# BIOCHEMICAL INVESTIGATIONS

# ON MANGO SAP AND SAP-INJURY

A THESIS

submitted to the

UNIVERSITY OF MYSORE

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Doctor of Philosophy

in

BIOTECHNOLOGY

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DECLARATION

I hereby declare that the thesis entitled "BIOCHEMICAL INVESTIGATIONS ON MANGO SAP AND SAP-INJURY" submitted to .the UNIVERSITY OF MYSORE, for the award of the degree of DOCTOR OF PHILOSOPHY in BIOTECHNOLOGY, is the result of research work carried out by me under the guidance of Dr. U. J. S. PRASADA RAO, Scientist, Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore-570 013, INDIA, during the period 1996-2001. I further declare that the results presented in this thesis have not been submitted for the award of any other Degree or fellowship.

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**CERTIFICATE**

This is to certify that the thesis entitled "BIOCHEMICAL INVESTIGATIONS ON MANGO SAP AND SAP-INJURY" submitted by Ms. Saby John K., for the award of DOCTOR OF PHILOSOPHY in

BIOTECHNOLOGY to the UNIVERSITY OF MYSORE is the result of research work carried out by her in the Department of Biochemistry and Nutrition, under my guidance during the period 1996-2001.

(Dr. U. J. S. PRASADA RAO)

Guide

THIS THESIS IS DEDICATED

TO

Mamma John

THE WIND BENEATH MY WINGS

Daddy John

FOR BELIEVING IN ME

Sajima

MY HERO

WordSmith

My BRIDGE ACROSS FOREVER

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CONTENTS

## LIST OF ABBREVIATIONS

| | |
|---|---|
| APS | Ammonium persulphate |
| CT AB | Cetyltrimethylammonium bromide |
| DEAE | Diethylaminoethyl |
| DOPA | 3,4 -Dihydroxyphenyl acetic acid |
| DTT | Diethyldithiothrietol |
| EDTA | Ethylenediaminetetra acetic acid |
| MIC | Minimum inhibitory concentration |
| PAGE | Polyacrylamide gel electrophoresis |
| POD | Peroxidase |
| PPO | Polyphenol oxidase |
| PVP | Polyvinylpyrrolidone |
| SDS | Sodium dodecyl sulphate |
| TEMED | Tetraethylmethylenediamine |
| Tris | Tris(hydroxymethyl) aminomethane |
| Km | Michaelis-Menten constant |
| [V] | Velocity of reaction |
| [S] | Substrate concentration |
| U | Units |
| Fig. | Figure |
| H | Hour( s) |
| min | Minute |
| sec | Second(s) |
| % | Percent |
| ~ | Approximately |
| °C | Degrees Celsius |

| | |
|---|---|
| p. | Page number |
| x g | times acceleration due to gravity |
| w/v | Weight by volume |
| kDa | Kilo Daltons |
| cm | Centimetre( s) |
| mm | Millimetre( s) |
| nm | Nanometre(s) |
| Kg | Kilogram (s) |
| g | Gram( s) |
| mg | milligram( s) |
| Ug | microgram(s) |
| M | Molar concentration |
| mM | Millimolar |
| uM | Micromolar |
| I | Litre(s) |
| ml | Millilitre(s) |
| ul | Microlitre(s) |

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SYNOPSIS

## INTRODUCTION

The mango, commonly referred to as the 'king of fruits' in the tropics, is relished the world over for its delicious flavor and luscious aroma. The world mango production stands at 23,852,000 MT. India is the world's largest producer of mango (12,000,000 MT). However, most of the fruit produced is consumed locally and India's contribution to the export market is minimal, primarily because post harvest damages are high and the shelf-life of the fruit is low.

One of the predominant post harvest problems faced by farmers is sap-injury. The Mango, in common with other members of. the family Anacardiaceae, has an extensive system of ducts or lactifers in both fruit and stem. Mango sap (latex) is contained within the ducts. The physiological role of mango sap is not known, however, it may have a defensive role against disease causing microorganisms and insect pests.

In the mature fruit, the sap maybe under considerable pressure and when the fruit is separated from the stem at the abscission zone during harvest, the sap spurts out. This sap is frequently deposited on the surface of the fruits, causing browning or blackening of the peel in the region of contact with the sap. This phenomenon is called sap-injury. Sap-injury not only reduces the consumer acceptance of the fruit, but also reduces the shelf life of the mangoes, as the regions of injury are more susceptible to bacterial and fungal attack. The constituents responsible for this injury have not been identified and its mechanism is also not clearly understood.

One of the methods being practiced by the growers to control sap injury involves desapping of the mangoes immediately after harvest. The sap thus collected as a by-product, is currently being wasted.

The primary objectives of this study have been to

a) identify the various components of mango sap of few Indian mango varieties

b) elucidate the mechanism of sap-injury and develop methods to control it

c) purify and characterise the enzyme polyphenol oxidase from mango sap

An outline of the studies carried out towards this end constitutes the subject matter of this thesis.

CHAPTER I. GENERAL INTRODUCTION

This chapter provides an overview of the current knowledge regarding the mango, with special reference to mango sap and sap-injury. Topics covered in this chapter include current production figures, historical aspects, distribution, growth and development of the fruit. Current' literature on important aspects like the biochemical indices of maturity, harvest and handling, post harvest physiology, storage, processing has been reviewed. This chapter also highlights the objectives and scope of the present investigation.

CHAPTER II. CHARACTERIZATION OF MANGO SAP

This chapter begins with a brief survey of available literature on saps, resins and latexes in plants, with particular emphasis on mango sap. The work described in this chapter is organised into three sections. The first section deals with characterisation of non-aqueous phase of mango sap. The studies leading to characterization of the aqueous phase are presented in the second section. The third section describes the screening of sap for antimicrobial activity.

Section. A: Characterisation of the non-aqueous phase of mango sap

The light, pale-yellow coloured, non-aqueous portions. of all the seven mango varieties were analysed by GC-MS and found to contain monoterpene hydrocarbons, viz. β-myrcene, cis- / trans-ocimene and limonene.

The major constituents of the sap non-aqueous phase from Totapuri, Raspuri, Seedling and Malgoa was p-myrcene, whereas in Baganapalli and Badami, ocimene was the major component, and in Mallika, limonene was the major constituent.a-Pinene, y-terpinene, a-copaene, p-caryophyllene and a-humulene were present in these varieties as minor components.

**Section B: Characterization of the aqueous phase of mango sap**

Mango sap was collected from seven different Indian mango varieties, viz., Raspltri, Badami, Seedling, Totapltn, Banganapalli, Mallika and Malgoa. The sap was separated into its constituent aqueous and non- aqueous phases and analysed to determine the nature of its constituents.

The viscous, colourless, aqueous phase was found to be composed of phenols, carbohydrates and proteins. A major part of the protein detected was found to be enzymatic in nature and predominantly it consists of polyphenol oxidase and peroxidase. The ash and metal contents of mango sap were also determined.

Section C: Study of the antimicrobial activity of mango sap

The antimicrobial activity of mango sap was studied using Bacillus cerells, Staphylococcus aureus, Escherichia coli and Pselldomonas aerllginosa. The non-aqueous phase exhibited antibacterial activity in the range of 0.1% -0.325% depending on the variety. Screening for antifungal activity was carried out using Aspergillus flavus, Aspergillus parasiticus, Fusalarium moniliforme, and Penicillium spp. Inhibition of growth was observed in the case of all the fungi tested. The extent of inhibition, however, varied in the case of different organisms.

CHAPTER III. CHARACTERIZATION OF SAP-INJURY AND ITS CONTROL

This chapter begins with a review of available literature on sap- injury in mango fruit. The various methods that have been employed by different workers to control sap-injury have also been summarised. The work presented is arranged in two sections. The first section deals with the mechanism of sap-injury and the second section outlines the attempts to control sap-injury.

Section A: Study of the mechanism of sap-injury

Application of sap and its: constituent phases on undamaged mangoes was followed by systematic scoring for sap-injury over a period of time. The non-aqueous phase was found to

cause maximum damage, whereas the aqueous phase caused very little or no damage. Application of pure terpenoids such as ocimene, myrcene and limonene, as well as solvents such as ether, acetone, methanol, on the surface of undamaged fruit caused the induction of sap-injury. Application of sap or any of its components or pure terpenoids or solvents on heat-treated peels at 100°C did not cause injury. Peel extract contained polyphenol oxidase, peroxidase enzymes, as well as a high amount of phenols. Results suggest that the non-aqueous phase of sap causes disruption of cellular structure and allows contact between polyphenol oxidase/peroxidase and their substrates in the peel, resulting in the formation of brown/black blotches on the peel, which are characteristic of sap-injury.

Section B: Control of sap-injury

Several wash solutions to control sap-injury were tested. Fruits treated with sap or its components, were washed with solutions of Tween-20, Tween-80, Triton-X 100, ascorbic acid, Surf@, Teepol@ and 'f water. In general Tween-80, Tween-20 and Triton-X 100 were found to be most effective, while others like ascorbic acid and water did not have much effect on sap-injury.

## CHAPTER IV. PURIFICATION OF POLYPHENOL OXIDASE FROM MANGO SAP

This chapter begins with a brief introduction on the enzyme polyphenol oxidase, its structure, reactions catalysed, isoforms, various sources, phylogenetic distribution in plants, physiological role and present and potential uses. Brief reviews on laccases also presented. This chapter comprises. of two sections, the first section deals with the purification of the enzyme polyphenol oxidase from the aqueous phase of mango sap and the second section deals with the characterization of the peak/ fractions obtained on chromatography.

### Section A : Purification of polyphenol oxidase from mango sap

On polyacrylamide gel electrophoresis (PAGE), the aqueous phase of Rasp!:lri mango sap showed one major band. The major 'band was found to exhibit both polyphenol oxidase and peroxidase activities. Preliminary attempts to separate the activities using various methods including affinity chromatography on Phenyl Sepharose CL-4B, gel filtration chromatography on Sephadex G-200 were unsuccessful. On subjecting the sap aqueous phase to ion-exchange chromatography using

DEAE-Sephacel with sodium chloride step-wise elution, the protein mixture was resolved into three peaks- the first peak (peak 1) exhibited only peroxidase activity, the second peak (peak 2) showed only polyphenol oxidase activity and the third peak (peak 3) exhibited both the enzyme

activities. All the 3 peaks showed single bands on native- PAGE and a slight difference was observed in the mobility of peak 3 fraction compared to the others.

Section B: Characterisation of peaks

On SDS-P AGE, all three peaks yielded a single band, each with a molecular weight of around 100,000. Each of the three peaks was characterized separately. The enzyme in peak 1 was found to be a peroxidase exhibiting optimum activity at pH 6.0. The Km of this enzyme for hydrogen peroxide was found to be 2.22 mM and for ortho- dianisidine it was found to be 1.33 mM. The enzyme displayed activity on a wide range of substrates including ortho-dianisidine, potassium iodide, tetramethyl benzidine and para-phenylenediamine. The enzyme was found to be more thermostable, upto 60°C.

The enzyme in peak 2 exhibited good polyphenol oxidase activity, with optimum pH being 6.0. The Km for this enzyme using catechol as substrate, was 12.5 mM. The enzyme was active on a wide range of substrates including catechol, 4-methyl catechol, diaminobenzidine. The enzyme activity was stable upto 60°C.

Peak 3 showed good polyphenol oxidase activity and and relatively less peroxidase activity. The optimum pH for polyphenol oxidase activity was 6.0 and was stable upto 60°C. The Km for this (enzyme using catechol as substrate was 8.33 mM. The enzyme had a wide substrate specificity and showed good activity on catechol, 4-methyl catechol, diamino benzidine, syringaldazine, p-phenylenediamine, p- naphthol and o-dianisidine. Among these, the last four substrates were found to be specific to peak 3 but not to peak 2. The substrate specificity results indicated that this enzyme may be a laccase.

CHAPTER V. SUMMARY AND CONCLUSION

The thesis concludes with a general discussion of the results presented. Sap-injury is a very important problem in post-harvest handling of mangoes. In an attempt to prevent sap-injury, mango fruits are often harvested with their stems (stalks) intact and subsequently desapped; mango sap thus obtained is a by-product of the mango during harvesting and is usually discarded. In this study attempts were made to understand the mechanism of sap-injury. We found that when mango sap spurts out and falls on the peel of the fruit, the non-aqueous components disrupt cellular integrity allowing contact between polyphenol oxidase/peroxidase and their polyphenol substrates in the peel, resulting in the brown/black blotches typical to sap-injury. Various wash solutions were prepared and applied to injured mangoes to test their

efficacy in controlling sap-injury. Neutral detergents like Tween and Triton-X 100 were found to be most effective in controlling sap- injury.

Attempts were made to attribute a value addition to mango sap. The aqueous phase of mango sap was found to be rich in oxidative enzymes like polyphenol oxidase and peroxidase that find various applications. The non-aqueous phase of mango sap was observed to be rich in terpenes, which are important aroma components.

Polyphenol oxidase, a very important oxidative enzyme, was purified and characterized.

A collective bibliography for all the chapters is presented after Chapter 5.

# CHAPTER 1

# GENERAL INSTRODUCTION

## 1.1. SECTION A: THE MANGO IN PERSPECTIVE

### 1.1. 1. Mango: The world scenario

The mango is the most important tropical fruit of Asia. Commonly referred to as the 'king of fruits [Purseglove, 1972], it currently ranks fifth in total production among major fruit crops, worldwide, after Musa [bananas and plantains], citrus, grapes and apples [FAa, 1997]. The world production of mangoes was estimated to be 23.4 x 106 MT. India ranks first in the world production of mango and contributes 54.2% of the total mango produced worldwide. Other prominent mango producing countries are China, Thailand, Indonesia, Pakistan and Mexico. Between 1971 and 1993, the production of mango, worldwide, has increased by nearly 50% [FAa, 1971; FAa, 1997]. Much of this increased production has occurred outside the traditional centres of mango culture, in South and Central America, Africa and Australia, and a significant proportion of the increased mango production is for export markets [Galan Sauco, 1993]. The high esteem, in which the fruit has always been held in Asia, is now apparently true for much of the world. Mangoes are now widely available as fresh fruit and in the form of frozen and processed products, not only in the tropics and sub-tropics, but also year-round in North America, Japan and Europe [Mukherjee, 1997].

### 1.1. 2. Mango in India

More than a thousand varieties of mango are available in India [Iyer, 1991]. Cultivation of mango in India, takes up an area of 12.2 x 106 Hectares. Andhra Pradesh heads the list of states where mango production is prominent, with 0.24 x 106 Hectares dedicated to the fruit, producing 2.9 x 106 MT per year. Bihar, Himachal Pradesh, Karnataka, Maharashtra, Orissa, Tamil Nadu, Uttar Pradesh and West Bengal are the other major contributors. In 1998-99 India exported 45.41 x 103 MT of mango as fresh fruit valued at 791.3 million rupees, and 455.49 x 103 MT of sliced anq dried mango, valued at 18.2 million rupees. This accounted for 41.5% of the income due to fresh and dry fruit export [Singhal, 2001]. India exports fresh mangoes to more than 50 countries. Over 90% of exports are directed to 7 countries viz., UAE, Saudi Arabia, Kuwait, UK, Singapore, Netherlands, Bangladesh [Dattatreyulu, 1997]. Since India is the richest source of quality mango varieties in the world, export of mango can potentially be increased through proper marketing and packaging practices. Processed products are likely to have better prospects than fresh fruit in the export market mainly because of the low shelf life of the fruit and also because its availability is restricted to one season. At present, mango nectar constitutes the major item of export, followed by mango chutney, pickles and mango slices in brine [Singh, 1990].

