase in turmeric leaves vs marker protein profile

Lane 1 - Marker proteins
Lane 2 - Purified protein of phenylalanine ammonia lyase

4.3.3.1. Kinetic studies

Substrate concentration

The initial velocity of PAL in the purified fraction as a function of the L-phenylalanine concentration, which is the primary substrate, is shown in Fig. 31. The molarity of the substrate ranged from 0.1mM to 1.0mM. There was a progressive increase in the activity with increase in phenylalanine concentrations. At higher molarity, the enzyme activity showed slight variation, indicating a steady level of the reaction velocity at this point. The K_m value was determined by drawing a double reciprocal plot with 1/v versus 1/s, as the slope of tangents to the curve (Fig. 32). Apparent K_m value for the reaction was estimated to be 0.33mM.

Temperature

Effect of temperature on the activity of enzyme was studied, ranging from 20°C to 45°C, at an interval of 5°C. Specific activity at each temperature was determined and was found to show a progressive increase upto 25°C ambient (888.9 x 10^{-2} units), which then declined (Fig. 33).

Effect of PH

Purified enzyme was used for determining the optimum pH of PAL. Specific activity was determined at pH ranging from 2.0 to 10.0. The maximum activity of PAL was seen at pH 8.8, with 876 x 10^{-2} units (Fig. 34), which then declined.

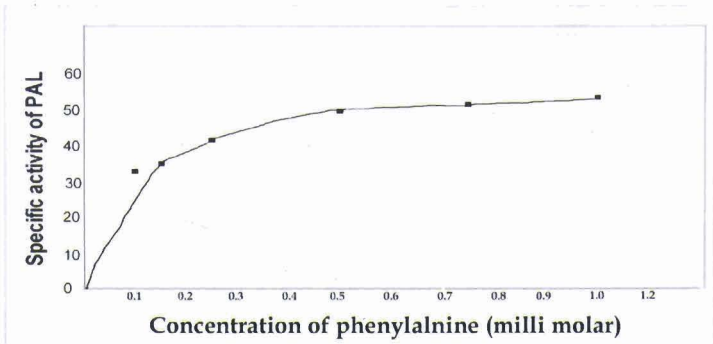

Fig. 31. Effect of exogenous addition of phenylalanine on PAL activity in the leaves of turmeric (Details as described in Materials and methods)

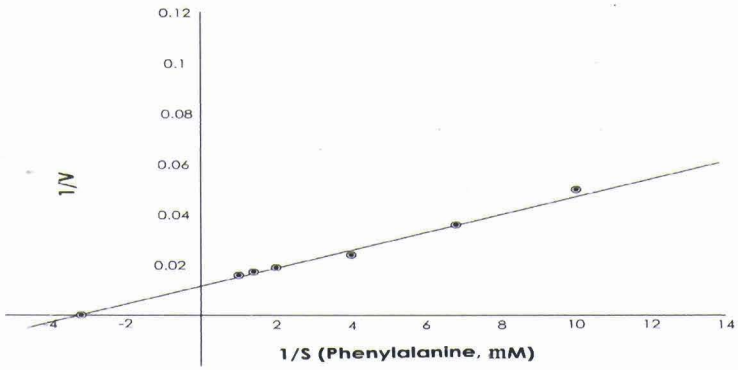

Fig. 32. Lineweaver-Burk plot for turmeric leaf PAL at different concentration of phenylalanine (Details as described in Materials and methods)

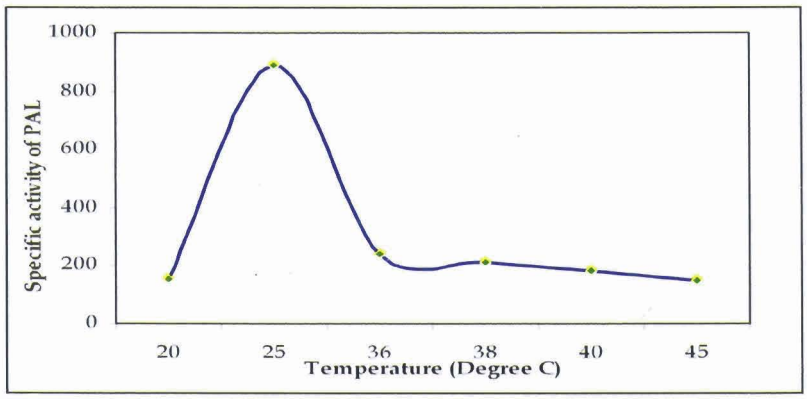

Fig. 33. Temperature - activity relationship of PAL from turmeric leaves (Details as described in Materials and methods)

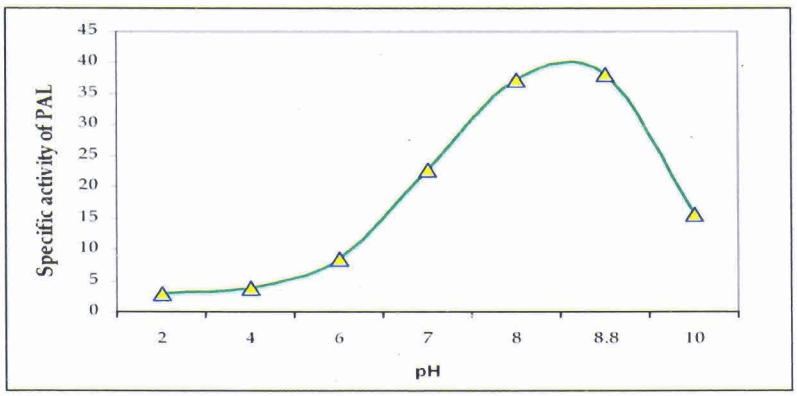

Fig. 34. pH-activity relationship of PAL from turmeric leaves (Details as described in Materials and methods)

Effect of inhibitors

Phenolic acids and their analogues inhibit PAL activity. Table 36 and Fig. 35 -38 summarise the results of inhibition experiments where cinnamic acid, ferulic acid, *p*-coumaric acid and chlorogenic acid were tested as inhibitors.

Trans- cinnamic acid, with molarities ranging from 0.03 to 1.0M was added to the medium for the assay. There was complete inhibition at higher concentrations of the compound (1M), which reduced the activity to near zero levels. Partial inhibition to the development of enzyme activity was seen at lower concentrations. In the case of ferulic acid, a linear decrease in the specific activity, indicating an increased level of inhibition, was observed in the leaves. In contrast to cinnamic acid, ferulic acid and coumaric acid did not completely abolish the activity and at lower concentrations gave only relatively weak modulation. However, exogenous addition of chlorogenic acid gave complete inhibition of the activity at all molarities tested (Table 37).

Table 36.Effect of inhibitors on specific activity of PAL

Inhibitor (M)	Protein (mg/ml)	PAL activity (Cinnamic acid released mg min^{-1})	Specific activity (Units x 10^{-2})	Percentage inhibition
Cinnamic acid				
0.03	0.6245	22.080	58.80	59.03
0.10		7.870	20.98	85.40
0.30		4.560	12.16	91.52
1.00		0.706	1.88	98.67
Coumaric acid				
0.10		35.200	93.80	34.64
0.50		11.700	31.20	78.26
1.00		3.200	8.50	94.08
Ferulic acid				
0.10		41.950	111.87	65.58
0.50		32.350	86.30	83.39
1.00		14.520	38.72	73.02
Chlorogenic acid				
0.10		1.2900	3.00	141.40
0.50		16.500	44.00	112.80
1.00		18.450	49.00	109.00
Without inhibitor (control)		53.820	143.52	