1.1. 3. Botanical aspects

As early as the sixteenth century, the name manga [Tamil] was used to refer to the mango [Singh, 1960] and the common English term and the botanical name Mangifera indica L., originate from this ancient name. Based on taxonomic and recent molecular evidence it is now apparent that the mango probably evolved within a large area including northwestern Myanmar, Bangladesh and northeastern India [Mukherjee, 1997]

Mangifera indica belongs to the dicotyledonous family Anacardiaceae, which consists of 64 genera, mostly trees and shrubs, some of which are poisonous. The tree itself is an arborescent evergreen and may attain a size of 50-60 feet. Its color varies between green through yellow to red. Mature specimens can survive for more then a hundred years. The flowers are borne on terminal pyramidal pannicles glabrous or pubescent; the inflorescence is rigid and erect and is widely branched, usually densely flowered with hundreds of small flowers, 5-10 mm in diameter. The flowers are small, monoecious and polygamous. Both male and perfect flowers are found in an inflorescence; the pistil aborts in male flowers. It is believed that the flowers are cross-pollinated by flies [Mukherjee, 1997; Singh, 1990; Lakshminarayana, 1980].

The fruit is a large, fleshy, resinous drupe. It varies in size, shape, colour and flavour. The mesocarp provides the edible pulp which is firm and can be fibrous or fibre-free, with a

flavour ranging from turpentine to sweet. Chlorophyll, carotenes, anthocyanins and xanthophylls are all present in the fruit, although chlorophyll disappears during ripening, whereas anthocyanins and carotenoids increase with maturity [Lakshminarayana, 1980]. The endocarp develops into a thick, tough, leathery, glandular covering of the seed and is termed the husk. The seed is ex-albuminous. It is solitary, large and flat, ovoid oblong and is surrounded by the fibrous endocarp at maturity. The testa is thin and papery.

The horticultural characteristics of principal mango cultivars in Southeast Asia are presented in Table 1.1.

Table 1.1. Horticultural characteristics of principal mango cultivars in Southeast Asia

| Cultivar | Flowering time[a] | Fruit season[a] | Fruit weight (g) | Shape[b] | Peel colour[c] | Peel thickness | Flesh colour | Flesh texture[4] | Seed size |
|---|---|---|---|---|---|---|---|---|---|
| Apple mango | 1-3 | 5-7 | 200 | RO | YO | thick | yO | T | medium |
| Arumanis | 5-8 | 8-12 | 400 | OOb | yG | thick | OY | VT | medium |
| Carabao | 9-1 | 1-6 | 200 | Ob | Y | thin | Y | VT | medium |
| Golek | 5-8 | 8-12 | 500 | Ob | GY | thin | OY | TM | medium |
| Maha 65 | 1-3 | 5-8 | >550 | Ob | YG | thick | Y | T | small |
| Nam Dorkmai | 11-1 | 3-5 | 300 | OOb | Y-oY | thin | oY | T | small |
| Pico | 11-2 | 2-6 | 200 | Ob | YO | thick | O | T | small |
| Rad | 11-1 | 3-5 | 200 | Ob | Y-oY | thick | Y | TM | medium-small |

[a]1 = Jan, 12 = Dec.

[b]RO = round-oval, O = oval, oOb = oval-oblong, Ob = oblong.

[c]Yy = yellow, Oo = orange, Gg = green

[d]T = tender, TM = more tender, VT = very tender. Capitals and small letters indicate major and minor colours respectively.

[Source: Kusumo et aI, 1984]

### 1.1. 4. Growth and development of mango fruit

Flowering in mango is preceded by the differentiation of the flower bud in the shoots. It takes about a fortnight for the tiny bud to develop and open into a flower. The period of differentiation varies from variety to variety and is also governed by the local climatic conditions [Singh, 1996], The hermaphrodite flowers of the mango inflorescence, after pollination and fertilization, set fruit. Fruit set is dependent on several factors including percentage of hermaphrodite flowers in a pannicle, pollination agents- insects, primarily houseflies- and the variety [Singh, 1996], Mango trees have an enormous potential to yield fruits.

Mature trees produce upto 1000 inflorescences each with 500-6000 flowers [Clarke and Clarke, 1987]. Fruit set is usually less than 10% and only 0.1-0.25% reach the harvesting stage [Purseglove, 1974; Jeron and Hedstrom, 1985]. The development of the mango fruit may be divided into four stages: (i) juvenile stage, up to 21 days from fertilization (rapid cellular growth); (ii) stage of maximum growth, 21-49 days from fertilization; (iii) maturation (respiration climacteric and ripening), 49-77 days from fertilization; (iv) senescence [Singh, 1960].

The growth pattern of the mango, unlike other stone fruits, appears to take the form of a simple, rather than double, sigmoid curve [Mukherjee, 1959; Lakshminarayanana, 1970]. Colouring and softening of the flesh is from seed outwards; at this stage the latter has become surrounded by a cartilagenous and, finally, strong endocarp. If allowed to ripen on the tree the later growth developments include the raising of the shoulders, and the elevation of the stem on a small mound surrounded by a hollow. The flesh ultimately becomes deep orange throughout. These readily observable changes have been used as a means of assessing the optimal picking date for immediate consumption or for storage.

During growth and maturation of mango, starch accumulation is the main chemical change in the pulp tissue [Leley et aI, 1943; Quintana et ai, 1984]. In developing mango fruits, acidity increased at early growth phase, reached a peak and then declined gradually until harvest [Wardlaw and Leonard, 1936]. Tandon and Kalra [1984] found that water-soluble pectins showed a steep rise after 70 days, reaching a maximum at 101 days of fruit growth. The ammonium oxalate-soluble fractions also showed a similar increase during fruit growth. The alkali- soluble fraction (protopectin) increased up to 70 days after fruit set but decreased thereafter until harvest. The physico-chemical attributes of I cultivars at the unripe (U) and ripe (R) stages are presented in Table 1.2.

### 1.1. 5. Nutritional value

Mango fruit contains 0.5-1.0% protein on a fresh-weight basis [Lakshminarayana, 1980]. In the case of Dashehari mango variety, a decrease in the soluble protein content was observed up to 44 days after fruit set, which increased again until 96 days [Tandon and Kalra, 1983]. Pathak and Sarada [1974] reported that lipid content in peel and pulp of five mango varieties ranged from 0.75 to 1.7% and 0.8 to 1.36%, respectively. Selvaraj et al [1989] reported that total lipid in seven commercial cultivars ranged between 0.263 and 0.671 % at harvest. The ascorbic acid content is considerably greater in the green, young fruit, although the ripe mango is an excellent source of the vitamin [Sidappa and Bhatia, 1954; Spencer et aI, 1956; Mattoo and Modi, 1969]. Gosh [1960] reported 36 mg of folic acid in 100 g of green fruit and Gopalan et al [1977] reported 0.08 mg of thiamin and riboflavin and 0.09 mg of niacin per 100 g of ripe mangoes. Ash content decreases during development with some increase near maturity, while crude fibre remains more or less constant [Kalra, et aI, 1995].

Table 1.2, Physico-chemical attributes of Southeast Asian cultivars at the umipe (U) and ripe (R) stages

| Cultivar | Ripe or Unripe | Soluble solids (°Brix) | Titrate acidity 9g/100g) | Total sugars (g/100g) | Starch (g/100g) | pH | Reference |
|---|---|---|---|---|---|---|---|
| Arumanis | U | 7.0 | 0.9 | 3.5 | 9.1 | 3.3 | Lam, 1980 |
| | R | 14.5 | 0.2 | 8.5 | 6.8 | 4.6 | |
| Carabao | U | 7.3 | 2.5 | 3.1 | - | 2.8 | Del Mundo et al, 1984 |
| | R | 19.0 | 0.3 | 26.6 | - | 5.1 | |
| Golek | U | 5.2 | 1.2 | 1.1 | 9.0 | 3.0 | Lam et al, 1982 |
| | R | 13.0 | 0.1 | 6.4 | 2.0 | 5.8 | |
| Malgao | U | 5.0 | 0.5 | - | 10.8 | 3.5 | Lam, 1980 |
| | R | 15.2 | 0.3 | 6.6 | 3.6 | 4.2 | |

[Source: Pantastico et aI, 1984]

1.1. 6. Diseases and insect pests

Mango fruits are prone to infections by several fungi and bacteria, and also infestation by various insect pests. As a result of this, all parts of the plant may be damaged. But those affecting flowers and fruits are the most serious ones and may sometimes result in loss of crop. Some of the important infections of mango are- anthracnose caused by Colletotrichum gloeosporioides, [Fizell and Peak, 1984; Jeffries et aI, 1990; Dodd et aI, 1992], Alternaria rot (Alternaria alternata) [Prusky et aI, 1992], powdery mildew (Oidium mangiferae and Oidiopsis spp.) [Schoeman et al., 1995], mango scab (Elsinoe mangiferae) [Ploetz et aI, 1994] and bacterial black Spot or Canker (Xanthomonas campestris pv. Mangijeraeindicae) [Snowdon, 1990]. There are many effective methods of disease control involving the use of systemic or protectant fungicides and bactericides [Dodd et aI, 1997].

The primary insect pests of mango are the mango hopper (Idioscopus clypealis) [Sen and Choudhuri, 1961], the mango mealy bug (Drosicha mangiferae) [Atwal, 1969], the mango fruit fly (Strumata frmlgineus) [Atwal, 1963], the mango seed weevil (Cnjptorhynchus mangiferae) [Babu, 1969], the red-banded mango caterpillar or mango seed borer (Nozorda albizonalis) [Pena, and Mohyuddin, 1997] and the mango bud mite (Aceria mangiferae) [Ochoa et aI, 1994]. Weevils and ants also cause e.xtensive damage to the crop [Pena and Mohyuddin, 1997]. Current mango pest' management practices largely involve the use of pesticides [Golez, 1991; Pena, 1993]. However, the control of fruit pests by chemicals alone has been complicated by the development of pest- resistance and other problems. Attempts to develop integrated pest management programmes are underway [Cunningham, 1984].

### 1.1.7. Physical and chemical indices of mango fruit maturity

Mango fruits traded commercially for consumption, as ripe fruits I are harvested green and ripened after harvest. If picked immature, however, fruits develop white patches or air patches and show lower amounts of Brix to acid ratio, taste and flavour, whereas over-mature fruits lose their storage life. Such fruits present a lot of problems during handling [Singh, 1996]. Mukherjee [1959] observed that mango fruits attained physiological maturity in about 90 days and the increase in size and weight almost stopped 4-5 weeks before harvest maturity in Dashehari, Langra, Fazli, Zafrani, Alphonso and Kishanbhog varieties. The number of days required for some important mango varieties to reach the recommended minimum stage of maturity for harvest are presented in Table 1.3.

Table 1.3. The number of days required for some important mango varieties to reach the recommended minimum stage of maturity for harvest

| Cultivar | Number of days | Calculated from | Coutry | Reference |
|---|---|---|---|---|
| Arumanis | 90<br><br>91 | Full bloom<br><br>Fruit set | Indonesia<br><br>Malaysia | Yuniarti, 1980.<br><br>Lam, 1980 |
| Carabao | 84<br><br>116 | Full bloom<br><br>Flower induction | Phillippines<br><br>Philippines | Mendoza, 1981.<br><br>Del Mundo et al, 1984 |
| Golek | 78<br><br>84 | Full bloom<br><br>Fruit set | Indonesia<br><br>Malaysia | Poernomo, 1972.<br><br>Lam, et al, 1982 |
| Malgon | 108<br><br>112 | First bloom<br><br>Fruit set | Indonesia<br><br>Malaysia | Suhardjo and Suhardi, 1981<br><br>Lam, 1980 |
| Nam Dorkmai | 100<br><br>93 | Full bloom<br><br>Fruit set | Philippines<br><br>Thailand | Narmuco Esguerra (unpublished).<br><br>Kasantikul, 1983. |

[Source: Kosiyachinda et aI, 1984]

It may be stated that a peak in starch content, TSS above 8 %, acidity around 1 % and TSS to acid ratio above 7 were indices for assessing maturity in Dashehari mango which were obtained 86 days after fruit set [Ananthanarayanan and Pillai, 1968].

1.1. 8. Harvesting of mango

Generally, in India, fruits are handpicked or plucked with a harvester, or branches are vigorously shaken to drop them. Fruits not accessible by hand are most often retrieved with poles equipped with a, severing blade and a bag. Recently, somewhat modified mango harvesters have been developed, used mainly in developed countries. Hydraulic driven lifts are used in the United States for picking mango fruits. It was further observed that decay loss, particularly due to stem-end rot, and the rates of respiration were minimum in fruits harvested with stalk. After harvesting, the fruits are heaped under shade to avoid direct sunlight. Injured, diseased,

immature, and ripe fruits are sorted out, and the fruits of similar maturity are packed together. Dropped fruits are packed separately [Kalra et aI, 1995].

### 1.1. 9. Packaging of the fruit

Proper packaging is an essential pre-requisite. In India, baskets of bamboo, pigeon-pea or mulberry with paddy straw as cushioning material have been used because of their easy availability and low costs [Naik, 1949]. This type of packaging was found to be unsatisfactory because of uneven ripening of fruits, more shrinking, bruising, etc.

Moreover, stacking was also a problem in baskets [Lakshminarayan et aI, 1971]. However, more uniform ripening and better-quality mangoes were observed when fruits were packed in ventilated wooden boxes [Gandhi, 1955]. Joshi and Roy [1986] packed Alphonso mangoes in corrugated fiber board (CFB) boxes with partitions and noted less bruising, slow ripening, reduced shrivelling, and less spoilage as compared to fruits packed in wooden boxes. Various cushioning materials, such as newspaper, paddy straw, dry and soft grasses, neem leaves, or wood wool have been tried for packing of mangos [Jain, 1961; Krishnamurthy, 1988]. Mango fruits are transported in various packings or loose in carts, trucks, and by rail [Anandaswamy, 1960].

### 1.1. 10. Storage and processing

Harvested fruit has the ability to respond metabolically to the environment in which it is stored. Various methods have been employed to extend the shelf-life and reduce losses. These can be classified as physical and chemical methods, and include storage of fruits at low temperature, sub-atmospheric storage, controlled-atmospheric storage, irradiation, heat treatment, and use of chemicals [Kalra et aI, 1995].

Mango fruit is processed and used at almost every stage of its growth. The range of products includes products derived from both raw as well as ripe fruit. Raw mango fruits are utilized for preparing raw mango powder (amchur), pickles, mango sauce or chutney, green mango beverage (panna). Raw mango slices are also preserved for use as a basic material for pickle and chutney manufacture (Kalra et aI, 1995). A large number of products are prepared from ripe mango fruit, the method?logy for which has been variously described for frozen and canned slices, pulp, jam, squash, juice, nectar, ready-to-serve (RTS) beverages, mango cereal flakes, mango leather, mango powder, mango toffee, and mango fruit bars [CFTRI, 1978; CFTRI, 1990].

## 1.1. 11. Post-harvest physiology of mango

Since most mangoes are harvested at a mature-green stage, post- harvest changes are principally concerned with events associated with ripening and senescence and with the effects of post-harvest handling techniques devised to control the occurrence and rate of these events [pantastico et aI, 1984]. According to Pantastico et al [1984] post-harvest life of mango can be divided into three phases:

a) storage life (or transportation life), which encompasses the period from harvest during which the fruit remains unripe and in a condition resistant to physical damage during normal handling,

b) ripening period, which designates the period from harvest until the fruit attains th~ stage of maximum consumer acceptability. This period encompasses the storage life period plus the final stages of ripening, and

c) shelf life, which starts when fruit is full-ripe and is the period in which fruit remains in an edible condition.

For 'Carabao' mango these phases have been found to occupy about four days, six days and five days respectively, at ambient temperature (about 30°C) [Lertpruk, 1983]. In general, mangoes take 6-14 days to ripen under ambient conditions, depending on the variety and environmental conditions. The mango is a climacteric fruit, and its period of ontogeny is characterized by a series of biochemical change initiated by the auto-catalytic production of ethylene and increase in respiration [Rhodes, 1980]. Burg and Burg [1962] reported that the respiratory peak in Kent and Haden mangoes during ripening coincided with ethylene evolution. They later estimated that 0.08 ppm of ethylene was present at the onset of the respiratory rise, and at the time of pre-climacteric respiratory minimum, it was sufficient to influence the metabolic activity in mango fruit cultivar Kent [Burg and Burg, 1965]. The principal change that occurred during ripening was the breakdown of starch to sugars [Kalra and Tandon, 1983]. There is a continuous decrease in acidity of fruits during ripening [Krishnamurthy et aI, 1971; Shashirekha and Patwardhan, 1976; Selvaraj et aI, 1989]. The ripening phenomenon is associated with loss of firmness. It appears that pectin polymers became less tightly bound in the cell wall during ripening, and the cell wall loosening involved hydrolysis of galactose containing polysaccharides [Seymour et aI, 1989]. Brinson et al [1988] reported net loss of arabinose, galactose, and galacturonic acid during cell wall degradation. An increase in soluble and a

decrease in insoluble proteins was reported during ripening of mango fruits [Tandon and kalra, 1983; Sharaf et al, 1989].

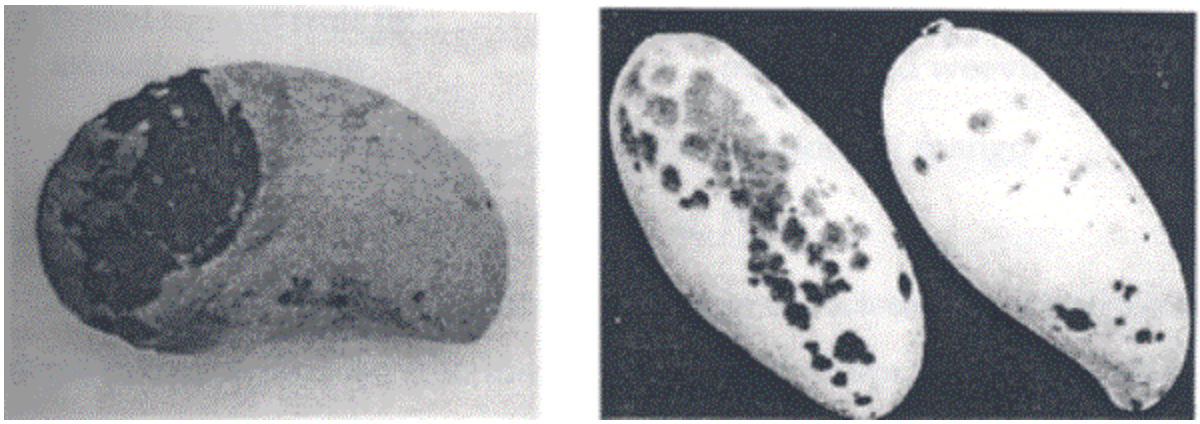
Ethylene synthesized in the fruit before the onset of climacteric activates the enzymes and inactivates the inhibitors present in unripe fruits [Mattoo, and Modi, 1969]. It was observed that catalase, and peroxidase increased several fold in ripening mangoes [Matto et aI, 1968; Singh and Chundawat, 1991]. Similarly, pectin esterase, polygalacturonase, polyphenol oxidase and cellulase, activities in ripening, mango marginally increased from harvest maturity to half-ripe stage, later their activIties decreased [Selvara) and Kumar, 1989]. Ibr Amylase activity also reached a peak in fully ripe fruits and declined hf, thereafter [Kalra and Tandon, 1983]. The increase in activities of glucose- 6-phosphate dehydrogenase, and NADP-dependent malic enzyme has been observed by Modi and Reddy [1967] during ripening of mango fruits. Lipase activity was found to be maximum at harvest, whereas lipoxygenase and alcohol dehydrogenase activities increased from harvest until half ripe or three-quarter-ripe stage and then declined. The aldehyde-forming activity was high at harvest maturity, decreased a little at half-ripe stage, and declined rapidly thereafter. The high activity of aldehyde-forming enzyme indicated the presence of C6-aldehydes (hexanal and trans-2-hexanal) in high concentrations to impart fruity aroma [Selvaraj, 1989].

## 1.2. SECTION B: POST-HARVEST DISORDERS OF MANGO

Post-harvest disorders are primarily of three kinds: Pathological, entomological and physiological disorders.

### 1.2.1. Pathological and entomological disorders

Post-harvest diseases of mango can be classified based on the time of initiation of infection: (1) where infection is initiated after severance of fruit from the tree, and (2) where fruit are infected while attached to the tree but the organism remains quiescent until after harvest when the fruit begins to ripen. The main diseases of mango due to post-harvest infection are stem-end rot caused by Diplodia natalensis Pole Evans and black rot caused by Aspergillus niger van Tiegh (Fig. 1.1. A) [Quimo and Quimo, 1974]. The main disease initiated during fruiting that develops post- harvest is anthracnose (Colletotrichum gloeosporioides Penz.), although it can also be spread after harvest by contact with infected material (Fig.l.l.B). Mango scab and sooty mould are other diseases that effect the mango fruit [Pordesimo, 1984].



A. Aspergillus rot of mango B. Anthracnose disease of mango

Fig. 1.1. Common post-harvest diseases of mango

[Source: Pordesimo, 1984]

The primary post-harvest pests of mango are fruit flies of the genus I Dacus (Fig. 1.2. A). The Oriental fruit fly, Dacus dorsalis Hendel, is the species most damaging to the mango fruit both in terms of actual damage to the produce and, more importantly, because of the restriction in trade created by quarantine requirements imposed by various importing countries. After fruit flies, the most important post-harvest pests of mango are the mango stone weevil (Sternochetus mangifera) and the mango weevil (5. gravis F.) (Fig. 1.2. B). The mango seed borer, Noorda albizonalis Hampson, is a serious pest of mango that attacks any stage of the fruit.

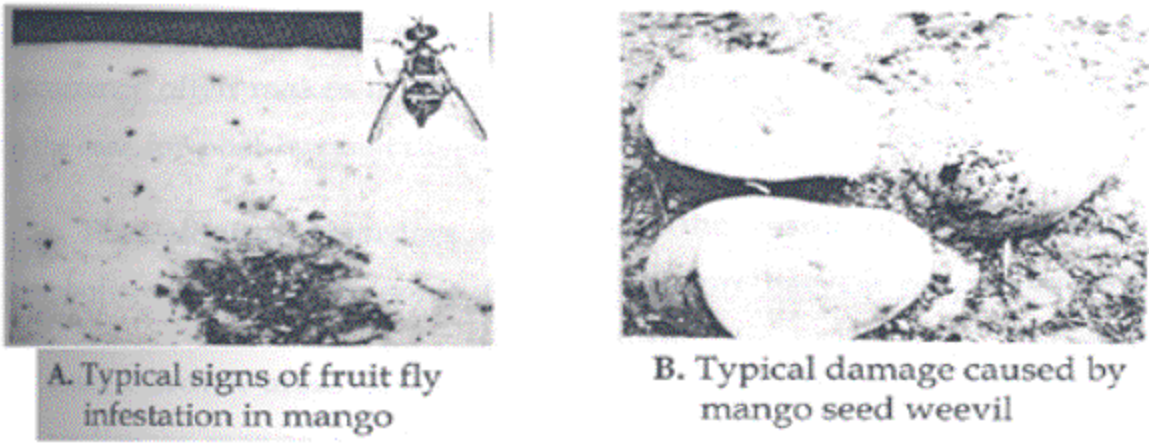



Fig. 1.2. Common post-harvest infestations of mango

[Source: Manoto et aI, 1984]

Diseases and pest infestations have been reported to be the greatest single cause of post-harvest loss in perishable crops [Coursey, 1972]. Various control measures have been examined to reduce the incidence of post-harvest rots and pest infestations. These include chemical treatment, hot-water dip, low temperature storage, gamma-irradiation and various combinations of these treatments. Chemical treatments can be applied either as pre-harvest spray or post-harvest dip while the other control measures are applied post-harvest [Pordesimo, 1984]. The sterile insect technique, fumigation and use of fly traps are being effectively employed in control of insect infestations in mango fruit [Manoto et aI, 1984].

1.2.3. Physiological disorders of mango

Exposure of mango fruit to various environmental, cultural and post-harvest handling treatments often leads to tissue abnormalities which are quite distinct from those induced by pathological and entomological agents. These types of abnormalities are generally termed as physiological disorders. The complexity of events leading to their occurrence often makes it more difficult to define the causal factors than is the case for post-harvest diseases or pests [Lizada et al, 1984].

Physiological disorders are usually the result of imbalances in metabolism induced by some factor in the pre-harvest or post-harvest environment that leads to cell collapse and the appearance of water- soaked brown areas on some part of the fruit. Pre-harvest factors that predispose mango to physiological disorders include growing location, orchard. condition, tree nutrition and conditions at harvest while post- harvest storage conditions such as temperature, oxygen and carbon- dioxide levels, packaging and surface coating treatments are contributing factors to the occurrence of the disorders [Subramanyam et al, 1971]. .

Conditions such as soft-nose [Young, 1960], tip-pulp [Verma, 1950], internal breakdown or soft-centre or spongy-tissue [Subramanyan et al, 1971] have been described in literature. Some of the physiological disorders are described below.

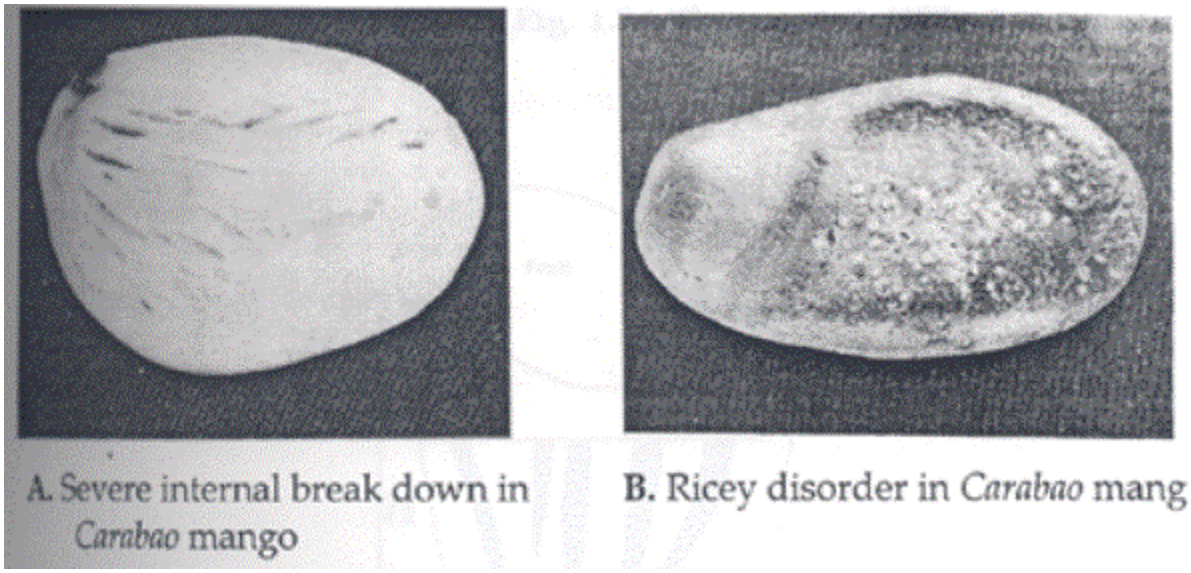
**Chilling injury** is manifested initially as a brown discolouration of the skin often accompanied by pitting but in more severe cases the skin colour becomes more pronounced and the flesh is affected. There is also uneven ripening with poor colour and flavour development and the fruit is prone to develop rots. It is generally accepted that storage of all cultivars of mango below 10°C renders the fruit susceptible to develop chilling injury although the time required to

show visible symptoms varies between cultivars [Lutz and Hardenberg, 1968]. It has been found that most cultivars can be safely stored for two to four weeks near 10°C and then ripened normally when subsequently returned to ambient temperature.

**Internal breakdown** has been observed in some varieties of mango subsequent to storage in polythene bags or polyvinyl chloride wraps. Waxing also invariably causes this disorder. Apart from failure of the peel colour to advance to a full yellow stage, affected fruit do not exhibit (external symptoms. Symptoms in the mesocarp range from pale flesh colour to the development of delineated starchy areas with airpockets, a condition termed spongy tissue. Severely affected fruits are characterized by a considerably reduced flesh firmness, uneven peel and pulp ripening, reduced total soluble solids and elevated titrable acidity (Fig. 1.3. A) [Gautham and Lizada, 1984].

**Lumpy tissue** (called heb in Thailand) is prevalent in several b. Southeast Asian varieties. This disorder is characterized by the development of indentations in the peel, which become wider and deeper as the fruit approaches senescence. The mesocarp of affected fruits contain white lumps. The necrotic areas have been found to consist of ruptured cells surrounded by intact cells loaded with starch [pakdeekulsumpum and Tongumpai, 1979]. Here are no reports of the aetiology of this disorder.

**Ricey disorder**, also of unknown aetiology, is commonly observed in 'Carabao' mango. Affected fruit show no external symptoms, but small lesions about the size of rice grains, delineated by tissue with a cotton- like appearance, can be observed in the mesocarp. The sensory attributes are generally unaffected (Fig. 1.3. B) [Lizada et aI, 1984].



A. Severe internal break down in *Carabao* mango

B. Ricey disorder in *Carabao* mang

Fig. 1.3. Common post-harvest physiological disorders of mango

[Source: Lizada et aI, 1984]

## 1.3. SECTION C: SAP-INJURY IN MANGO

### 1.3.1. Sap-injury

Sap-injury is an important post-harvest problem in mango. The mango fruit has a network of branching fruit ducts, which are present in both fruit and stalk and penetrate the transition zone between the fruit l:8se and its stalk. Several large fruit ducts continue into the stalk but end a short distance beneath the abscission zone (Fig. 1.4.). These fruit ducts contain a viscous, caustic liquid referred to as mango sap [Joel, 1981]. Flow of sap between fruit and stalk is a part of the growth process. The amount of sap exuded varies with cultivar, maturity and production area [Ledger, 1991]. As the fruit matures and ripens, sap flow decreases and eventually stops. Formation of an abscission layer blocks further sap flow and the fruit falls from the tree [Brown et aI, 1986]. If this sap comes in contact with the peel of the fruit, it causes a darkening or browning of the peel in the region of contact. This discolouration of the peel is referred to as sap-injury or sapburn-injury (Fig.1.5) [Brown et al, 1986; Loveys et al, 1992].