Values are mean of three observations

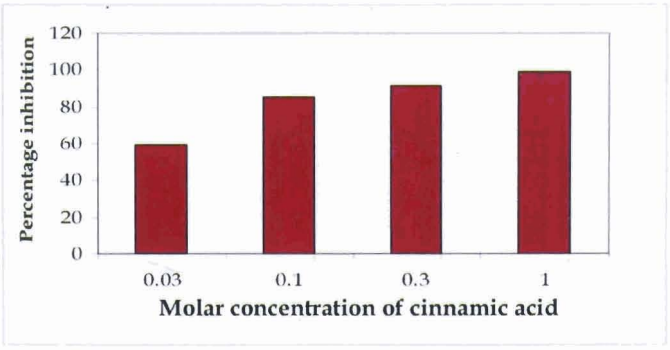

Fig. 35. Inhibitory effect of cinnamic acid on PAL activity

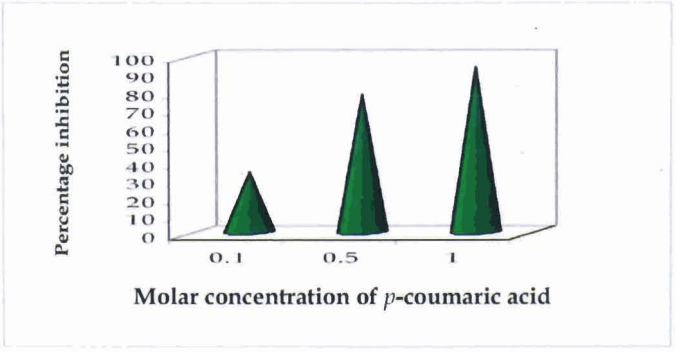

Fig. 36. Inhibitory effect of *p*-coumaric acid on PAL activity

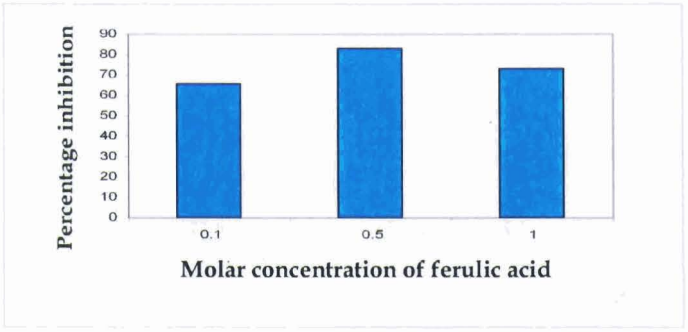

Fig. 37. Inhibitory effect of ferulic acid on PAL activity

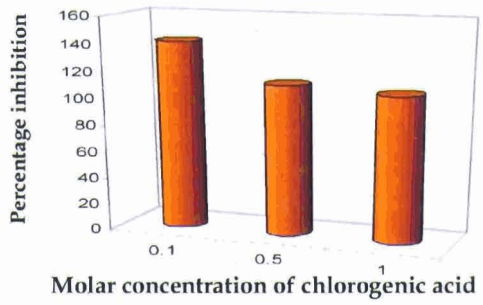

Fig. 38. Inhibitory effect of chlorogenic acid on PAL activity

Light studies

PAL activity of etiolated turmeric seedlings (grown in dark, plate 11) exposed to red (plate 10), blue and white light is given in Table 37 and Figs. 39, 40 and 41. The exposure time was 72 hrs and the samples were taken at 6hr-intervals and compared with control. Exposure of the seedlings to red light showed two peaks - one at 18 hrs and another at 48 hrs. (Fig. 39). Fig.40 showed the effect of continuous white light in which an early peak at 18 hrs is followed by a much greater peak at 48 hrs. Fig.41 showed the behavior in blue light, in which an early peak at 12 hrs is seen. It should be noted that continuous white light leads to greater increase in extractable PAL activity than by any of the other irradiation treatments used. It is possible that the greater energy of white source may be responsible. In all cases a lag phase of 12 hrs. is seen. A 3-fold increase in the activity was seen at 48 hrs after exposure. However, the seedlings kept in dark expressed a peak at 12 hrs, after which negligible activity was seen.

Table 37.Effect of different source of light on PAL activity

Serial No.	Period of exposure (hrs)	Specific activity of Pal (units x 10^{-2})			
		Red light	Blue light	White light	Dark control
1	0	0.074 ± 0.004	0.075 ± 0.004	0.075 ± 0.004	0.0750 ± 0.004
2	6	3.550 ± 0.16	1.240 ± 0.082	3.650 ± 0.14	0.1239 ± 0.006
3	12	4.890 ± 0.16	8.820 ± 0.29	6.270 ± 0.095	3.2400 ± 0.05
4	18	8.500 ± 0.51	6.270 ± 0.142	8.820 ± 0.085	0.2500 ± 0.027
5	24	7.330 ± 0.44	4.750 ± 0.16	7.800 ± 0.10	0.3700 ± 0.02
6	30	14.090±0.098	3.990 ± 0.32	16.180 ± 0.079	0.0570 ± 0.007
7	36	14.540 ± 0.35	2.070 ± 0.066	18.890 ± 0.12	0.0550 ± 0.001
8	48	25.510 ± 0.41	0.650 ± 0.053	27.330 ± 0.064	0.0054 ± .0036
9	60	4.960 ± 0.15	0.700 ± 0.085	8.970 ± 0.095	0.0000
10	72	4.290 ± 0.13	0.530 ± 0.17	8.130 ± 0.12	0.000

Values given are mean of three observations

Statistical analysis was done using ANOVA and the data were found to be highly significant. The level of significance was tested using DMRT. ANOVA of the values for red, blue, white and dark are given in Tables 38a, b, c and d.

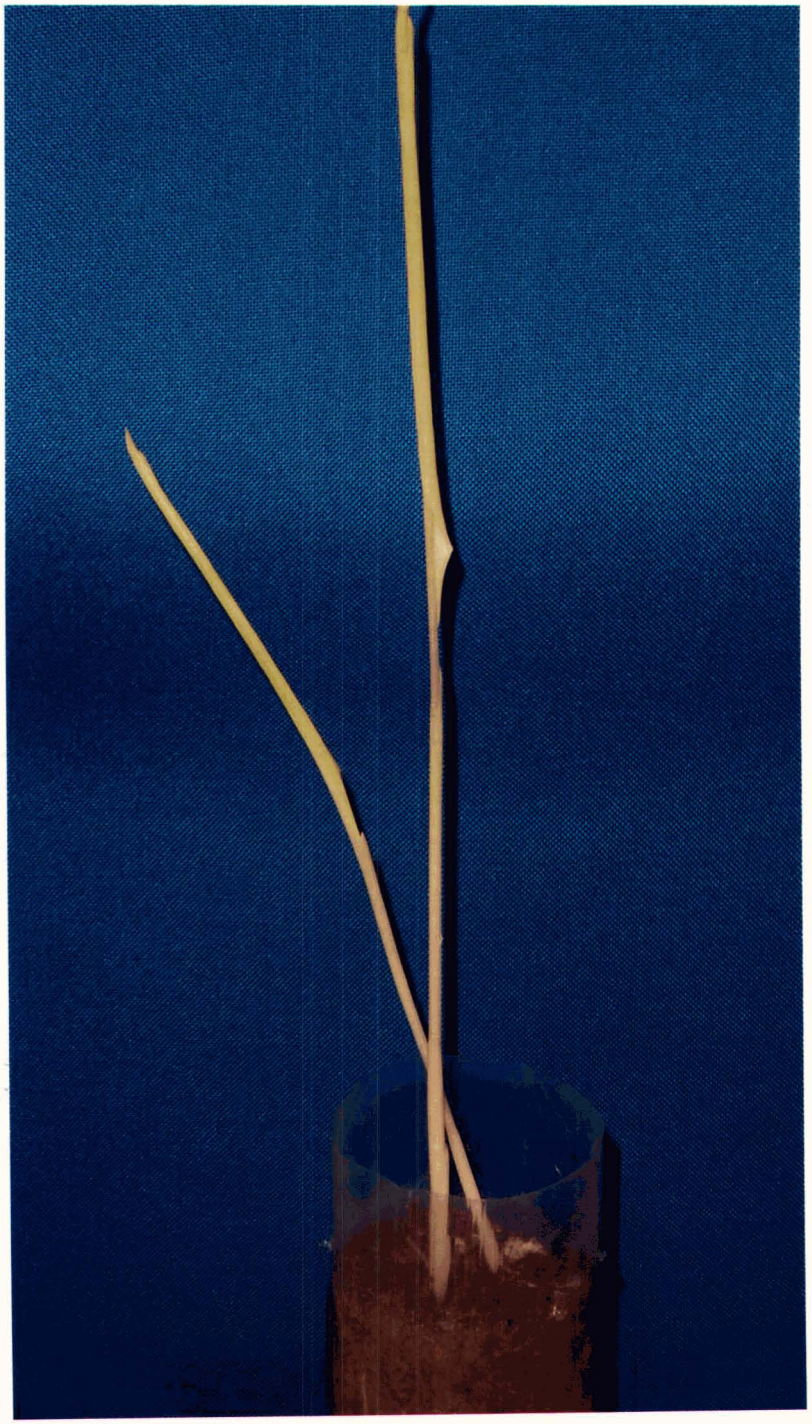

Plate 10. Etiolated turmeric seedlings

Plate 11. Exposure of etiolated turmeric seedlings to red light

Table 37a. ANOVA for red light

Dependent Variable: RED

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
HR	1489.385	9	165.487	1953.085	0.000 (HS)
Error	1.695	20	o.085		
Total	3800.308	30			
Corrected Total	1491.079	29			

a R Squared = 0.999 (Adjusted R Squared = 0.998). HS= Highly Significant

Table 37b. ANOVA for blue light

Dependent Variable: BLUE

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
HR	233.251	9	25.917	1045.825	0.000 (HS)
Error	0.496	20	0.025		
Total	485.960	30			
Corrected Total	233.747	29			

a R Squared = .998 (Adjusted R Squared = .997). HS= Highly Significant

Table 37c. ANOVA for white light

Dependent Variable: WHITE

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
HR	1731.626	9	192.403	20985.825	0.000 (HS)
Error	0.183	20	0.009		
Total	5110.161	30			
Corrected Total	1731.810	29			

a R Squared = 1.000 (Adjusted R Squared = 1.000). HS= Highly Significant

Table 37d. ANOVA for dark source

Dependent Variable: DARK

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
HR	26.826	9	2.981	8019.127	0.000
Error	0.007	20	0.000		
Total	32.189	30			
Corrected Total	26.834	29			

a R Squared = 1.000 (Adjusted R Squared = 1.000). HS= Highly Significant

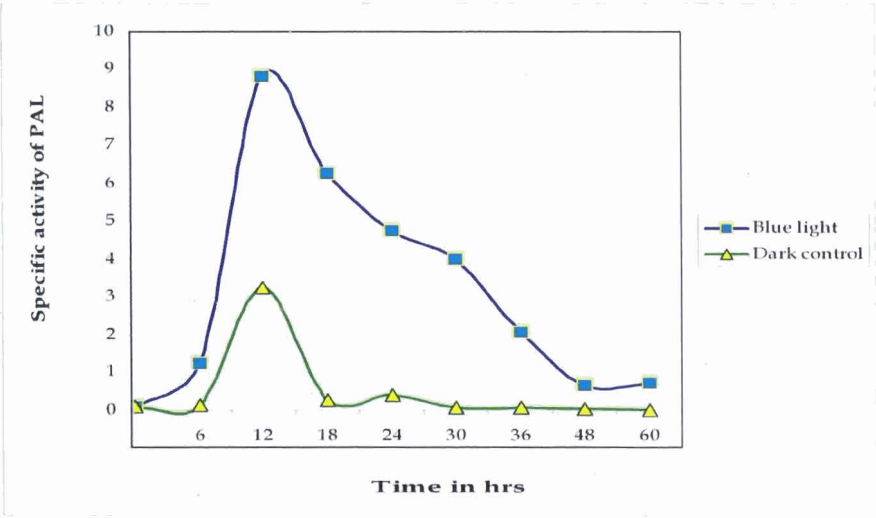

Fig. 39. Effect of continuous irradiation of blue light on PAL activity in leaves of etiolated turmeric plants

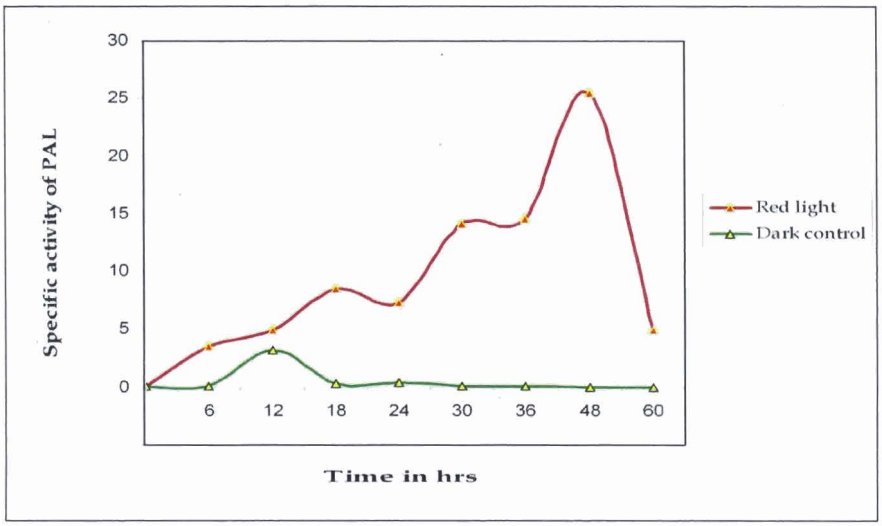

Fig. 40. Effect of continuous irradiation of red light on PAL activity in leaves of etiolated turmeric plants

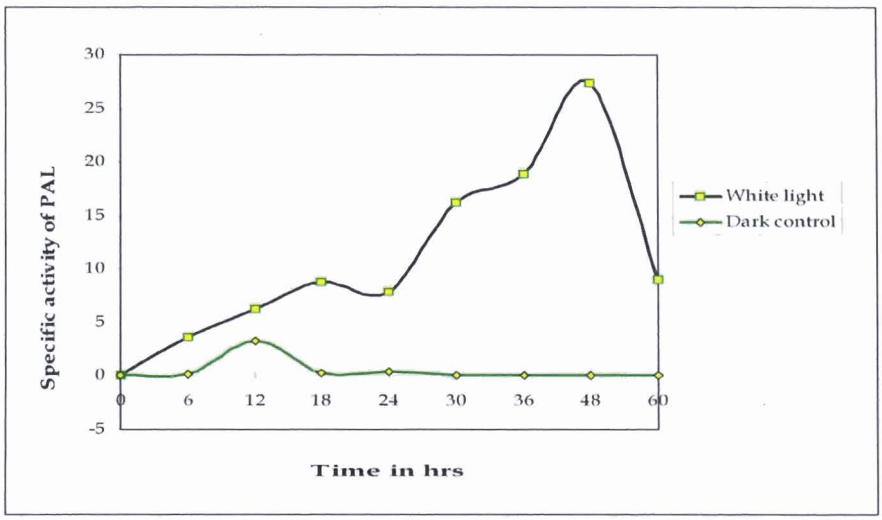

Fig. 41. Effect of continuous irradiation of white light on PAL activity in leaves of etiolated turmeric plants

4.4. Amplification of PAL gene

Good quality RNA was obtained from *Curcuma* leaves following the protocol of Johnson *et al.*, (2005). RNA was subjected to reverse transcription using PAL specific primer. Upon electrophoresis in 2% agarose gel, a distinct bands was observed of size approximately 400bp.

DISCUSSION

Neema Antony "Investigations on the Biosynthesis of Curcumin in Turmeric (Curcuma longa L.)" Thesis. Indian Institute of Spices Research , University of Calicut, 2005

DISCUSSION

CONTENTS

DISCUSSION

Biosynthesis involves the *in vivo* production of compounds from the simpler ones and involves a number of enzyme systems and intermediates. In higher plants like turmeric, the rate and occurrence of the reactions may differ based on the availability of the precursors and intermediates and also regulation at the gene level which in turn depend on the necessity of the cell for particular metabolites. However due to the paucity of enzyme studies in this area, the details of biosynthetic pathways remains largely unknown or speculative, especially the biosynthetic control mechanism and their relation to the growth of the plant.

The experiments carried out in the present study have led to some conclusions, which are explained in this chapter, specifically on the rate determining factors. Discussions are mainly confined to the relative variations in primary and secondary metabolites, the formation of the curcuminoids as related to the availability of precursors and intermediates and activity of the regulatory biosynthetic enzyme during rhizome development. Three tracer experiments conducted during the investigation also give insights on the prime precursor for the biosynthesis and the major intermediates involved, in addition to arriving at the most probable pathway for the biogenesis. The discussion also centers on the localisation, purification and kinetics of the major rate-determining enzyme Phenylalanine ammonia lyase (PAL), which initiates the biosynthesis.

5.1. Primary metabolites

5.1.1. Starch

Starch is the major reserve carbohydrate in curcuma species as reported by Uphof (1968). Apart from starch as the major polysaccharide, Gonda *et al.* (1990) has isolated another acidic polysaccharide from the rhizomes, which may also add to the total content. The level starch was found to be progressively increasing as the plant matures. The lower percentage of starch at the early stages might be due to the utilization of plant carbon source for the synthesis of metabolites other than starch, which is then followed by the accumulation of starch, before full maturity. This is supported by the findings that curcuminoids and other secondary metabolites showed higher values in the early stages of plant growth, which require carbon source for their synthesis as reported elsewhere in this thesis. At later stages, both secondary metabolites as well as starch reached a plateau.

The study could indicate a similar pattern for varieties Prabha and Prathibha, while Suguna and Sudarsana, which are early maturing varieties, forming, another group. Var. Alleppey however, behaved differently.

Regarding dry recovery, after 180 DAS, marked increase was seen, which was also reflected in the changes in starch and other secondary metabolites. Early maturing varieties, Suguna and Sudarsana, had low dry recovery as compared to other varieties, studied.

5.2. Secondary Metabolites

The major secondary metabolites of commercial importance in turmeric are oleoresin, essential oil and curcuminoids, which impart the characteristic aroma, flavour and color to the rhizome. In addition, phenolic

compounds, which are the precursors for these metabolites bears equal significance.