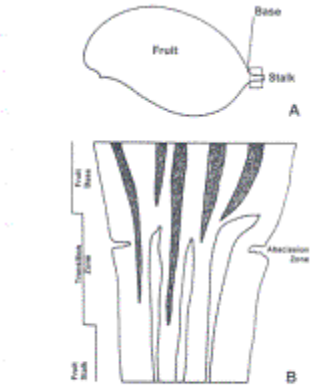



**Fig. 1.4.** Ducts in mango fruit and stem
*[Source: Joel et al, 1978]*

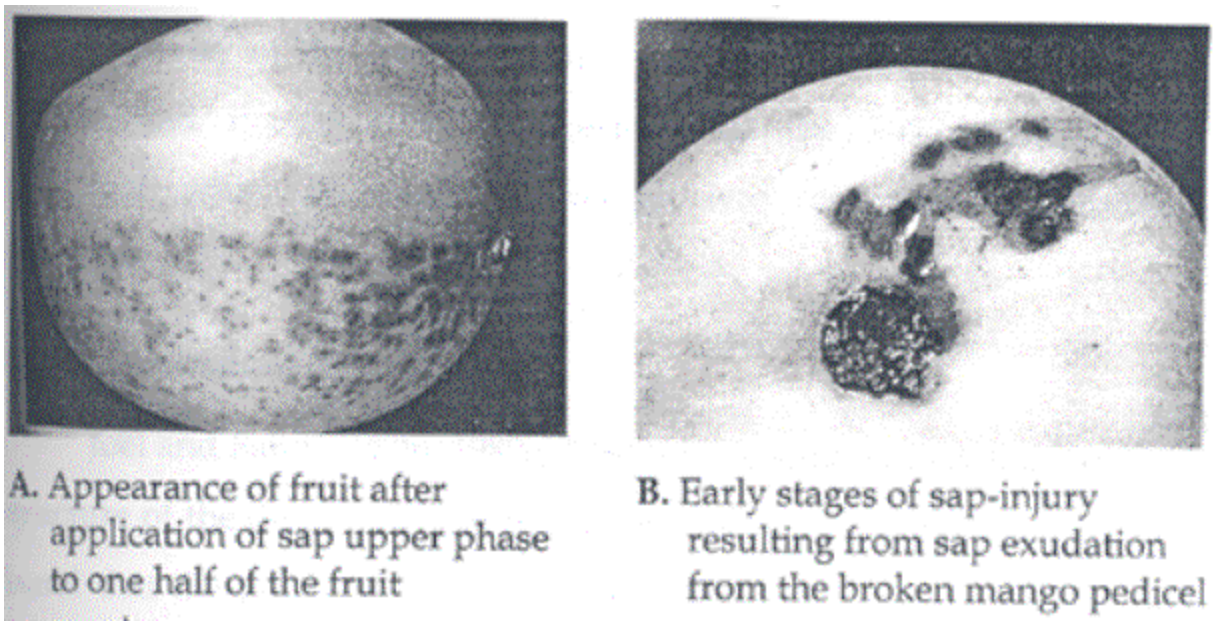



Fig.1.5. Sap-injury on Kensington

[Source: Loveys et al, 1992]

1.3.2. Factors influencing sap-injury

Though sap-injury is a superficial damage, it reduces consumer acceptance and decreases shelf life of the fruit. Johnson and Coates [1993] reported that sap-injured skin could be invaded by Aspergillus spp., especially in hot conditions. Thus, it is a very important post-harvest handling and marketing problem of the mango industry, particularly in the case of fresh fruit. Brown et al [1986] reported that sap obtained from the fruit harvested in the afternoon (2.00 pm) caused more sap-injury than that collected from fruit harvested in the morning (7.00 am) and this was attributed to a dilution of injurious components of sap at higher fruit turgor. These authors also reported that the 'spurt sap' causes more injury than 'ooze sap'. Loveys et al [1992] reported that the organic layer of sap may be responsible for sap-injury. These authors found that the Kensington variety of mango was more susceptible to sap-injury than the Irwin variety. Thus the susceptibility of mango fruit to the injury as well as the severity of the injury seems to vary depending on the cultivar. O'Hare and Prasad [1992] suggested that the 5-(12-heptadecenyl)-resorcinol, which is present in mango, was responsible for causing sap-injury.

1.3.3. Composition of mango sap

Joel and Fahn [1980b, 1980c, 1980d] have studied in detail the ultra- structure of the duct system in the shoots, stem and fruit of mango. They found that the proteins and carbohydrates in the shoot ducts originated from the cell walls of  is integrating cells during the lysogenous process of duct formation [Joel and Fahn,1980b]. On the other hand, proteins and carbohydrates in the fruit sap of mango were found to be produced by special secretory epithelial cells lining the lumen of the ducts Uoel and Fahn, 1980d]. The mode of mucilage production is summarized in Fig. 1.6. Joel and Pahn [1980d] observed that shoot ducts differ from fruit ducts in the absence of mucilage-secreting epithelial cells. This observation is in agreement with the findings of Vasistha and Siddiqui [1938] and Ulmert [1970] that the sap of mango fruit was found to contain more protein and carbohydrate than shoot sap. Pantastico (1975) reported that mango sap has a wide variety of constituents including tannins, enzymes, resins and terpenes. Joel et al [1978] and Robinson et al [1993] have reported the presence of the enzyme laccase mango sap. Bandhyopadyay et al [1985] reported the presence of 5-substituted resorcinol and they suggested that the resorcinol derivatives of sap were responsible for causing mango dermatitis. The presence of the enzyme polyphenol oxidase or laccase in mango sap was reported by Joel et al [1978] and Robinson et al [1993].

Loveys et al [1992] have studied the components of mango sap in the Kensington Pride and the Irwin varieties of mango. They observed that mango sap could be separated, by centrifugation, into an upper oily phase, yellow-brown in colour, which represented 10% of the total sap volume; and a lower phase that was milky and viscous in nature. Their analyses of the upper phase of mango sap showed that in both cultivars the majority of the peaks in the un-derivatized samples were terpene-like compounds. In Kensington variety, the major component was identified as terpinolene and in Irwin, as car-3-ene [Table 1.1].

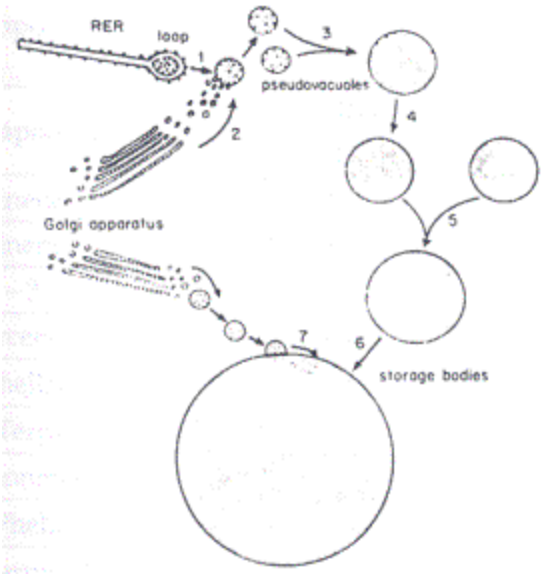



Fig 1.6. Production of protein -carbohydrate mucilage in mango fruit ducts.

[Source: Joel and FaM, 1980d]

Gholap and Bhandyopadyay [1977] characterized the chemical components responsible for the aroma of green mango from the sap of the Alphonso and Batali varieties. They reported the composition of an ether extract of mango sap from the Alphonso and Batali varieties of mango. They found that the major component in each case constituted (3 %% of the total volatiles and had a similar green mango aroma note, though there was a significant difference in retention time (Rt) between them. The major component of the Alphonso variety was identified as as-ocimene and that of Batali variety was B-myrcene. They concluded that although the constituents responsible for the green aroma of raw mango of two different varieties were both acyclic hydrocarbons and identical subjectively in their aroma, they differed in molecular structure.

Table 1.1. Components of Kensington (K) and Irwin (I) sap upper phase % Relative abundance

| Compound | % Relative abundance | |
|---|---|---|
| | K | I |
| $\alpha$ - Pinene | 0.84 | 0.68 |
| Sabinene | 0.26 | - |
| Car -3 -ere | 5.87 | 89.8 |
| Myrcene | 1.91 | - |

| | | |
|---|---|---|
| α - Phellandrene | 0.45 | 0.51 |
| α - Terpinene | 2.31 | 0.26 |
| Limonene | 1.30 | 1.97 |
| Phellandrene | 0.95 | 0.80 |
| β - terpinene | 0.25 | - |
| γ - cymene | 0.05 | 0.19 |
| Terpinolene | 83.7 | 3.33 |
| α - Copaene | - | 0.14 |
| α - Gurjunene | 0.15 | 0.08 |
| β - Caryophyllene | 1.35 | 1.67 |
| α - Humulene | 0.62 | 0.79 |
| ρ - Cymene-8-ol | 0.07 | - |

1.3.4. Methods used to control sap-injury

a) Physical methods

Different practices are followed in different parts of the world to control sap-injury in the harvested mangoes. In certain parts of India, the mangoes are de-sapped for 15-30 min on bamboo structures. In some parts of the world including India, one of the common practices was the inversion of fruits in soil for 15-30 min after harvest. This practice may, however, lead to infection of the fruit by soil-borne pathogens. Johnson et [1993] demonstrated that soil bleeding of mango caused a significant increase in post-harvest infection and also the earlier appearance of stern-end rot.

It is reported that the standard harvesting practice in Australia is to clip the fruit from the tree with a long stem (5 to 10 cm). The long stems are then broken off by hand in the packing shed and the fruit are de-sapped by placing them stem end down for 20-30 min. However, r during the shifting of fruits from the orchards to the packing sheds, care must be taken to keep the stalk intact. If the stalk is broken during the shifting, it contaminates the fruits with sap and causes sap-injury [Ledger, 1991].

b) Chemical methods

In order to control sap-injury in mango fruits, several methods have been suggested. Plugging the stem-end of the fruit with chemicals after de-stalking, washing the fruit with 1 % sodium

bicarbonate or 1 % aluminium potassium sulphate or applying surface coatings to the fruit prior to de-sapping, were a few of the methods that were attempted. Brown et al [1986] used sodium bicarbonate and aluminium potassium sulphate powders to plug the cut end of the fruit to prevent sap flow and thereby sap-injury. However, this method did not control sap-injury. The same workers also tried washing the sap-contaminated fruit with 1 % sodium bicarbonate and 1 % aluminium potassium sulphate. They reported that washing the fruit with 1% aluminium potassium sulphate controlled sap-injury to a certain extent.

Lim and Bowman [1995] used different paraffinic oils to pre-coat the fruits that were harvested with stalk, before de-sapping. They reported that this method was successful in controlling the sap-injury. As this method involves harvesting of mangoes with stalk, breakage of the stalk may take place while the fruits are in the containers and the released sap, resulting in sap-injury.

Lizada et al [1986] used hot water treatment to control sap-injury. They reported that subjecting the freshly stained mangoes to hot water treatment (51-55°C, 10 min) minimized the sap- injury, whereas subjecting the fruits to this treatment after the sap had dried, aggravated the problem.

In order to control sap-injury, therefore, in-depth knowledge of the components of mango sap in the different local varieties is essential. There are no reports so far of the exact composition of mango sap in general, and in particular from Indian mango varieties. The role of mango sap in defense of the fruit against various pest infestations and infections has been long suspected. However, it is not known whether mango sap exhibits anti-microbial activity.

## OBJECTIVES AND SCOPE OF THE INVESTIGATION

The mango, commonly referred to as the 'king of fruits' in the tropics, is relished the world over for its delicious flavour and luscious aroma. The world mango production stands at 23,852,000 MT. India is the world's largest producer of mango (12,000,000 MT). However, most of the fruit produced is consumed locally and India's contribution to the export market is minimal, primarily because post-harvest damages are high and the shelf-life of the fruit is low.

One of the major post-harvest problems faced by farmers is that of sap-injury. The mango, in common with other members of the family Anacardiaceae, has an extensive system of ducts or lactifers in both fruit and stem. Mango sap or latex, which is contained within the ducts, is a viscous liquid having a low pH and a high oil content. The physiological role of mango sap is not known; it may have a defensive role against disease causing microorganisms and insect pests. In the fruit, the sap is under considerable pressure, and when the fruit is separated from

the stem at the abscission zone during harvest, the fruit lactifers are severed. The sap contained within them spurts out, and is frequently deposited on the surface of the fruits, causing browning or blackening of the peel in the region of contact with the sap. This phenomenon is called sap-injury. Sap-injury not only reduces the consumer acceptance of the fruit but also may also reduce the shelf-life of the mangoes, as the regions of injury may be more susceptible to bacterial and fungal attack.

Some of the methods suggested for control of sap-injury require harvesting the mangoes with intact stalk followed by (a) de-sapping by breaking the stalk and dipping in soil; (b) de-sapping the fruit on bamboo Structures; (c) plugging the cut end of the stalk with certain chemicals like sodium bicarbonate or aluminium potassium sulphate; d) giving the fruit a protective coating of wax or paraffinic oil, before de-sapping. All these methods have their own disadvantages. Moreover, in India, mangoes are usually harvested without proper precautions. As a result, fruits are often contaminated with sap. Therefore, sap-injury is a major problem. Some investigations on composition of mango sap and sap-injury have been reported in the literature, pertaining mainly to the Australian and US varieties. No such studies on mango sap or on sap-injury in Indian mango varieties have been reported as yet. Therefore, the major Objectives of the present investigations are:

1) To study the composition of the sap of a few Indian mango varieties;

2) To elucidate the mechanism of sap-injury in mango fruits, with special reference to components of mango sap, and of the mango peel, involved in sap-injury;

3) And to develop suitable methods to prevent, or control, sap-injury in mango fruits;

Knowledge of the composition of mango sap will have two-fold benefits. Firstly it will help us to better understand the mechanism of sap-injury and thereby enable us to devise methods to control it. Secondly identification of the useful compounds in mango sap might contribute to the utilization of an otherwise wasted by-product of the mango industry. Farmers would then be encouraged to collect it as a useful by-product, which will not only be economically beneficial to but also reduce the incidence of sap-injury. Understanding the mechanism of sap injury is not merely of academic interest, but is essential for controlling of injury. Once the composition of the sap is known and a clear understanding has been acquired of the nature of the components -in both sap and peel -that are actually responsible for the injury it may be possible to develop methods to prevent or control sap-injury.

## 2.1. INTRODUCTION

### 2.1.1 Nature of latexes in different plants

Many plants produce latex or sap, a viscous liquid which is usually white, but in a few plants it may be yellow, orange, or red in colour. All latexes are emulsions or aqueous suspensions of varying proportions of insoluble materials which may contain alkaloids, terpenes, oils, gums, resins, phenolics, proteins (including enzymes), sugars, and long-chain hydrocarbons [Vasistha and Siddiqui, 1938; Barton and Seoane, 1956; Dawson, 1956; Corsano and Mincione, 1965; Ansari et aI, 1967; Bhatia, et ai, 1967, Billets et aI, 1977; Joel et aI, 1978]. Special cells called laticifers, which represent a complex internal secretory system, are known to produce latex in many higher plants. Laticifers are known to occur in several 'families including Anacardiaceae (eg" Mangifera indica- mango, Anacardium occidentale- cashew), Apocynaceae (eg" Plumeria obtura, Nerium oleander), Asclepiadaceae (eg" Hoya bicarinata, Calotropis gigalltea), Euphorbiaceae (eg" Ricinus communis- castor, Euphorbia Pulcherri ma- poinsettia), Caricaceae (eg" Carica papaya, Carica candamarcencis- mountain papaya), Moraceae (eg., FiciiS carica- common fig, Atrocarpus altis- breadfruit) and Papaveraceae (eg" Argemone mexicana- prickly poppy, Papaver somniferum- opium poppy) [Purseglove, 1968]. Many commercial gums, including rubber, balata, guayule, gutta-percha, opium, chicle and chewing-gum are products made from refined latex [Purseglove, 1968; Tessier et al, 1976],

The physiological role of sap/latex is not entirely understood. In some plants, latex is exuded at the site of wounds, forming a protective sores which heal slowly [Epstein et aI, 1997; Behl and Captain, 1979]. These include the Renghas trees of Malaya, belonging to the genera Gluta g. G. renghas L.), Melanorrhoea and Melanochyla. They contain red sap, which blackens on exposure. The irritant seems to be a volatile aromatic substance, as even sheltering under the tree can cause irritation glove, 1968]. The sap of the Toxicodendron species including the ivy, poison-oak and poison-sumac, contains oleoresins called mols which initiate a cell-mediated, delayed, hypersensitivity Reaction in humans [Baer, 1983]. Symptoms are usually severe and range from hemorrhoids and headaches [Mitchell, 1990] to asthma-like symptoms [Campbell, 1983] and photodermatitis [Lampe et aI, 1.968].