5.2.1. Phenolic acids

Phenolic acids are the major intermediary precursors for phenylpropanoid biosynthesis (Bolwell *et al*, 1986; Creasy, 1987; Jorrin and Dixon, 1990; Sato *et al*, 1982, Stafford and Ibrahim, 1992). Conversion of cinnamate to *p*-coumarate and further to caffeate and ferulate is the established series (Torssell, 1983; Van Sumere and Lea, 1985; Swain, *et al*, 1979), which form the initial part of the biosynthetic pathway of curcuminoids as suggested by Geissman (1969). The major occurrence of these three phenolic acids, as evidenced by TLC and HPLC, with minor amount of sinapic acid (detected only through HPLC), which is the immediate product after ferulate in the phenolic acid metabolism in turmeric, leaves clearly support the precursor role of these components. The negligible presence of sinapic acid in the leaves combined with a higher rate of formation of the earlier metabolites give additional evidence for the channeling of these compounds towards curcumin biosynthesis. This also indicates the higher levels of phenolic intermediates in the leaves, which could be correlated with the higher activity of PAL in leaves, especially at 120 DAS.

5.2.2. Oleoresin

Oleoresin, rightly called "the essence of spice" constitutes the high volatile oils, coloring principle, fixed oils and resins. Between 120 and 210 DAS, the rhizomes contained higher amount of oleoresin, at which stage the other secondary metabolites viz. essential oil and curcuminoids also occur in higher proportion. In roots, however, maximum content of oleoresin was observed at 150 DAS, indicating a probable role of storage site for roots, which may later be translocated to rhizomes. The higher values for

curcuminoids at this stage (150 DAS) support this contention. Among the varieties, higher values were seen for Suguna and Sudarsana at 150 DAS (17% and 14% respectively), at which stage, the major reserve carbohydrates had shown a decline. This may be due to the diversion of their precursors for the formation of oleoresin.

Statistical analysis indicated that varieties Suguna and Sudarsana formed a group having least value in the case of oleoresin as per DMRT. Highest essential oil was seen in var. Suguna, with varieties Prabha, Prathibha and Alleppey forming a group having lower levels of oleoresin.

5.2.3. Volatile oils

Volatile oils, which contribute aroma to spices, are a mixture of predominantly sesquiterpene ketones and alcohols. But in turmeric, the contribution of aroma by these components appears to be less, as compared to other spices.

Not much work has been done on the maturity changes of volatile oils till date. At 180 DAS and 210 DAS, significantly higher concentrations were seen in rhizomes among the varieties studied. In leaves, there was a gradual decline of the volatile oil percentage, due to the translocation of the various components of the oil towards the precursors for flavanoids and phenyl propanoid synthesis.

Before the rhizomes mature, higher concentrations of volatile oils were seen in the roots, in Suguna and Sudarsana. This may probably be due to the poor vegetative growth, which is a characteristic of the varieties.

5.2.4. Curcuminoids

5.2.4.1. Distribution

As has been reported by several workers, curcuminoids exists, as a complex pigment consisting of curcumin I (C), curcumin II (DMC) and curcumin III (BDMC). The variations in the content of the three forms are responsible for the varied effect in the fields of medicine and pharmacology. It has been noticed by Perkins (2002) that chemopreventive property of curcuminoids is specifically due to the higher concentration of curcumin I. This is due to the stimulated activity of caspase-8, which initiates Fas signaling pathway of apoptosis, inhibiting cell proliferation. In order to correlate the rate-determining enzyme with curcuminoids biosynthesis, the latter was estimated in rhizomes and roots of different varieties/accessions of turmeric during plant growth. The pattern of distribution of the three curcuminoids within themselves and among varieties/accessions was also analysed.

As reported by Perotti (1975), the distribution patterns of the three curcuminoids are in the ratio 60:20:20 in most of the *Curcuma* species. The results of the present study indicate that there are variations in the above pattern.

In the released varieties and also in the other high curcumin accessions, the distribution of curcumin I varied from 65 to 70%, whereas the accessions with low curcumin levels showed variations ranging from 50 to 60%. However, curcumin II and curcumin III did not show a specific range, and almost kept a steady level upto 90 DAS. However, some of the varieties deviated markedly within stages. Conversion of curcumin III to II and curcumin II to curcumin I involves two consecutive methoxylation processes. While this serial methoxylation of curcuminoids were analysed, it was

observed that the rate of methoxylation from curcumin III to curcumin II was higher in the early stages, which reflected in a high percentage of curcumin I. In addition, the lower concentration of curcumin II, as is observed in all cases studied may be due to the fact that conversion of curcumin III to curcumin II may be comparatively slow and that of curcumin II to I may be at a faster rate which could result in low accumulation of II. Except for var. Prabha, which showed a reverse trend.

The methyl donor for methoxylation is S-Adenosyl methionine in both cases. The primary methoxylation to convert curcumin III to curcumin II appears to be a slow process, either due to the slow conversion to curcumin II followed by a faster rate of conversion to curcumin I or due to the higher rate of condensation reactions prior to curcumin III formation. The faster conversion of curcumin II to I appears to be natural, since the presence of one methoxyl group in the benzene ring of curcumin II (DMC) may accelerate / stimulate the chances for the second methoxylation leading to formation of curcumin I.

This gives an additional clue for the serial methoxylation in the conversion of curcumin III → curcumin II → curcumin I. These variations will also help in the diversified end uses for the three forms, as is reported, in the field of medicine.

In short, curcumin I is a good source from all varieties except Prathibha, which showed a higher level only in the immature stages. As for curcumin II (DMC), a better source appears to be varieties Alleppey, Prabha and Suguna. Regarding curcumin III (BDMC), varieties Suguna and Sudarsana are found to be best sources.

5.2.4.2. Total Curcuminoids

Varieties Alleppey, Prabha and Prathibha showed lower levels during the very early stage of germination (30 DAS), but a distinct rise in the levels was observed after one month (3-fold), indicating higher rate of synthesis of precursors and related enzymes. This is also supported by the higher PAL activity observed in turmeric leaves, which is a constant source of phenolic precursors, required for phenyl propanoid biosynthesis.

A concomitant decrease in the level of curcuminoids and PAL activity was seen at 120 DAS. The fact that PAL subjected to feedback inhibition by cinnamic acid and other phenolic acids in a competitive manner is evident from the lower levels of curcuminoids and PAL activity at this stage. The intermediary phenolics are thus constantly being utilized in the ensuing biosynthetic pathway leading to a higher level of the pigment, in the following stages.

It is quite clear from the results that there is an interplay between the levels of phenylalanine, its' direct product cinnamic acid and the phenolic intermediates resulting in the varied response towards curcumin synthesis. Several workers have reported the competitive binding of phenylalanine and the regulation of PAL activity leading to phenyl propanoid biosynthesis (Mavandad *et al.*, 1990, Appert *et al.*, 1994).

In varieties Suguna and Sudarsana, higher levels of curcumin was observed during the very early phase of germination (5.6 - 5.7%). A varied pattern was seen during the different growth stages, but after 120 DAS, drastic reduction in the pigment levels was noticed, which coincided with a lower level of PAL activity at this stage. As explained earlier, the intermediary enzymes supported by PAL contribute actively towards these

changes. These varieties are characterized by poor vegetative growth and low dry recovery and are early germinating ones. Another feature to be noticed in these varieties is the higher levels of oleoresin and essential oil percentage, which are the major secondary metabolites. The higher percentage of curcuminoids, as reported here, is reflected in the higher oleoresin content, observed in these varieties. Moreover, this also points out to the fact that there exists a higher metabolic rate leading to enhance levels of secondary metabolites.

In roots, the maximum concentration of curcuminoids was seen in all the varieties at 150 DAS, which showed a decline afterwards. Variety Prabha had the highest levels. Otherwise, no specific conclusion could be drawn from the results.

5.3. Tracer studies

Studies on incorporation of labeled compounds is a pre requisite for any biosynthetic work, which can lead to identification and an understanding of the availability of the precursors involved in the whole process. It can also give valuable information on the site of biosynthesis and the partitioning of the intermediates involved for various other metabolic pathways.

5.3.1. Incorporation studies using ^{14}C -CO_2

This experiment was conducted as a basic study, as has been done in other crops, prior to the actual labeled studies to trace the path of labeled carbon incorporated and to sketch the site of synthesis and translocation of the metabolites of interest *in vivo*. Only roots and rhizomes were analyzed for curcumin since leaves and pseudostems were not found to be sources for the pigment. This indicated a serial translocation of the precursor for curcumin

synthesis towards root, and further to rhizomes. The higher level of incorporation in roots combined with rapid translocation towards rhizomes on curcumin is another possible explanation of the results observed. This also agrees with the high concentration of total curcumin in the roots during the same period (3-4 months).

5.3.2. Root absorption studies using 1-^{14}C -phenylalanine

Phenylalanine is reported to be the primary precursor in phenylpropanoid biosynthesis (Margna *et al*, 1989, Stafford and Ibrahim, 1992). In the experiment on phenolic acid incorporation, higher ^{14}C - phenylalanine was observed in roots, as the mode of absorption in this experiment was only through roots. In rhizomes, a higher rate of incorporation was seen at 48 hrs, which declined when the source of ^{14}C - phenylalanine was withdrawn. After withdrawal, labeled compound was seen in leaves and pseudostem upto one month, probably due to translocation from roots.

Majority of phenylalanine absorbed through roots and other parts is mainly utilised for protein synthesis (Margna *et al.*, 1989) minor proteins being channalised towards phenolic biosynthesis. The values for incorporation in rhizomes are very much less than expected, due to the above possibility. Apart from protein synthesis in the plant, the partitioning of labelled phenylalanine towards phenyl propanoid pathway takes place at a faster rate, as it is in a highly active metabolic stage, and thus offering another reason for the lower incorporation of the labelled compound in rhizomes, leaves and pseudostem.

Separation of curcuminoids by TLC and the measurement of ^{14}C -label in it using LSC confirmed presence of labeled compound in curcumin from

rhizomes and roots. In the present case, the ^{14}C -label was detected in the rhizomes and roots up to 4 months in contrast to labelled phenolic acids, which were detected only for a short term (one month). This indicates that the conversion of phenylalanine to curcuminoids occurs at a higher rate in presence of the substrate i.e., phenylalanine, confirming the same as the initial precursor in the biosynthetic pathway. The reduction in the incorporation detected during subsequent stages might be due to the dilution of the labeled compound due to increase in the bulk volume of the vegetative parts during plant growth and also due to the withdrawal of the source of labelled compound. Interestingly, a higher incorporation was seen in rhizomes at the fourth month, which might be due to the availability of labelled phenylalanine, originating from the degradation and turnover of protein.

5.3.3. Root absorption studies using 2-^{14}C-malonyl CoA

Malonyl Co A has been suggested to be involved in both the postulated pathways for curcumin biosynthesis. In the phenylpropanoid pathway, the central methylene group is provided by malonyl CoA with two symmetry-related cinnamate molecules. In a study conducted by Roughly and Whiting in 1973, the fractional distribution of activity of labelled malonyl CoA was found to be more in the cinnamate part (0.31) with negligible incorporation in the methylene group (0.06). This indicates that malonyl CoA synthesized *in vivo* is channeled to other metabolic pathways viz. phenylalanine metabolism or towards the synthesis of other carbon compounds. The rate of conversion (*in vivo*) of acetate to malonate may be more, part of which will get absorbed in the central methylene group and the rest diverted towards fatty acid synthesis.

The erratic incorporation of labelled malonyl CoA and the poor distribution in the curcumin extracts, rules out any possibility of the second

pathway of curcumin biosynthesis viz. the acetate pathway, where five acetate / malonate units is said to form the second cyclic C_9 unit of curcumin.

5.4. Phenylalanine Ammonia Lyase

5.4.1. PAL activity during rhizome development

Most of the biosynthetic pathways are basically controlled by a regulatory enzyme, which functions according to the cells' demand. These enzymes usually catalyse the initial (rate limiting) step in the biosynthetic pathway and have been controlled by the feed back inhibition of the excess immediate product. These inhibitions are usually competitive and will be released as soon as the scarcity of the product occurs.

PAL as the bridge between primary metabolites and natural product biosynthesis, is a potential site for pathway regulation. Since PAL is the first reaction in the biosynthesis from phenylalanine of a wide variety of phenylpropanoid compounds, the fluctuations in the PAL levels are thought to be a key element in the control of phenylpropanoid biosynthesis. Smith *et al.*, 1977 reports that increase in PAL levels are only transient and follow a rapid decay to basal or new basal levels. Many reports have demonstrated a correlation between change in the levels of phenylpropanoid biosynthetic enzymes and product accumulation of specific end products. (Hahlbrock and Scheel, 1989; Amrhien and Zenk, 1971; Shields *et al* (1982); Robbins *et al.*, 1985).

In the present study, sixty days after sowing, there is a marked increase of the enzyme activity, resulting in an accumulation of t-cinnamic acid, which is the immediate product. The enzymic level remained steady for 30 days, with a concomitant production of t-cinnamic acid. The decrease in the levels

of PAL after 90 DAS point to the fact that cinnamic acid might regulate the *de novo* production by inhibiting the expression of PAL-gene including activation of a inactive precursor protein (Lamb and Rubery, 1976).

Trans-cinnamic acid appears to act as an *in vivo* modulator of the synthesis of phenylpropanoid pathway enzymes (Bolwell *et al.*, 1988). Alternatively, a post transcriptional control is also suggested by Dixon *et al* (1980), where cinnamic acid modulates PAL-levels in tissue treated with high concentration of inhibitors of mRNA synthesis. It was also shown by Varner and Ramachandra (1964) that phenylalanine as well as cinnamic acid repressed the synthesis of PAL. Most probably, cinnamic acid is the active agent rather than phenylalanine and could function as a natural repressor of synthesis. Both Cinnamic acid and *p*-coumaric acid inhibited PAL activity in *vitro* (Koukol and Conn, 1961).

The fact that there is a decline in the PAL activity during later stages indicates a higher rate of utilization of cinnamic acid and other phenolic acids leading to secondary metabolites and in this particular case, the curcuminoids. Curcuminoids are synthesized from trans-cinnamate through or the hydroxylation and subsequent methoxylation, modulated by a series of enzymes, resulting in a number of intermediary products. In the initial stages, these may act as activators, which might perturb the normal feed, back control by stimulating the channeling of carbon into the phenylpropanoid pathway. Thus the initial increase in the enzyme activity reflected on an increase in the rate of *de novo* enzyme production against a low background production of enzyme removal. The subsequent decay in the enzyme activity might be result of a decline in production of the enzyme concomitant with a marked increase in the rate of removal of the enzyme activity. This indicates a dual feed back modulation of PAL *in vivo* following endogenous production of cinnamic acid.

Apart from the aspects of *de novo* synthesis and re degradation of PAL, the substrate phenylalanine itself may be a limiting factor for the variations observed during the rhizome development. It was proposed by Bate *et al* (1994) that the potential capacity of PAL to deaminate phenylalanine tended to be much higher than the rate of phenolic synthesis.

The higher PAL activity in the early germination phase as is observed here (60 DAS and 90 DAS) indicates a higher rate of deaminating capacity. This is sufficient enough to contribute to the curcuminoids synthesis at later stages, where a decline was observed. This suggests a predominance of characteristic synthetic and then degradative processes characteristic of the turnover of plant protein (Vickery *et al.*, 1940). It is seen here that a direct relation exists between the PAL activity and the level of curcuminoids during the growth stages, which confirms the observations made earlier.

5.4.2. Localization of PAL in turmeric

Density gradient centrifugation followed by marker enzyme analysis gave indication of the presence of PAL in the microsomal fraction. In the first experiment, the cell fractionation for PAL was carried out. PAL activity was present in the soluble fractions.

This is in conformity with the findings of Kalghatgi and Subba Rao, 1976. Endoplasmic reticulum was also suggested as a site for phenylpropanoid and flavanoid metabolism in petal tissue by Hrazdina et *al* (1984). These authors also suggested the occurrence of a membrane bound multienzyme complex associated with the entire pathway from phenylalanine to flavanoids. *In vitro* demonstration of the phenypropanoid core pathway reaction sequence using phenylalanine alone as a substrate substantiated that

at least the core enzymes existed as a multienzyme complex (Deshpande *et al*, 1993, Archnine and Blancoflor, 2004).

Although Schopfer has suggested that presence of PAL in particles may be attributed to artifacts that form when PAL is treated with dilute buffer, the enzyme remained in the microsomal fraction after washing (Amerhein and Zenk, 1971). Similarly the present study also showed that resedimentation or relayering of the fractions could enhance the activity to 80%, which confirmed the evidence for a particulate form of PAL. Since phenylalanine is present in the cytosol in the free amino acid pool, there must be a flux of these substrate molecules out of the cytosol onto the particles (Camm and Towers, 1973). This is also supported by the work of Hrazdina and Wagner, who suggest that the entire pathway from phenylalanine to flavanoids is membrane (ER) associated and this metabolism is facilitated by a multi enzyme complex. PAL appears to be located in the lumen of the membrane. These authors have also reported ER as a site of phenyl propanoid metabolism in Hippeastrum (Hrazdina and Wagner, 1984).

5.4.2.1. Sub fractionation of microsomes

Three peak activities were obtained for PAL, corresponding to three zones - each approximately ranging between 23-25%, 29.5% and 34-35% sucrose. These zones are attributable to the organelles: endoplasmic reticulum (ER), proplastids and plasma membrane. Highest activity was found to be associated with endoplasmic reticulum (less denser fraction) and this fraction within the gradient was characterized by isopyinic density gradient centrifugation.

Czichi and Kindl in 1977 reported the occurrence of cinnamic acid hydroxylase also along with PAL in cucumber cotyledons. They have

suggested that the co-occurrence of PAL and cinnamic acid hydroxylase in ER at density 1.10g/cm^3 renders these organelles peculiarly suited as compartment in which both the enzymes are commonly controlled, probably as membrane-bound enzyme associate or as micro compartment. These authors (1975) had earlier reported that cinnamate, as an intermediate in hydroxy cinnamate formation remains bound to the membrane enzyme complex. They have also proposed a model of membrane-bound multi enzyme complex for the conversion of aromatic amino acids into phenols.