#### 2.1.2.1. Latex in mango

The mango tree, like other members of the family Anacardiaceae, has a prominent duct system that is present in both fruit and stem (lnveys et aI, 1992] and contains sap (resin/latex). The resin ducts of the mango fruit appear as a longitudinal, multi-layered network [Fig 2.1],

which is both, branching and anastomosing in all directions and is completely covered by an epidermis [Joel, 1980a].

The chemistry and biochemistry of the sap secreted by the fruit ducts of mango have not been studied in detail so far. However, very little data regarding various components of sap is available. Loveys et al [1992] were able to account for 70% of the upper phase of saps of both Kensington and Irwin varieties of mango in terms of terpene content. The water content of the sap of Kensington variety was found to be only 3 % (w/v). Vasistha and Siddiqui have reported the presence of 'protein~carbohydrate mucilage' in mango sap as early as 1938. From dry mango sap they isolated and studies a resin (mangiferen), a resinous acid (mangiferic acid), and a resinol (Mangiferol). The degradation and oxidation products of mangiferen suggested that the chemicals are more related to the abietic acid series of resins, and support the view of resins being condensation products of isopren.

Fig 2.1. Resin ducts in mango [Source: Joel et ai, 1980a]

Other workers have dwelled on the irritant nature of certain sap components. Mango sap can cause a skin rash on humans, contact dermatitis, which has for many years been associated with the handling and consumption of mangoes. Keil et al [1946] and Gallagher [1953] demonstrated that the consumption of freshly picked unripe mango could cause contact dermatitis similar to that caused by other fruits of the [Blank, 1957; Ruehle and Ledin, 1960]. Bandhyopadyay et al

[1985] reported the isolation and identification of a mango dermatitis allergen, alkenyl resorcinol, a non-volatile constituent of mango sap.

In India, mangoes are harvested at a stage when they are mature as yet unripe. At the time of harvest, a good amount of sap flow still and when the fruit is severed from the stem at the abscission, sap spurts out from the abscission point ("spurt sap") and subsequently continues to exude out from the stalk end ("ooze sap"). During' harvesting or post-harvest handling, the mango fruits are invariably exposed to mango sap. This usually occurs when sap from the stalk end runs down the side of the fruit or spurt sap from other harvested fruit falls on the surface of the mango. It may also appear as irregular blotches on the peel where fruits have been in contact with one another [Brown et aI, 1986; Loveys et aI, 1992]. Sapbum-injury, being an important post harvest problem, has gained the attention of several workers, and concerted efforts have been made to control it [Brown et aI, 1986; Loveys et aI, 1992; Ledger, 1991; O'Hare and Prasad: 1991, Lim and Kuppelweiser, 1993; Lim and Bowman, 1995].

Joel [1980a] reported that the sap was present in the duct system and duct system in mango may playa vital role in protection against the fruit fly. Essential oils of various plants were known to possess anti- microbial activity [Caccioni et aI, 1998]. Hahn and Appleman [1952] found that Streptococcus faecalis was completely destroyed with cold pressed orange oil at a concentration of 1000 ppm in frozen orange juice. The terpene fraction of orange oil at 250 mg % was found to be inhibitory to the spotes of Aspergillus, Rhizopus, and Fusarium [Patel et aI, 1983]. The sennal oils of Leptospermum scoparium (manuka) and Kunzea ericoides (kanuka), which were rich in terpenes, possess significant anti-microbial k'tivity [Porter and Wilkins, 1998]. It is therefore likely that the mango sap, which is rich in mono-terpenes, may possess significant anti-microbial activity. In this study we have investigated the anti-bacterial and anti-fungal activities of the aqueous and non-aqueous phase of mango sap.

Varietal variations in terpenoid composition of mango sap are likely, as indicated by Loveys et al [1992]. The composition of sap from different Indian varieties of mango has not been studied. Sap-injury is a major post harvest problem universally, and controlling this problem will require an in depth knowledge of the components of mango sap in the different local varieties. Towards this end, we have attempted to study, in detail, the composition of the aqueous and non-aqueous phases of mango sap. The role of mango sap in defense of the fruit against various pest infestations and infections has been long suspected. However, to date, there are no reports of anti-microbial activity in mango sap. During the course of this investigation, we have examined the effect of mango sap on the growth of a few common bacteria and fungi.

## 2.2. MATERIALS

### 22.1. Plant materials

Fruit and sap of mango varieties Badami, Raspuri and Seedling, grown on the campus of Central Food Technological Research Institute, Mysore, India; Malgoa, Totapuri and Mallika varieties obtained from the Ramakrishna Vidyashala, Mysore, India; and Banganapalli variety grown in Chirala, India, were used in this study.

### 2.2.2. Microbial cultures

Cultures of Aspergillus flavus, A. parasiticus, Fusaritlm moniliforme, Penicillium spp., Bacillus cereus, Staphylococcus aureus, Escherichia coli and Pseudomonas aernginosa were obtained from the Department of Food Microbiology, CFTRI, Mysore, India, were used in the study.

### 2.2.3. Chemicals

n-Alkane hydrocarbons were procured from Aldrich Chemical Company; Catechol, o-dianisidine, gallic acid, azocasein, sodium polygalacturonate (citrus), pectin (citrus), TEMED, Coomassie brilliant blue R-250, Trizma base, p-mercaptoethanol, linoleic acid, were obtained from Sigma Fine Chemicals, St. Louis, USA. H202, glycine were obtained from Qualigens Fine Chemicals Mumbai, India; Chloramphenical rose Bengal agar, nutrient agar, ammonium persulphate were obtained from Hi Media, Mumbai, India; Folin -Ciocalteau reagent was obtained from SOH, Mumbai, India. All other reagents were of analytical grade.

## 2.3. METHODS

### 2.3,1. Collection of mango sap

Mature mango fruits were harvested with pedicels (about 2 inches k>ng) intact. Subsequently, the pedicels or stalks were detached from the fruit at the abscission zone and the sap was collected into glass tubes for c.1e min. For certain experiments, mangoes were harvested at three different stages of development and sap was collected. These stages were defined based on the time interval from initiation of flowering; stage I -10 weeks; stage II -14 weeks; stage III -harvest maturity.

### 2.3.2. Separation of aroma components

Two methods were used to obtain aroma components from sap. One is separation of non-aqueous phase by centrifugation and the other by ether extraction of whole sap.

a) Centrifugation: Mango sap was separated into upper non-aqueous lower aqueous phase by centrifugation at 3000 x g for 5 min at room temperature [Loveys et aI, 1992]. The non-aqueous phase was stored at 4°C, whereas the aqeous phase was stored at 4°C and later used for characterization studies.

**b) Extraction:** To the freshly obtained whole sap taken in a separating funnel, 2 equal volumes of peroxide-free distilled diethyl ether was added. This was shaken well, and then allowed to settle. The upper ether layer containing the extracted aroma components was removed, distilled and the distillate was stored at -20°C until use.

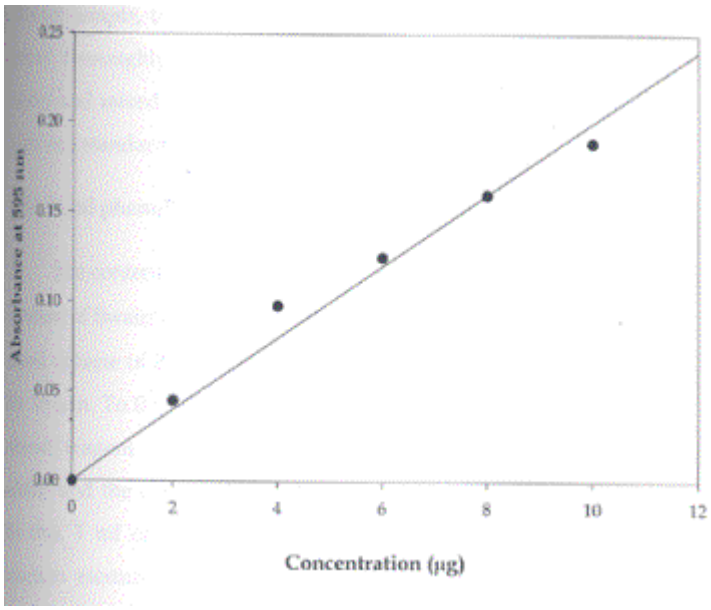
2.3.3. GC-MS analysis

The aroma components of the sap from different varieties were using a Schimadzu 17 A-GC chromatograph equipped with a QP-5000 (quadrapole) mass spectrometer. The samples were diluted times with acetone and 1 μl was injected. A fused silica capillary SPB-1 (30 m x 0.32 mm i.d., film thickness 0.25 μm), coated with polydimethyl siloxane, was used. Helium was the carrier gas at a flow rate of 1 ml/min; injector temperature was 250°C; detector temperature was 250°C and initial oven temperature 50°C for 3 min. The temperature was later increased to 250 °C at the rate of 2°CI min. This temperature was maintained for 5 min. splitting ratio 1:50 ionization voltage, 70 e V.

Retention indices for all the compounds were determined according to the Kovats method using n-alkanes as standards (Jennings n Shibomoto, 1980]. The compounds were identified by comparison of Kovats indices and by co-injection with an authentic specimen; and also by matching their fragmentation patterns in mass spectra with those of NI5f library and published mass spectra (Jennings and Shibamoto, 1980; Adams, 1989].

2.3.4. Protein estimation

The protein content in aqueous phase of mango sap was determined using the dye-binding method of Bradford [1976]. Since the sample was viscous, it was diluted ten times with 0.05 M sodium phosphate buffer (pH 7.5), centrifuged, and the supernatant used for protein estimation. Bovine serum albumin was used as a protein standard




(Fig. 2.2). Standard graph for protein estimation by Bradfords dye-binding method

2.3.5. Carbohydrate estimation

The carbohydrate content in the sap was determined by the phenol-sulphuric acid method [Dubois et aI, 1956]. To 0.1 ml of suitably diluted sample, 0.4 ml of water, 0.3 ml of 5% phenol were added and mixed thoroughly. To this, 1.8 ml of concentrated sulphuric acid was added and mixed. The colour obtained was read at 480 nm. Glucose was as a standard (Fig. 2.3).

2.3.6. Total phenolics in mango sap

The content of total phenolics in mango sap was determined by the method of Swain and Hills [1959]. Mango sap (300 μl) was mixed with an equal volume of 80% ethanol and this was centrifuged at 7000 x g at 4°C for 15 min. To 0.5 ml of this ethanolic extract, 8.5 ml of water, 0.5 ml of phenol reagent (Folin-Ciocalteau reagent, diluted 1:2 with water), was added and the contents were incubated at room temperature for 3 min. To this, 1 ml of saturated sodium carbonate was then added and the reaction mixture was incubated at room temperature for 60 min. The absorbance was recorded at 675 nm. Gallic acid in 80% ethanol was used as a standard (Fig. 2.4).

2.3.7. Determination of ash and metal content in mango sap

Ash and metal contents were determined according to the described in AOAC [1990]. The sample was accurately weighed in a clean silica crucible. The contents of the crucible were dried on a hot plate (100-2DDOC) and after the sample stopped emitting smoke, the crucible was kept in a muffle furnace.

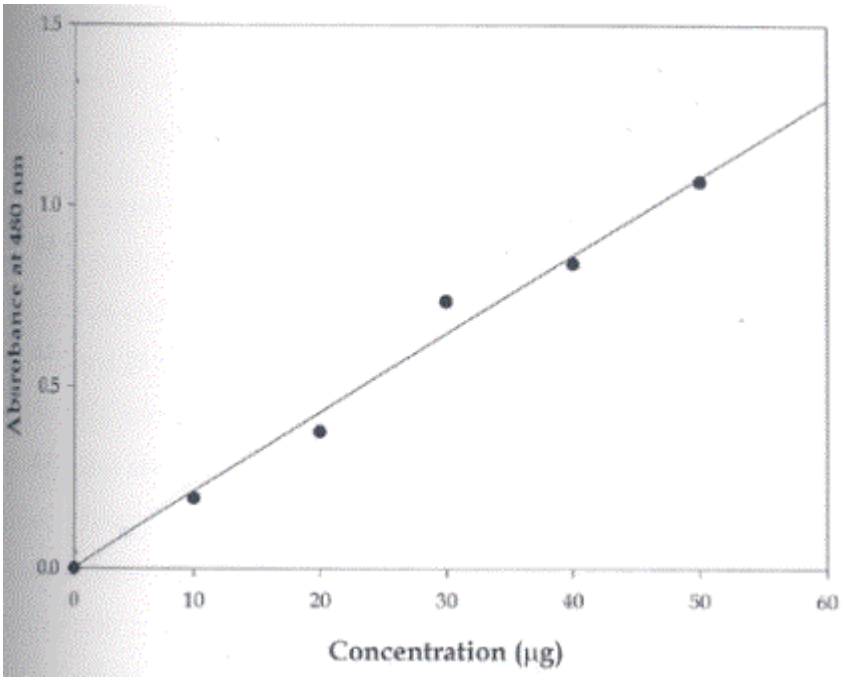



**Fig 2.3 Standard graph for the estimation of carbohydrate content by phenol-sulphuric acid method**

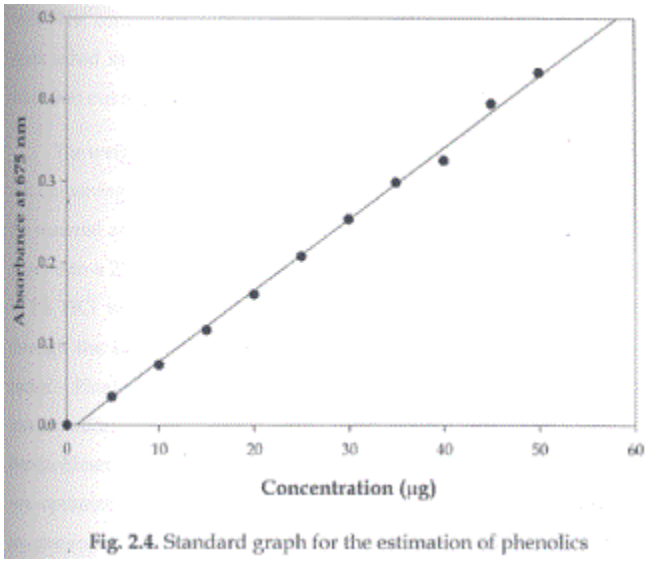



Fig. 2.4. Standard graph for the estimation of phenolics

Fig 2.4 Standard graph for the estimation of phenolics

The temperature of the muffle furnace was slowly raised to 250-300°c. Later the temperature was raised to 450°C and ashing was continued. After 5 hours, the crucible was removed from the furnace, cooled to room temperature and the contents moistened with water. Concentrated nitric acid (0.5 ml) was added and the contents were mixed well. The contents of the crucible were then dried on a hot plate and ashed in a furnace till carbon free ash was obtained. The crucible taken out and cooled to room temperature.