In contrast, Amershein and Zenk (1971) found that the two enzymes – cinnamic acid p-hydroxylase and PAL were not associated with one and the same type of membrane, in the microsomes of *Fagopyrum esculentum*. Hence they did not consider a co-operation between these two enzymes.

In the present study in turmeric, where the phenyl propanoid pathway is mainly channeled towards curcumin biosynthesis, the occurrence of the major rate-limiting enzyme, PAL, in the pathway has been found to be localized in the microsomal fraction. The enzyme could be detected in the portion of sucrose gradient, approximately corresponding to density of ER, proplastids and plasma membrane.

It should be noted that without compartmentation, a clear separation of both sequences - protein synthesis and phenyl propanoid biosynthesis - is impossible, since significant concentration of L-phenylalanine has to be maintained always for protein synthesis. This is supported by the findings of Margna *et al.*, (1989) that a correlation between an increase in PAL activity and a decrease in the pools of free L-phenylalanine could be demonstrated only in a few cases. It has been conclusively proved that phenyl propanoid and flavanoid metabolism takes place in complexes on the cytosolic face of the ER (Stafford, 1981; Hrazdina and Wagner, 1984; Dixon *et al.* 1988; Stafford,

1990). A number of microsomal cytochrome P450 enzymes occur in these pathways, which may serve to anchor complexes to the ER membrane. These observations corroborate with the present findings on the presence of PAL associated with ER in turmeric.

5.4.3. Purification

The subcellular localisation of PAL could reveal its presence in the sub-microsomal fractions especially endoplasmic Reticulum, proplastids and plasma membrane. To gain further insight on the molecular forms of PAL, attempts were made to purify the enzyme to homogeneity.

Jorrin *et al.*, (1991), partially purified PAL from sunflower (*Helianthus annus L.*) hypocotyls and detected the presence of mono, tri-and tertrameric forms of PAL by molecular gel filtration on Sephacryl S-200, using different elution conditions. Tanaka and Uritani (1977), purified PAL from cut injured sweet potato which gave a molecular weight from 2,85,000 to 3,20,000 consisting of subunits having molecular weight 80,000 and sedimentation coefficient 11.6 to 11.9 S, respectively.

In turmeric, PAL could be purified 157-fold after Ammonium sulphate fractionation, followed by anion exchange chromatography and gel filtration. The enzyme was found to have a MW of approximately 156,000 and a subunit molecular weight of approximately 39,000. This suggests that the holo enzyme consists of a tetramer of similar or identical subunits. This is in agreement with the tetrameric nature of PAL in all organisms examined to date. The thick single band obtained after SDS-PAGE also supports this. The enzyme is similar in its physical properties to that purified from angiosperm sources, but differs from PAL preparations from bean and alfalfa in its' apparent lack of isozymes. These data are consistent with the values for PAL

from other sources viz., leaf mustard (Lim *et al.*, 1998) and mustard cotyledon (Lim *et al.*, 1997).

By purifying PAL through $(NH_4)_2SO_4$ fractionation, DEAE-cellulose chromatography, Sephadex G-200 and Q-Sepharase chromatography, from the cytosolic fraction of leaf mustard (*Brassica juncea var.integrifolia*), the enzyme was found to consist of four subunits, each having an estimated MW of 40,000 on SDS-PAGE, with an optimal pH and temperature of 9.0 and 45°C respectively. Another study conducted by the same authors in mustard leaf, identified a second form of PAL (PAL II) with a molecular mass of about 37,000 Da. with optimum pH and temperature of 8 and 45°C respectively. As is seen from the results, turmeric leaf PAL closely resembles leaf mustard PAL, in molecular weight and seems to be entirely different in its molecular form from sunflower and sweet potato (Tanaka and Uritani, 1977), parsely (Appert *et al.*, 1994), bean (Da Cunha, 1988) and maize (Havir *et al*, 1971) which exhibited a higher subunit molecular weight. In any case, irrespective of the molecular mass, PAL from all these crops, including turmeric existed as a tetramer in its native form.

The fractions containing PAL activity were concentrated using centrifugal ultra filtration devices (Centre prep 30: Amicon). SDS-PAGE was carried out using the concentrated protein, as described in Materials and Methods. The banding pattern of the proteins separated during the various stages of purification showed a thick fast moving band which was consistently in all the lanes, indicating the different stages of purification (Plate 8).

The reference proteins used in determining the molecular weight are Rabbit phosphoryalse b (MW 97,400), Bovine serum albumin (MW 66,200), Glutamate Dehydrogenase (MW 55,000), Ovalbumin (MW 42,700), Aldolase

(MW 40,000), Carbonic anhydrase (MW 31,000), Soya bean trypsin Inhibitor (MW 21,500) and Lysozyme (MW 14,400). Electrophoresis of the purified enzyme gave a thick single stained protein band corresponding to a subunit molecular weight ranging from 38,000 to 40,000, indicating that the enzyme is an oligomer consisting of four subunits.

Isoforms of PAL have been chromatographically resolved in extracts of a number of angiosperm species, in which different stresses appear to induce increase in specific isozyme forms (Bolwell *et al*, 1988; Jorrin and Dixon, 1990). In contrast, PAL from turmeric could not be resolved into distinct isoforms by anion exchange or gel filtration chromatography. This is evident from the occurrence of a single major peak on purification by gel filtration and also the presence of a single band during electrophoresis. In addition, PAL displayed normal Michaelis- Menten Kinetics (Fig. 31) suggesting that turmeric leaf PAL is constitutively present as a single form.

5.4.4. Kinetic studies

In order to investigate the kinetic properties of an enzyme, it is essential to have a purified form to get reproducible results and to avoid artifact. With the above purification fractions for PAL from turmeric leaves, it was possible to make a few observations on the major kinetic parameters.

5.4.4.1. Effect of substrate concentration

In turmeric, *in vitro* studies on the changes in the substrate concentration showed an enhanced PAL activity, with a linear increase with the supply of phenylalanine. Thus exogenous supply of phenylalanine at lower levels in the incubation medium could increase cinnamic acid concentration while at higher levels, the activity remained stationary

irrespective of the substrate concentration, exhibiting a hyperbolic curve (Fig.).

Km value

Several reports are available on the negative rate cooperativity of PAL for its substrate L- phenylalanine (Havir *et al.*, 1971), although this may simply reflect the combined kinetics of a number of isoforms, each exhibiting classical Michaelis- Menton Kinetics (Levitzki and Koshland, 1969).

Da Cunha (1988) in their studies on the purification, characterization and induction of PAL in *Phaseolus vulgaris* has reported a K_m value of 1.25 mM for L-Phenylalanine. In yet another study on *Phaseolus vulgaris*, Bolwell *et al.*, (1985) could resolve the enzyme into four forms by chromatofocusing, with apparent PI values of 5.4, 5.2, 5.1 and 4.9. Even though a preparation containing all four forms exhibited apparent negative rate co-operativity with respect to substrate, the individual forms displayed normal Michaelis-Menten kinetics, with K_m values of 0.077mM, 0.122mM, 0.256mM and 0.302mM in order of decreasing apparent PI value.

Gupta and Acton in 1979 purified PAL from mustard (*Sinapis alba* L.) to homogeneity. The enzyme constituted 0.01% of the total cellular protein with a pH optimum of 8.6. The K_m value of the enzyme was noted as 0.151-0.154 mM. Negative cooperativity was not detected in the concentration range tested.

In the present study in turmeric,, the enzyme could be purified to 157-fold, and negative cooperativity was not seen with the substrate (L-phenylalanine) tested. The K_m value was found to be 0.33mM exhibiting Michaleis -Menten kinetics. This was also reported for PAL from radish cotyledons (Fourcoy, 1980), in *Rhizoctonia solani* (Kalghatki and Subba Rao,

1975), *Phaseolus vugaris* (Bolwell *et al.*, 1985) and also from mustard (Lim *et al.*, 1998).

The deamination reaction in *vivo* is also controlled by another cellular mechanism, other than the catalytic activity at the enzymic level. This is the control at the level of substrate supply, i.e., phenylalanine as the endogenous source. According to Margna (1977) the deminating capacity of plant tissues is always sufficient to consume all phenylalanine left over from protein synthesis which becomes available to the enzymic action by PAL during normal cell metabolism.

5.4.4.2. Effect of pH

The position of pH optima at saturating substrate concentration is determined by the ionization of the groups of the enzyme- substrate complex. From the studies on pH-activity relationship, it is seen that with L-phenylalanine as the substrate, the optimum pH was found to be 8.8, similar to the value obtained from potato (Jangaard, 1974). The pH optima were also found to be similar in *Phaseolus vulgaris* (da Cunha, 1988) in mustard (*Sinapis alba* L) cotyledon. (Gupta and Acton, 1979), in jack pine (*Pinus banksiana*) and also in wheat *Hordium vulgere* (Koukol and Conn, 1961).

5.4.4.3. Effect of Temperature

Studies on temperature optima for PAL activity indicated maximum activity at 25°C under the specified conditions. At higher temperatures, the enzyme was shown to be inactivated, with negligible activity. As mentioned above PAL on purification, resembles in its molecular weight with that from irradiated mustard cotyledons and also with that of mustard leaf PAL (*Brassica juncea* var. *integrifolia)* (Lim *et al*, 1997, Gupta and Acton, 1979).

However the optimum temperature (25°C) was exhibited only by the enzyme from mustard cotyledons (*Sinapis alba* C.).

Da Cunha (1988) during his studies on PAL purification from *Phaseolus vulgaris* also reported that the enzyme could be specifically eluted at 20°-25°C during sephacryl S-200 gel filtration, confirming the presence of a non-degradable form at 25°C. In most other crops, the temperature optima was found to be 45°C. Strong exceptions to these findings were reported by Ohshima *et al* (1991). In this case, a thermostable PAL was purified from *Thermo actinomyces intermedius*. The enzyme was not inactivated even at 50° and 70°C and also at pH varying from 5 to 8. In another report by Appert *et al* (1994) in Parsely (*Petroselium crispum* Nym.), the four PAL isozymes exhibited similar Km values and identical temperature (58°C) and pH (8.5) optima. However, the present study on PAL from turmeric leaves indicated similar kinetic parameters as for mustard cotyledons, including the temperature optima.

5.4.4.4. Effect of inhibitors

There exists much experimental evidence that cinnamate and hydroxy cinnamate derivatives may alter the level of many PAL responses (Edward *et al., 1990;* Minamikava and Uritani, 1965). Lamb (1979) reported that modulation of enzyme activity by pathway intermediates occurred by a rapid post-transcriptional mechanism, by affecting either the processing or translation of PAL –mRNA. By using density labelling, it was shown by Shields and coworkers in 1982, that cinnamate inhibited *de novo* synthesis and also stimulated the removal of the pre existing enzymes.

In turmeric leaves, PAL activity was inhibited partially at lower concentrations and completely at higher concentrations of cinnamic acid. It appears that cinnamic is a competitive inhibitor binding to the active site of

PAL. In the model proposed by Hanson and Rose (1975), a hydrophobic pocket, which interacts with a benzene ring, plays an important role. This model explains the inhibition shown by cinnamic acid and its hydroxyl and methyl derivatives *viz.*, coumaric, caffeic and ferulic acids. Hanson and Havir (1970) suggested that the active site of PAL can take a more stable confirmation, which is the one binding with the reaction product viz; cinnamic acid.

It has also been established that PAL is sensitive to a PAL specific proteolytic system (Creasy, 1987), which is apparently activated by the PAL reaction product cinnamate (Bolwell *et al*, 1986, Shields *et al*, 1982). This can also be explained by the feedback inhibition of PAL mediated by various phenolic compounds (Sato *et al*, 1982). Turmeric leaf PAL activity was also inhibited only at milli molar concentrations as in jack pine (*Pinus banksiana*), as reported by Campbell and Ellis (1991). These data are in contrast with those for PAL from other species such as yeast, sweet potato, and pea (Sato *et al*, 1982) and alfalfa (Jorrin and Dixon, 1990), in which phenolic compounds are effective in inhibiting PAL at concentrations as low as 100µM. It may be, that, in turmeric leaves, which are rich in phenolic acids to initiate pigment synthesis, modulation of PAL activity by low concentrations of these metabolites is not a metabolically feasible mechanism. Exogenous supplies of the intermediates of phenylpropanoid biosynthesis inhibited initial development of enzyme activity. Thus, cinnamic acid and other hydroxylated cinnamic acids gave partial inhibition to the development of enzyme activity at increasing concentrations. In contrast, addition of chlorogenic acid exhibited inhibitory effect on PAL activity at all concentrations. The results seen here thus indicated that PAL, the first enzyme in phenylpropanoid metabolism, may be affected by phenolic acids formed in the pathway, as was reflected in the partial inhibition shown by ferulic and *p*-coumaric acids.

5.4.4.5. Light

PAL is an enzyme, intensively investigated in molecular photo morphogenesis. (Camm and Towers, 1973; Wellmann and Schopfer, 1975). This has also received a great deal of attention as a result of Zucker's discovery (1965) of the increased activity of PAL in potatoe slices incubated in light. The stimulatory effect of light in either etiolated or excised tissue had since been shown to be quite general although exceptions are known. In several species, continuous illumination leads to changes in the extractable activity of PAL leading to increase in flavanoid changes (Engelsma, 1970; Attridge and Smith, 1967; Zeier, 2004).

The present studies demonstrated that in etiolated turmeric seedlings, exposure to red light caused two peaks, one at 18 hrs and the other at 48hrs., due to the photo activation of phytochrome, as reported in Alaska peas by Attridge and Smith (1967). Mohr (1974) examined the mode of action of phytochrome (continuous far-red light) in increasing levels of L-phenylalanine ammonia-lyase activity in cotyledons of developing mustard seedlings (*Sinapis alba* L.), Here, bandwidths and density shifts of isopycnically banded enzyme show that in darkness the enzyme was synthesized *de novo*, continuously turning over (half-life approx. 3 h) and that maximum labeling achievable was reached at 12h. 3.2-fold (6h), 5-fold (12h) and 10-fold (24h) light-mediated increases in enzyme activity were accompanied by a similar pattern of labeling as observed in darkness. The response to red light treatment is not immediate. In all experiments, a lag phase of 6hrs is seen. This indicates the existence of a characteristic induction and repression sequence. Although evidence for the participation of enzyme synthesis in response to exposure to light has been proved in other crops (Zucker, 1965; Schopfer and Mohr, 1972) such evidence has not yet been obtained for turmeric. The two peaks as are seen here might be due to two

isoforms of PAL, as suggested by Havir and Hanson (1968) and Minamikawa and Uritani (1965) and the two forms are sequentially induced.

Blue light also gave a single, but not so predominant peak at 12 hrs for PAL. Lois *et al* (1989) has shown that blue light mediated by a flavine photoreceptor will cause cis- trans isomerism of hydroxy cinnamic acids in *Cucumus* seedlings, which correlates with the increased PAL levels. Kubasek *et al.*, 1992 also reported that the induction of PAL is mediated by a specific blue light receptor. PAL is actually controlled by a feed back system in which the trns-isomers are more inhibitory than cis-isomers. The lower activity seen after blue light illumination might be due to the enhanced conversion to the trans-form of cinnamic acid, resulting in PAL inhibition.

Exposure to white light also causes a lag phase of 6hrs, similar to that observed under the effect of red irradiation with two peak activities, which are the results of a phytochrome mediated response. It is demonstrated that the white-light mediated stimulation of PAL activity involves stimulation of the rate of *de novo* production of active enzyme (Lamb and Rubery, 1976). In *Sinapis alba* seedlings, the lag phase is taken as the time required for the derepression of the gene for PAL.

Seedlings kept in dark also showed a peak at 12hrs, but the catalytic power is somewhat smaller than that found for illuminated seedlings, showing that in dark also phenylpropanoid metabolism is active even though to a lesser degree as is seen in rose cell suspension cultures (Lois *et al.*, 1989). These results agree reasonably well with published reports (Kendrock and Frankland, 1968). It is clear that all the light treatments brought about an early rise in extractable enzyme activity, which reached a peak at 12hrs, followed by a further increase reaching a maximum at 48hrs. A characteristic feature of these light induced increases in the enzyme activity is the lag phase

of 12hrs. A lag phase has also been observed by Mohr and his colleagues (1974) in the photo induction of PAL in *Sinapis alba* seedlings, where it has been taken to represent the time required for the derepression of the gene for PAL.

Thus exposure of turmeric seedlings to the three forms of light evokes varied response to PAL, either through *de novo* synthesis or through a derepression factor or through feed back inhibition.

5.5. Amplification of PAL Gene

Primers specific to PAL gene were synthesized and this primer was used for tagging PAL specific gene from *Curcuma*. The expected size of the PAL gene was around 1000 bp. Results indicated the presence of cDNA bands of 400 bp approximately. This band may be considered as that of PAL and the sequence might be truncated. Also, the reason may be attributed to the fact that the same primer sequence may be present in some other protein in that species. Cloning and sequencing of the cDNA fragment has to be carried out for further confirmation.