The weight of the crucible with its ash content was recorded. To the IF containing crucible, 10 ml 6M HCI was added, warmed to dissolve the material and the contents were filtered through a Whatman 41 filter paper into a 25 ml volumetric flask. To transfer the remaining ash, 10 ml of 1% HCl was added to the crucible and was warmed and filtered through the same filter paper into the volumetric flask that was used. Finally, the volume was made up to 25 ml with 1 % HCI. The concentration of metals in this solution was measured using a model 3110 Atomic Absorption Spectrophotometer which was optimized for wavelength and standard curve using standards. The recommended parameters are slit width 0.7 nm (for iron 0.2 nm); flame -air acetylene. The wavelength used for iron was 248.3; zinc, 213.9; copper, .324.8; manganese, 279.5; magnesium, 285.2; potassium, 766.5; sodium, 589 and for lead, 217 nm.

2.3.8. Polyphenol oxidase assay

PPO activity was assayed using catechol as substrate [Coseteg and Lee, 1987]. The 1 ml reaction mixture contained varying amounts of appropriately diluted enzyme, 0.1 ml of 0.5 M catechol and the remaining volume was made up with 0.05 M sodium phosphate buffer (pH 6.0). One unit of polyphenol oxidase activity was defined as the amount of enzyme that caused increase in absorbance of 1.0 per minute at 420 nm.

2.3.9. Peroxidase (POD) assay

Activity of POD was measured using H202 and o-dianisidine as mtrates [Aparicio -Cuesta et al, 1992]. The 1 ml reaction mixture contained varying amounts of the appropriately diluted enzyme, 0.1 ml of 0.25% o-dianisidine and 0.1 ml of 1% H202 in 0,05 M sodium acetate buffer, pH 6.0. One unit of peroxidase activity was defined as the amount i of enzyme that caused an increase in absorbance of 1.0 per minute at 420 nm.

2.3.10. Protease assay

Serine protease content was estimated using the azocasein hydrolysis method [Sarath et al, 1989]. To 0.45 ml of 0,05 M Tris-HCI buffer (pH 8.0), 0.05 ml of azocasein solution (25 mg/ ml) was added and little solution was pre-incubated at 37°C for 10 min. To this, 0.2 ml of appropriately diluted sample was added and it was incubated at 37°C for 30 min. To the reaction mixture 0.5 ml of 10% trichloroacetic acid was added and it was kept in ice for 10 min. This was centrifuged at 8000 x g for 10 min and the supernatant was transferred into a fresh tube. To the supernatant, 0.04 ml of 10 M sodium hydroxide was added and it was maintained at room temperature for 5 min. The absorbance of this solution was measured at 440 nm. An increase in absorbance of 1.0 was regarded as 1 unit of activity. In case of cysteine protease assay, the procedure followed was the same as above except phosphate-cysteine- disodium EDT A buffer was used instead of Tris-HCI, pH 6.0 (AOAC, 1975].

2.3.11. Amylase Study

Gelatinised soluble starch (1 %) was prepared in 0.05 M sodium hate buffer (pH 6.0), 50 μl of appropriately diluted enzyme sample added to 1 ml of this solution and the reaction mixture was hubated at 37 °C for 30 min as per the procedure described by Bernfield [1955]. The reaction was stopped by the addition of 1 ml of initrosalicylate (DNS) reagent. The reducing sugar released was estimated by the DNS method [Luchsinger and Comesky, 1962]. One unit amylase activity was defined as the amount of enzyme that catalyses the liberation of reducing sugar equivalent to 1 μ mole per hour.

2.3.12. Polygalacturonase Assay

The reaction mixture consisted of 0.2 ml of appropriately diluted enzyme in 0.15 M NaCI, 0.2 ml of 0.2 M Tris-acetate buffer (pH 4.5), 0.1 mJ of 0.01 M CaCl2 and 0.5 ml of 1 % polygalacturonate. The reaction was carried out for 1 hour at 37°C. It was stopped by heating at 100°C for 3 min and 0.5 ml of each solution was analysed for reducing groups using the dinitrosalicylate method [Luchsinger and Come sky, 1962]. One unit of activity is the amount which catalyses the formation of 1 μ mole of reducing group per hour [Pressey and Avants, 1976].

2.3.13. Pectinmethylesterase (PME) Assay

The rate of citrus pectin de-methylation was measured at room temperature by titration with 0.025 N NaOH. 50 ml of 1% (w Iv) pectin in 0.1 N NaCl was used as substrate and adjusted to pH 7.0 before the addition of 1.0 ml of enzyme extract. One unit of PME activity was defined

as the amount of enzyme capable of catalyzing the consumption 1μmole of base per hour under assay conditions [Priya Sethu et al, 1996]

2.1.14. Lipoxygenase Assay

The linoleic acid substrate was prepared according the method kribed by Shiiba et al [1991]. The reaction mixture consisted of 0.05 M sodium acetate buffer (pH 5.5, 0.95 ml), linoleic acid (7.5x10-3M, 30 μl) and enzyme extract (20μl). Enzyme activity was expressed in terms of hydroperoxide formed (1 μmole) per min using an extinction value of 25x104 M-Icm-l.

2.3.15. Catalase Assay

Catalase was assayed by the method of Aeib [1984]. The 1 ml reaction mixture contained 0.9 ml of 0.05 M potassium phosphate buffer, pH 7.0 and 0.1 ml of appropriately diluted sap. The reaction was started with the addition of 5 μI of 2.3 M H202 (11.46 mM). One unit of enzyme activity was defined as the quantity which degrades 1 μmole of H202 per minute under standard conditions at room temperature.

2.1.16. Stability of PPO and POD in mango sap

Aqueous phase of mango sap was stored for different time periods separately at 4 °C and room temperature. Aliquots were drawn from these and assayed for the activities of PPO and POD at regular intervals of time.

2.1.17. Polyacrylamide gel electrophoresis

The sap aqueous phase was subjected to native gel electrophoresis according to the procedure described by Laemmli [1970]. The gel was cut into 3 parts. One part each was subjected to protein staining, polyphenol oxidase staining and peroxidase staining as described below.

a) Protein staining

The protein bands in the gel were visualized by staining with Coomassie blue R-250 [Laemmli,1970].

b) polyphenol oxidase staining

The enzyme polyphenol oxidase was detected in the gel using the procedure described by Lee [1991]. After electrophoresis, the gel was washed twice (for 5 min each) in 0.05 mM sodium acetate buffer, pH 6.0, in a petri-dish with gentle shaking. The gel was transferred into the freshly prepared solution containing 275 mg of catechol and 25 mg of p-phenylenediamine in 50 ml of 0.05 M sodium acetate buffer (pH 6.0) and maintained with regular shaking until the bands developed to the required intensity. The reaction was stopped by the addition of 5 % acetic acid, and washed several times with water to remove traces of substrate. The gel was stored in 5 % ethanol.

c) Peroxidase staining

After electrophoresis the gel was washed twice (for 5 min each) with 0.05 M sodium acetate buffer, pH 6.0. The gel was transferred to a solution containing 1 mg/ ml of diaminobenzidine in the above buffer. The colour reaction was started by the addition of 0.05 ml of 6% H202 (Hoffman, 1970]. The gel was allowed to remain in the solution till the lI8MIs were visible. The reaction was stopped by transferring the gel to 5% acetic acid and stored in 5 % ethanol.

2.13.18. Determination of anti-bacterial activity

The anti-bacterial activity of aqueous and non-aqueous phases of sap from different varieties was tested against different Gram +ve and Gram -ve bacteria by pour plate method [Negi et aI, 1999]. Bacterial strains, Bacillus cereus, StaphylococClIS aureus, Esclzerichia coli and Pseudomonas aeruginosa were maintained at 4°C in nutrient agar. Strains were grown in nutrient broth at 37°C for 24 hours. To obtain synchronization of cells, it was again sub-cultured in nutrient broth (37°C for 48 hours) for use as inoculum. The cells were harvested by centrifugation (1200 x g, 5 min) and diluted suitably in saline solution. Cell suspension (103 cfu/ml) of each bacterium was used for inoculation. Different concentrations of mango sap were introduced into sterile melted and cooled nutrient agar (20 ml) in separate flasks. Bacterial suspension (0.1 ml) was added into each flask and the medium was pour-plated aseptically. In the case of the control only agar and bacterial suspension were used. The growth of each bacterium was observed after incubation at 37°C for 20-24 h. The minimum inhibitory concentration was reported as the lowest concentration of the sap required for inhibition of complete growth of the bacterium being tested [Naganawa etal,1996].

2.3.19. Determination of anti-fungal activity

Anti-fungal activity of the non-aqueous phase of mango sap was tested against Aspergillus jlavus, A. parasiticus, Penicillium spp. and Fusarium moniliforme. The fungal strains were maintained at 4°C in potato dextrose agar. Strains were grown in potato dextrose agar at 26°C for 5 days and spore suspension (103 spores/ml) of each fungus was prepared in sterile water and used for inoculation. The anti-fungal activity was evaluated by the spore germination method [Paster et aI, 1999]. Different concentrations of mango sap were introduced into sterile melted and ax>led chloramphenicol rose bengal agar (20 ml) in separate flasks. Spore suspension (0.1 ml) was added into each flask and the medium-was pour- plated aseptically. In the case of the control only spore suspension was used. The number of spores germinated was counted at regular intervals of 24 h up to five days and germination of spores was expressed as percentage of the control.

2.3.20. Statistical evaluation

The sap-injury data were subjected to statistical analysis using the method of Steel and Torrie [1980].

## 2.4. RESULTS AND DISCUSSION

### 2.4.1. SECTION A: COMPOSITION OF THE NON-AQUEOUS PHASE OF MANGO SAP

Sap collected from different Indian mango varieties viz, Badami, B4nganapalli, Malgoa, Raspuri, Seedling, Mallika, Totapuri was a viscous liquid with a pH around 4.0 and aroma characteristic of the raw mango &nit. The collected .sap had a tendency to separate into two phases -an upper light, pale-yellow coloured, non-aqueous layer and a lower viscous, colour less, aqueous layer. Centrifugation of the sap was performed to accelerate the process of its separation into the constituent phases as in the case of Kensington and Invin mango varieties [Loveys et aI, 1992].

#### 2.4.1.1. Sap content and its constituent phases in different mango varieties

The sap yield varied in different varieties. As can be seen from Table 2.1, Malgoa, Seedling, Mallika and Banganapalli varieties yielded 25.2 ml, 22.5 ml, 21.0 ml and 18.5 ml of sap per 10 kg of fruit respectively, whereas much lower amounts were obtained from Badami, Raspuri and Totapuri varieties (10-11 ml per 10 kg of fruit).

The ratio of the non-aqueous phase to the aqueous phase was found to be different in different varieties (Table 2.1). The Seedling and Totapuri varieties had non-aqueous to aqueous ratios of about 1:2, and Mallika and Badami had about 1:3 and 1:4 respectively, indicating the presence of relatively large amounts of non-aqueous phase in these varieties, whereas Malgoa, Banganapalli and Raspuri varieties yielded

Table 2.1 Aqueous and non-aqeous phases of mango sap

| Sl. No. | Variety | Volume (ml/10 kg of mango) | | | Ratio (no |
|---|---|---|---|---|---|
| | | Whole sap | Non-aqueous phase | Aqueous phase | aqueous /aqueous |
| 1 | Totapuri | 11.0 | 3.7 | 7.3 | 1:2 |
| 2 | Seeding | 22.5 | 6.8 | 15.7 | 1:2:3 |
| 3 | Mallika | 21.1 | 5.4 | 15.7 | 1:3 |
| 4 | Badami | 10.0 | 2.0 | 8.0 | 1:4 |
| 5 | Raspuri | 11.0 | 1.4 | 9.6 | 1:7 |
| 6 | Banganapalli | 18.5 | 1.5 | 17.0 | 1:11 |
| 7 | Malgoa | 25.2 | 1.8 | 23.4 | 1:13 |

2.4.1.2 Composition of non-aqueous phase

The non-aqueous portions of all the 7 mango varieties were analysed by GC-MS and found to contain mainly monoterpene hydrocarbons, viz. β-myrcene, cis-/trans-ocimene and limonene [Figs. 2.5b, 2.6]. The major constituent of the sap from Totapuri, Raspuri, Seedling and Malgoa was β-myrcene (~50-80%) whereas in Banganapalli, trans-ocimene (91 5), in Badami cis-ocimene (83%) and in Mallika, limonene (61%) were the major constituents (Table 2.2.). α-Pinene, trans-allo ocimene were present in considerable amounts certain varieties. β-Pinene, γ-terpinene, α-copaene, β-caryophyllene and α-humulene were present in these varieties as minor components.

Earlier, Gholap and Bandhyopadhyay [1977] have reported the presence of only cis-ocimene in the sap (latex) of Alphonso (synonymous with Badami), and p-myrcene in the Batali mango varieties. Loveys et al [1992] have reported the presence of terpinolene (84 %) and car-3-ene (90%) as major terpenoids in the saps of Australian Kensington variety and the lr1vin

variety of Florida, respectively, as major components. But neither of these compounds was detected in any of the Indian mango varieties studied here.

Monoterpene hydrocarbons were reported to be the major aroma constituents of mango [Hunter et a11974; Bandhyopadhyay and Gholap, 1979; Macleod and Troconis, 1982; Idstein and Schrier, 1985; Macleod et aI, 1988; Tamura et aI, 2001]. Bandhyopadyay and Gholap [1978] reported the presenc;e of cis-ocimene and p-myrcene in the pulps of Alphonso and Totapuri mango varieties respectively. Interestingly, in the present study the saps of these two varieties were found to contain cis-ocimene and p-myrcene as the major aroma components [Table 2.2.]. A previous report by MacLeod et al [1988] indicated the presence of terpinolene as the major aroma component of the pulp of Kensington variety of mango and subsequently, Loveys et al [1992] reported terpinolene in the sap of the same variety. These studies indicate that the pulp and sap of a particular mango variety share similar terpenoid compounds.