SUMMARY AND CONCLUSION

Neema Antony "Investigations on the Biosynthesis of Curcumin in Turmeric (Curcuma longa L.)" Thesis. Indian Institute of Spices Research , University of Calicut, 2005

SUMMARY AND CONCLUSION

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The present study confines to the investigations carried out on the biosynthesis of curcuminoids in turmeric (*Curcuma longa* L.). Rational importance of the crop mainly centers on its medicinal and pharmacological properties as well as being a natural dye in the food industry. India being the largest producer and consumer of turmeric adds double importance to the problem, with respect to its export potential. Very recent findings on the chemopreventive properties, its role in prevention of AIDS and in the control of Alzheimer's disease strikes a golden note in the history of turmeric.

The present study mainly emphasis on the *in vivo* synthesis of the coloring pigment in the crop. Since the structural elucidation of curcumin by Lambe in 1910, much work has been done on the chemical and pharmacological aspects of the compound. But reports on the biosynthesis of the compound are very meagre. Keeping in view the two pathways suggested in early sixties and seventies as mentioned in the introduction, present study was undertaken.

As a preliminary step, the relative concentration of other metabolites – essential oil, oleoresin and phenolic acids as well as starch during plant growth were determined which gave a basic information on the rate of biosynthesis of the compounds during rhizome development and provided an indication on the availability of other precursors required for biogenesis.

Regarding oleoresin and essential oil, an increase is observed during the initial stage (upto 120 DAS), in contrast to starch, the major reserve carbohydrate in turmeric, which exhibited a steady increase up to maturity. Along with secondary metabolites, the total curcuminoids also showed higher levels during the initial growth of the plant. As rhizomes matured, there was

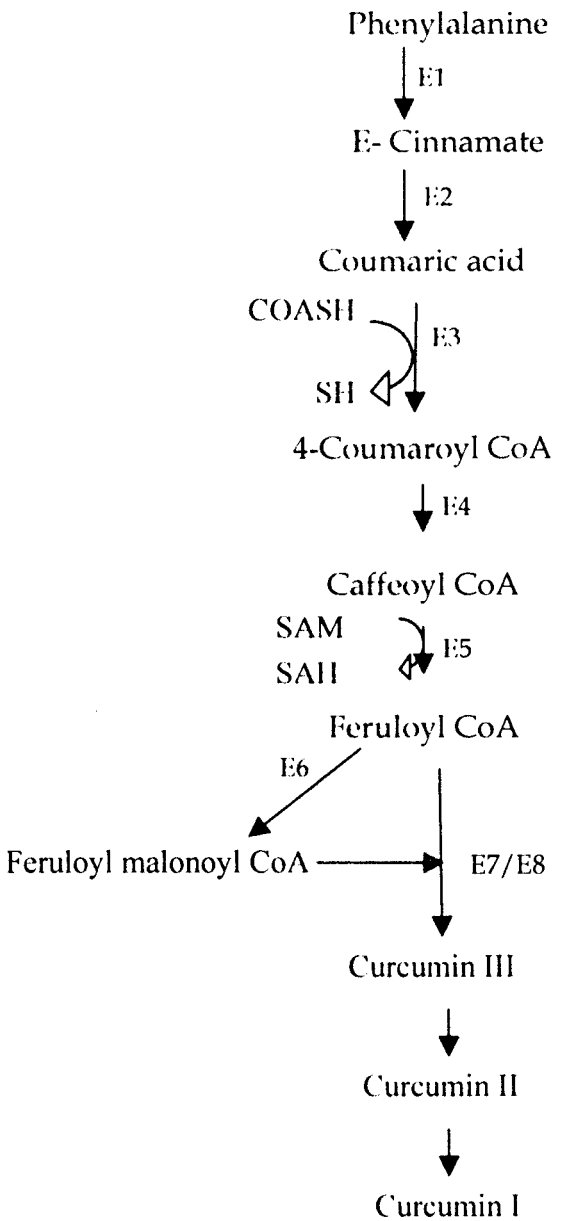
an enhancement of starch synthesis resulting in the relatively lower percentage of curcuminoids. However, 3-fold increase in the level of the pigment was seen in 3 to 4 month seedlings.

Any biosynthetic investigation needs experiments through tracer studies involving labelled precursors / intermediates. As such, three experiments were conducted using i) ^{14}C-Carbonate ii) 2-^{14}C-Malonyl CoA and iii) 1-^{14}C-Phenylalanine.

Based on the previous studies on the distribution of curcuminoids, the tracer experiments were planned during the early phase of plant growth i.e., within 1-2 months. The first experiment using ^{14}C-carbonate gave a clue on the translocation of the pigments from the root to the rhizome. Second experiment using 2-^{14}C-malonyl CoA, which is the intermediary precursor resulted in a non -significant incorporation of the label in curcumin thereby ruling out the second postulate suggested by Roughly and Whiting in 1973.

The third experiment was conducted to check the possibility of occurrence of the phenyl propanoid pathway suggested by Geissman in 1973. The first committed step in the biosynthesis of phenylpropanoid skeleton in higher plants is the deamination of L-phenylalanine to form t-cinnamate which is catalyzed by the enzyme phenylalanine ammonia lyase. The enzyme activity was monitored during rhizome development, which indicated higher levels during initial phase. Based on this, the incorporation studies were done during the early stage. The incorporation of the label was seen in phenolic acids, the intermediary precursor in biogenesis, indicating the conversion of a part of ^{14}C-phenylalanine to phenolic acids in the vegetative parts. In addition, the presence of ^{14}C- label in curcuminoids confirmed the inclusion of phenolic acids into curcumin molecules. This comes out as an evidence for

Proposed pathway for curcumin biosynthesis in *Curcuma longa* L.



E1	:	Phenylalanine ammonia lyase
E2	:	Cinnamate 4-hydroxylase
E3	:	4-Coumarate.. CoA ligase
E4	:	4-Coumarate CoA 3- hydroxylase
E5	:	5- hydroxy ferulate CoA methyltransferase
E6	:	Feruloyl CoA Malonyl transferase
E7	:	NADP-dependent Feruloyl malonoyl dehydrogenase
E8	:	Curcumin synthase

the phenyl propanoid pathway involving phenylalanine as the primary precursor and phenylalanine ammonia lyase as the rate-determining enzyme.

The major phenolic acids identified through TLC and HPLC were found to be, *p*-coumaric acid, caffeic acid and ferulic acid. This gave an additional evidence for channelisation of the major portion of ferulic acid toward curcumin biosynthesis.

Results on HPLC analysis of curcuminoids in a few high and low curcumin varieties/accessions could be correlated with PAL levels. However, the percentage distribution of curcuminoids followed a general pattern 65:20:15 for curcumin I, II and III eventhough slight variations were observed between the accessions. These variations might be due to the difference in the rate of serial conversion of the three forms of curcumin. Existence of curcuminoids in these three forms will definitely help in formulating diversified end uses for turmeric in the field of medicine.

In short, curcumin I was found to be a good source at maturity from all varieties studied except var. Prathibha, which showed high values in the immature stages. Varieties Alleppey, Prabha and Suguna appear to be better sources of curcumin II (DMC). As for curcumin III (BDMC), Suguna and Sudarsana were found to be good sources.

The association of Phenylalanine Ammonia Lyase (PAL) with phenolic acids, oleoresin and curcuminoids conclusively prove it to be the key regulatory enzyme in curcumin biogenesis. Accordingly the enzyme was localized, sub-fractionated and later purified for further studies. This included the kinetic parameters as well as effect of other external factors.

Localization of PAL in turmeric leaves using density gradient centrifugation could reveal the presence of PAL in the microsomal fraction. Subfractionation confirmed the distribution of PAL in the endoplasmic reticulum, proplastids and plasma membrane.

PAL could be purified from turmeric leaves to 157-fold after ammonium sulphate fractionation followed by anion exchange chromatography over DEAE cellulose and gel filtration chromatography using sephacryl S-200. The purified enzyme was subjected to electrophoresis, which gave a single thick band corresponding to a molecular weight of approximately 39,000 KDa. This suggests the holoenzyme to be a tetramer of similar or identical subunits, which is in agreement with the tetrameric nature of PAL in all organisms examined to date.

The purified enzyme was found to have an optimum pH of 8.8 with temperature optima at 25°C ambient. The K_m value of the enzyme was found to be 0.33 mM. Cinnamic acid and its hydroxyl and methyl derivatives, especially coumaric, caffeic, ferulic and chlorogenic acids were found to be strong inhibitors of PAL. Rate of inhibition were in the order cinnamate > *p*-coumarate > caffeate > ferulate > chlorogenate.

As PAL is highly sensitive to light, exposure of etiolated turmeric seedlings to various forms of light, mainly white, red and blue showed varied response. Two peak activities were seen in the case of red and white light. Maximum activity as is seen in white light, might be due to two isoforms of PAL of temporary occurrence. Lower activity of PAL during exposure to blue light was observed with only one peak, which might be due to the enhanced conversion of the trans-cinnamic acid, which is more inhibitory to PAL as compared to the cis form.

Attempt was also made to sequence the mRNA using RT-PCR. The expected size of the PAL is around 1000 bps. But the results had shown that the band has got around 400 bps length, indicating a truncated sequence.

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