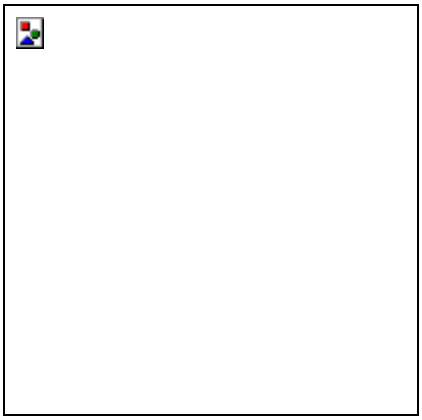


Fig 2.5a. GC profiles on non-aqueous phase of sap from different mango varieties

I. Mallika; II. Raspuri; III. Malgoa; IV. Banganapalli;

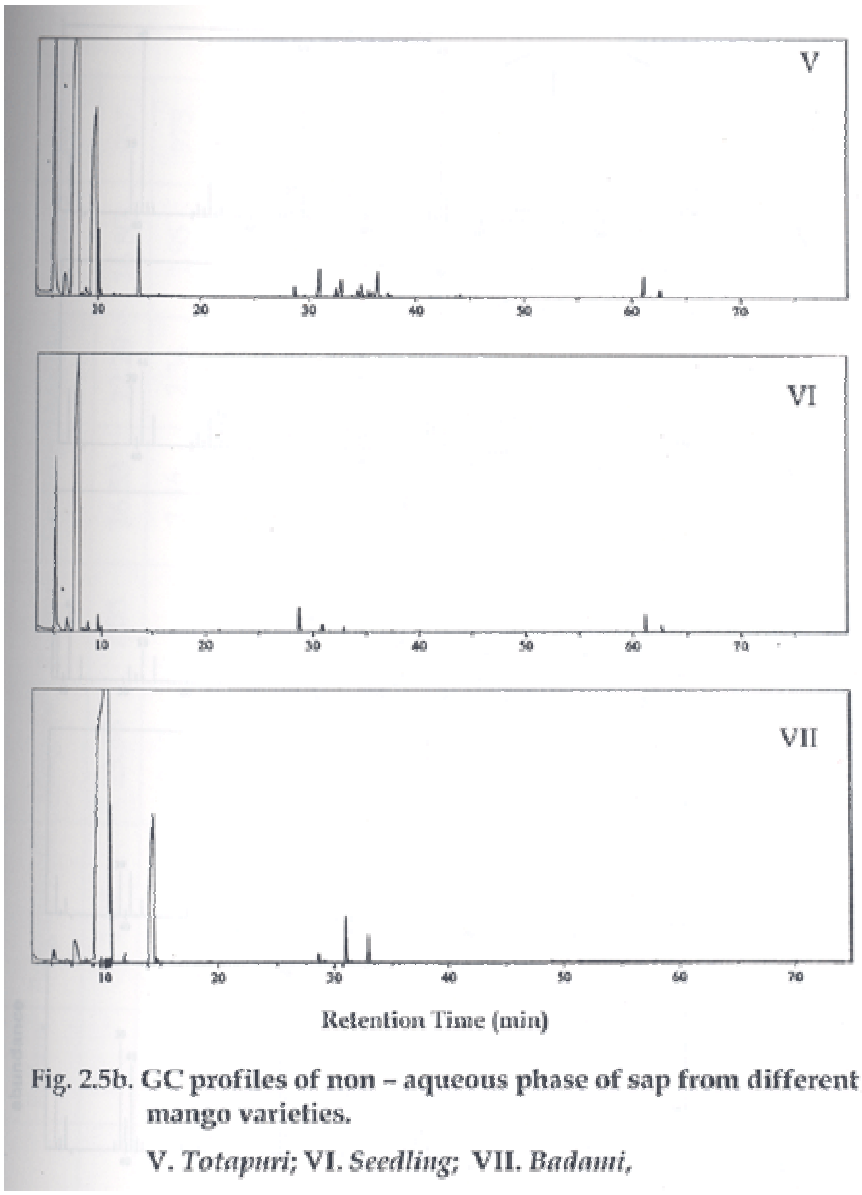



**Fig. 2.5b. GC profiles of non – aqueous phase of sap from different mango varieties.**

**V. *Totapuri;* VI. *Seedling;* VII. *Badami,***

Fig.2.5b. GC profiles of non-aqueous phase of sap from different mango varities

V. Totapuri; VI. Seedling; VII. Badami

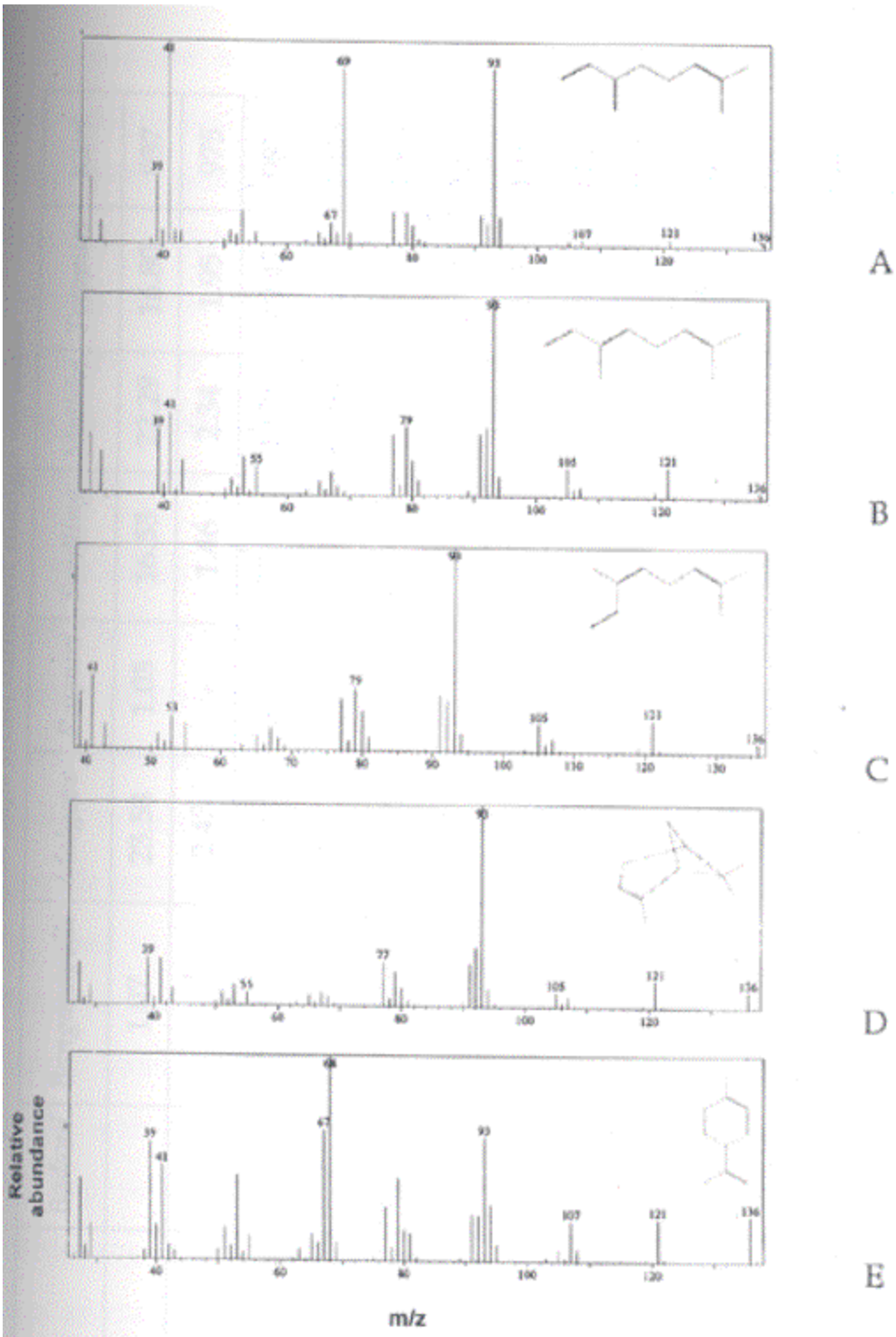



Fig. 2.6. Mass spectra of major terpenoids present in non - aqueous phase of mango sap

A. β-myrcene, B. *Cis*-ocimene, C. *Trans*-ocimene,

D. α - Pinene, E. Limonene,

Fig 2.6. Mass spectra of major terpenoids present in non-aqueous phase of mango sap

A. β-myrcene, B. Cis-ocimene, C. Trans-ocimene, D.α - Pinene, E.Limonene

Table 2.2 Percentage compostion of terpence components in saps from different mango varieties

| Variety RT (min) | Compound | Badami | Banganapalli | Malgova | Raspuri | Seeding | Malliaka | Totapuri | $KI^{cal}$ |
|---|---|---|---|---|---|---|---|---|---|
| 5.13 | α-Pinene | | 1.07 | 23.58 | 1.03 | 16.53 | 22.29 | 16.80 | 937 |
| 6.44 | β-Pinene | - | - | 2.43 | - | 1.46 | 2.34 | 1.95 | 975 |
| 7.17 | β-Myrcene | 2.07 | 0.32 | 58.89 | 66.17 | 77.57 | 10.38 | 49.42 | 987 |
| 8.32 | Limonene | - | - | - | - | 0.25 | 61.04 | 0.39 | 1025 |
| 9.53 | Cis-Ocimene | 82.83 | 1.37 | 10.68 | - | - | - | - | 1044 |
| 9.87 | Trans-Ocimene | - | 90.95 | 1.21 | 22.28 | 0.54 | - | 22.78 | 1049 |
| 9.98 | γ-Terpinene | 2.54 | - | - | 1.63 | 0.21 | - | 1.24 | 1055 |
| 13.95 | Transallo Ocimene | 11.56 | - | 0.90 | 1.53 | - | - | 2.53 | 1125 |
| 28.68 | α-Copaene | - | - | 0.28 | 0.22 | 1.20 | - | 0.28 | 1378 |
| 31.01 | β - Caryophyllene | 1.50 | 2.96 | 1.49 | 2.03 | 0.37 | 2.67 | 0.87 | 1414 |
| 32.54 | α-Guaiene | - | - | - | - | - | - | 0.27 | 1441 |
| 32.98 | α-Humulene | - | 1.65 | 0.83 | 1.11 | 0.20 | 1.29 | 0.55 | 1448 |
| 34.56 | Allo – Aromadendrene | - | - | - | - | - | - | 0.22 | 1474 |
| 34.85 | γ - Gurujunene | - | - | - | - | - | - | 0.35 | 1479 |
| 35.86 | δ - Cadinene | - | - | - | - | - | - | 0.20 | 1495 |

In addition to terpenoids, the mango fruit also contains several 8S, alcohols and lactones [Hunter et aI, 1974; Bandhyopadyay and Gholap, 1979; Macleod and Traconis, 1982; Idstein and Schrier, 1985; Macleod et aI, 1988]. The raw mango aroma is essentially due to Hpenoids, the aroma of the ripe fruit is due to the other aroma axnponents. It is probable that esters, alcohols and lactones develop during the ripening process whereas sap imparts the raw mango aroma to the unripe fruit. .

The aroma components of the ether extract of whole sap were also subjected to GC-MS analysis. The results indicated that there is no significant difference between the composition of the non-aqueous phase and that of the ether extract. This implies that simple centrifugation is sufficient to extract all the aroma components present in sap. Certain terpenoids such as β-myrcene, ocimene, limonene, α- and β-pinene are being used in low concentrations (ppm) as aroma components in various processed foods like baked goods, frozen dairy products, meat products, condiments, relish, soft candies, gelatin, puddings, non-alcoholic and alcoholic beverages, cheese, chewing gum etc [Burdock, 1995]. Sap non-aqueous phase can thus be used directly in various confectionary items, bakery products and beverages, to impart the raw mango aroma. Besides this, the individual components of sap can be used as raw materials for nature-identical aroma chemicals.

### 2.4.2. SECTION B: COMPOSITION OF THE AQUEOUS PHASE OF MANGO SAP

Sap collected from six mango varieties viz, Raspuri, Badami, Seedling; Banganapalli, Totapuri, Malgoa, were separated into non-aqueous phase and aqueous phase. As reported earlier (P.51) the amount of aqueous phase present in sap varied from variety to variety (Table 2.1.). The aqueous phase of sap was subjected to the analysis of protein, enzymes, carbohydrate, total phenols, and metal contents.

#### 2.4.2.1. Protein content

The protein content in the aqueous phases of sap from different mango varieties was estimated. Highest amount of protein was found in the Raspuri variety (4.3 mg/ ml) and lowest in the Banganapalli variety (2.0 mg/ ml) (Table 2.3).

Table 2.3. Protein, carbohydrate content, activities of PPO, POD, serine and cysteine proteases in sap aqueous phase of mature mango

| Variety | Content (mg/ Iill ) | | | Specific Activity (U/mg protein) | | | |
|---|---|---|---|---|---|---|---|
| | Total Phenolics | Carbo-hydrate | Protein | PPO | POD | Protease | |
| | | | | | | Serine | Cysteine |
| Raspuri | 0.160[d] | 309[b] | 4.3[d] | 186.5[d] | 786.7[e] | 5.2[d] | 0.17[b] |
| Badami | 0.127[c] | 302[b] | 3.1[b] | 214.1[e] | 456.3[b] | 4.Q[c] | 0.38[d] |
| Seedling | 0.049[a] | 343[c] | 2.9[b] | 169.8[b,c] | 451.4[b] | 4.3[c] | 0.22[c] |
| Totapuri | 0.049[a] | 260[a] | 3.5[c] | 165.6[b, c] | 560.5[c] | 2.6[b] | 0.22[c] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Banganapalli | 0.108[b] | 314[b] | 2.0[a] | 147.3[a] | 401.4[a] | 2.4[a, b] | 0.12[a] |
| Malgoa | 0.045[a] | 348[d] | 2.2[a] | 177.7[c,d] | 711.0[d] | 2.1[a] | 0.37[d] |
| SEM (±) | 0.03 | 4.15 | 0.05 | 4.79 | 5.42 | 0.04 | 0.03 |

Values shown in the table are mean of four values. Different alphabets shown in the same column differ significantly at level $P < 0.05$.

2.4.2.2. Polyphenol oxidase activity

Among the six varieties studied, PPO activity was found to be the highest in sap of the mature Badami mango (214 U/mg protein), while Banganapalli had the lowest (147 U/mg protein) (Table 2.3.). The PPO activity in sap is much higher than in most fruits and vegetables reported so for [Galeazzi et aI, 1981; Das et aI, 1997; Cho and Ahn, 1999]. The specific activity of PPO in Banana was reported to be 15.4 U / mg protein [Galeazzi et aI, 1981}, in pine apple 0.73 U/mg protein [Das et aI, 1997], in potato 0.21 U/mg protein [Cho and Ahn, 1999]. PPOs have gained attention primarily due to their role in browning of tissues subsequent to bruising, or during the preparation of many fruit/vegetables for processing [Vamos-Vigyazo, 1981]. The browning is the result of enzymatic oxidation of phenols and eventual non-enzymatic polymerisation of the quinones formed into tannins or "melanins". However, studies have indicated that this enzyme may playa vital role II in the biochemical processes leading to fruit ripening and plant senescence in general as a means of controlling ethylene biosynthesis [Vamos-Vigyazo, 1981; Yang, 1967]. The role of this enzyme in sap is not known. It has been observed that all members of the Anacardiaceae, i\1cluding Shinus molle, Rhus and Mangifera, contain extracellular laccase Uoel, 1978].

2.4.2.3. Peroxidase activity

The activity of POD in sap of mature mango was found to be highest in Raspuri (787 U / mg protein) followed by Malgoa and the lowest in Banganapalli (401 U/mg protein; Table 2.3.). Badami and Seedling had similar specific activities (around 450 U/mg. protein). Totapuri had a specific activity of 560 U / mg protein. There are reports of POD activity in many of the common fruits [V amos- Vigyazo, 1981], however, the presence of POD in mango sap has not been reported so far, In the presence of $H_2O_2$, POD catalyses the oxidation of substrates such as phenols, aromatic-, primary-, secondary-and tertiary-amines, Ieuco dyes, ascorbic acid and indole. Despite its ubiquity and extensive study, the physiological role of POD remains obscure. PODs have been implicated in plant senescence, physiological breakdown of fruits and

vegetables, and in the deteriorative changes in flavour, texture, colour, and nutritional value in processed fruits and vegetables [Vamos- Vigyazo, 1981]. Miller et al [1989] reported the increase in peroxidase activity in cucumber fruit under mechanical stress.

2.4.2.4. Protease activity

The sap from all the varieties studied exhibited both serine protease and cysteine protease activities. The serine protease activity in r sap of mature mango ranged from 2.1 to 5.2 U/mg protein (Table 2.3.). It was the, highest in Raspuri followed by Badami and seed seedling. The varieties r Totapun; Banganapallz and Malgoa had sImIlar activIties (around 2 U / mg protein). Differences were also noticed in the activities of cysteine proteases in sap from different mango varieties. It was found to be the highest in Badami and the lowest in Banganapalli (Table 2.3.). The presence of protein hydrolyzing enzymes in fruit is well documented and the properties of some proteases, for example, papain from papaya latex, have been extensively studied [Kang and Warner, 1974; Park et aI, 1979; Smith and Kimmel, 1960]. Protease activity has been reported to play an important role in protein turnover and particularly during the final stages of senescence when protein catabolism is responsible for increases in free amino acids and amides [Dilley, 1970].

2.4.2.5. Activities of other enzymes

Very low activities of lipoxygenase (0.037 to 0.048U/mg protein), amylase (2.5 U/mg to 4.3 U/mg protein) and polygalacturonase (1.1 to 1.3 µM/h/mg protein) were detected. Catalase and pectin methyl esterase were not detected.

In general, the Banganapalli variety had the lowest protein content as well as the lowest enzyme activities compared to other varieties. Raspuri had the highest amount of protein .content and also pad higher enzyme activities.

2.4.2.6. Carbohydrate content in sap aqueous phase

The carbohydrate content in sap aqueous phase of mature mango ranged from 260 mg/ml in Totapuri to 348 mg/ml in Malgoa. In the other varieties it was about 300 mg/ ml sap (Table 2.3). The preliminary studies on carbohydrate from Raspuri sap indicated that it was a non-starch high molecular weight polysaccharide which was not dialysable out of a dialysis bag with a 12,000 cut off. It had a very little amount of free sugars (0.45 mg/ ml). It is interesting to note that in the mango varieties Malgoa, Banganapalli and Seedling both the whole sap content

as well as the carbohydrate content are higher (Tables 2.1.; 2.3.). It has been noticed that sap oozes out even after 24 h in these three varieties. The physiological role of this carbohydrate in sap is not known. It is likely that it provides the osmotic pressure required to draw moisture from the fruit into the duct system. It can be speculated that the carbohydrate may be responsible for maintaining the high pressure of fluid in the ducts, so that sap continually oozes out through the ienticels and forms a thin protective film on the surface of the fruit. The high pressure maintained may also allow sap to flood damaged/wounded areas as a defense to control infestation.

2.4.2.7. Total phenolic content in mango sap aqueous phase

The amount. of total phenolics ranged from 0.045 mg/ ml in Malgoa to 0.160 mg/ml in Raspuri (Table 2.3). Phenolic compounds are widely distributed in the plant kingdom and are particularly prominent in fruits where they are important in determining colour and flavour. It has been suggested that they were originally by-products of the metabolism of aromatic amino acids [Neish, 1964]. Plant phenolics have long been suspected of playing a role in the disease resistance of plants, and this function has been accounted for in a number. of ways. It has been reported that there is a rapid rise in polyphenol concentration following an infection, also, there is often a considerable amount of phenolic oxidation concurrent with this rise, that is mediated by both host and parasite PPO. The oxidized phenolics are more potent anti-fungal agents than the non-oxidized phenolics, and may also playa role in the hypersensitivity reaction which consists of rapid death of a few cells resulting in the confinement of

the pathogen to a restricted area of high PPO and oxidized phenol content [Cruickshank, 1964].

2.4.2.8. Ash and metal contents in sap aqueous phase

The ash content in sap aqueous phases of different varieties ranged from 0.3 % to 1.05 %. It was the lowest in Malgoa and the highest in Raspuri (Table 2.4.). The metal content in aqueous phase of mature mango was determined. The content of potassium was found to be the highest in sap of all mango varieties and ranged from 588 ppm to 1588 ppm. The next most abundant metal was magnesium, followed by sodium. Considerable amounts of copper, iron, zinc and magnesium were also detected. Since PPO is a copper containing enzyme and POD is a heme enzyme a major part of these metals may be bound to these enzymes. It has been reported that mango fruit pulp was rich in potassium, sodium and magnesium [Gopalan et aI, 1989].

Table 2.4. Ash and Metal content in aqueous phase of mango sap

| Metal | Variety | | | | | | SEM |
|---|---|---|---|---|---|---|---|
| | Raspuri | Badami | Seedling | Totapuri | Banganapalli | Malgoa | 1 |
| Ash* | 1.05[e] | 0.93[d] | 0.76[c] | 1.03[e] | 0.69[b] | 0.30[a] | 0.02 |
| Iron[#] | 2.4[d] | 1.3[a] | 1.8[c] | 2.6[d] | 3.4[e] | 1.5[b] | 0.05 |
| Zinc[#] | 0.2[b] | 0.3[c] | 0.3[c] | O.I[d] | 0.2[b] | 0.9[d] | 0.01 |
| Copper[#] | 2.8[d] | 1.8[c] | 1.5[b] | 0.6[a] | 1.7[b,c] | 0.5[a] | 0.03 |
| Manganese[#] | 0.7[a] | 1.5[b] | 1.4[b] | 5.4[d] | 4.8[c] | 14.4[e] | 0.05 |
| Magnesium[#] | 501[b] | 505[b] | 560[c] | 756[d] | 1078[e] | 418[a] | 5.83 |
| Sodium[#] | 143[a] | 190[b] | 24O[d] | 148[a] | 229[c] | 144[a] | 3.29 |
| Potassium[#] | 1588[f] | 926[d] | 800[c] | 667[b] | 1100[e] | 588[a] | 5.95 |

"- %; # -ppm

V alues ~hown in the table are mean of three determinations. Different alphabets shown in the same row differ significantly at level $P < 0.05$.

2.4.2.9. Electrophoresis of saps

Native polyacrylamide gel electrophoresis (PAGE) of the aqueous phase of sap from different mango varieties was carried out. Only one major protein band was observed in the sap of each variety (Fig.2.7). However, they differed in their mobilities. Protein band of Badami sap had a higher mobility than that of Raspuri. A significant difference was noted in the case of Totapuri sap proteins. In this variety, the sap proteins were separated into three bands, which had equal intensity. Thus, the protein patterns of sap exhibit to some extent varietal differences. Native PAGE profile of the sap indicated that there are very few proteins in mango sap. Most of the bands visible on native gel with Coomassie staining also stained for PPO and POD activities (Fig 2.7.). This suggests that these enzymes comprise a major part of the sap proteins. The same bands stained for both PPO and POD activities, indicating that either the same protein is showing two enzyme activities or different proteins are co-migrating in the gel.

2.4.2.10. Changes in protein, carbohydrate contents and enzyme activities at different stages of fruit development

The protein and carbohydrate contents of mango sap were found to decrease as the fruit matured, in all varieties studied. The decrease in protein content from stage I to stage III was around 35 to 40% in all varieties (Fig. 2.8.). The decrease in carbohydrate content from stage I to stage III was around 20 to 25%, but in Banganapalli the decrease was only 8% (Fig. 2.9.). In the case of PPO and POD enzyme activities, the general trend was towards a decrease in activity as the fruit matured (Fig. 2.10.; Fig. 2.11.). With respect to PPO the decrease in specific activity in saps

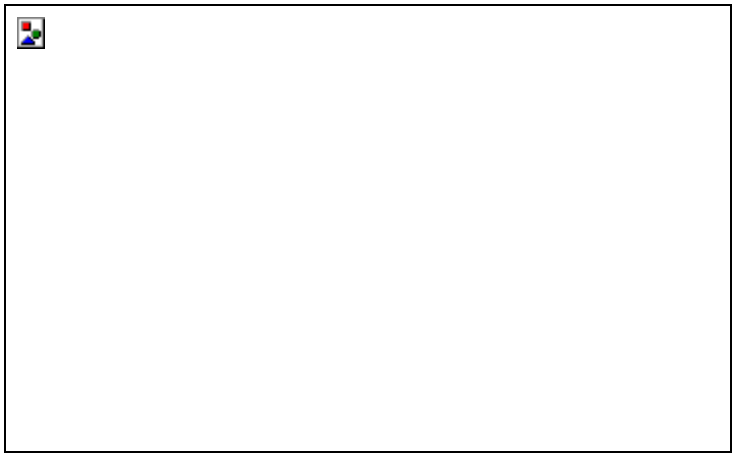


Fig 2.7: Native polycrylamide gel electrophoresis of the aqueous phase of sap of different varieties of mango

A. Polypheno oxidase stain; B. Protein stain; C. Peroxidase stain

R. Raspuri; B. Badami; S. Seedling; T. Totapuri; Bp. Banganapalli; M. Malgoa

from stage I to stage III ranged between 12% in Totapuri, and 57% in Malgoa. In Raspuri and Banganapalli a decrease of around 35 % was observed, whereas in Seedling and Badami, the decrease was 27% and 19% respectively. In the case of POD the decrease in specific activity from I stage I to stage III ranged between 19% in Banganapalli and 54 % in Badami. In Raspuri, Seedling, and Malgoa, the decrease was about 35%. There are no reports available on the PPO and POD activities in mango fruit at different stages of fruit development. However, in apple and grape from tender fruit and mature fruit a decrease of 17 and 5% respectively were reported [Marques et aI, 1995]. Coseteng and Lee [1987] also reported a decrease in PPO activity in apple from the early stage of maturity until harvest. Veda et al [2000] reported the increase in amylase activity as mango fruit matured.

The serine protease activity was very high at the mature stage compared to the initial tender stage (Fig. 2.12.). However, in Totapuri sap the enzyme activity was not as high as with other varieties. In the case of cysteine protease activity, no definite trend was observed. In the varieties Raspuri, Totapuri and Banganapalli, a decrease, and in Seedling and Malgoa, an increase in activity was observed with increase in maturity (Fig. 2.13.).

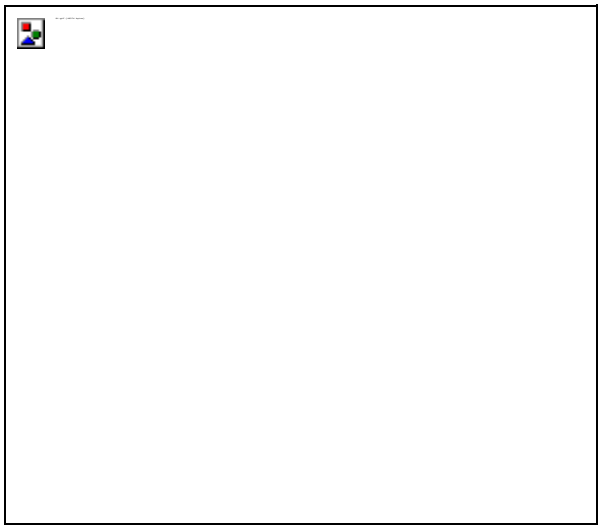


Fig 2.8. Protein content in the aqueous phase of mango sap at different stages of fruit development

Mean of four values ± SD

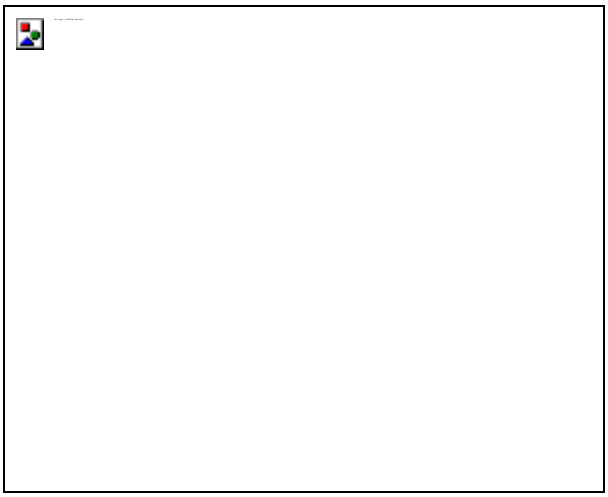


Fig 2.9 Carbohydrate content in the aqueous phase of mango sap at different stages of fruit development, Mean of four values ± SD

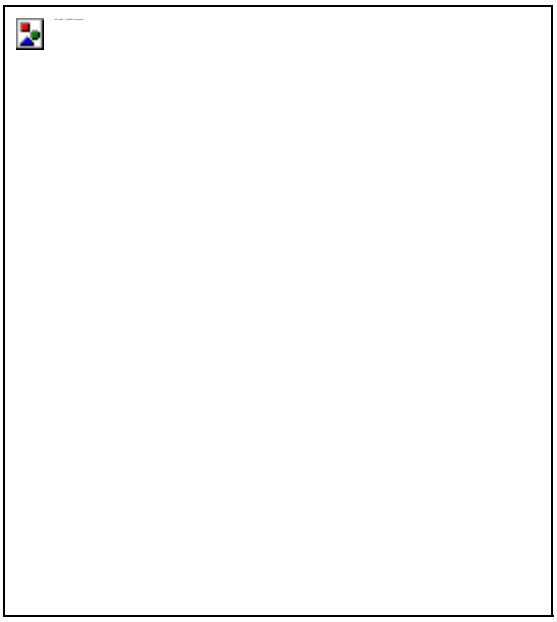


Fig 2.10 Polyphenol oxidase content in the aqueous phase of mango sap at different stages of fruit development, Mean of four values ± SD

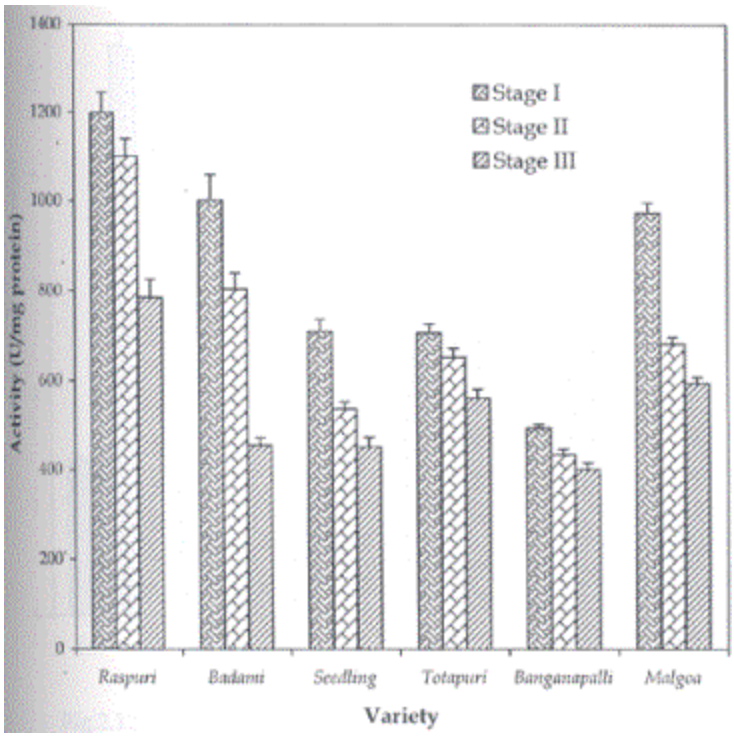



Fig 2.11 Peroxidase content in the aqueous phase of mango sap at different stages of fruit development, Mean of four values ± SD

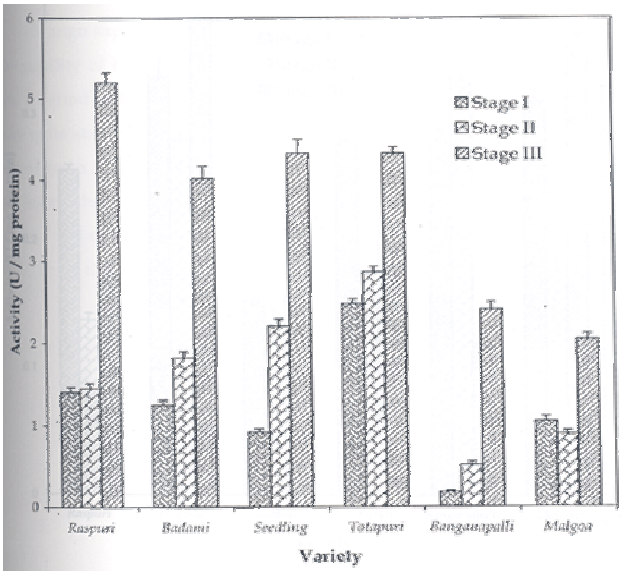



Fig 2.12 Serine protease activity in the aqueous phase of mango sap at different stages of fruit development

Mean of four values ± SD

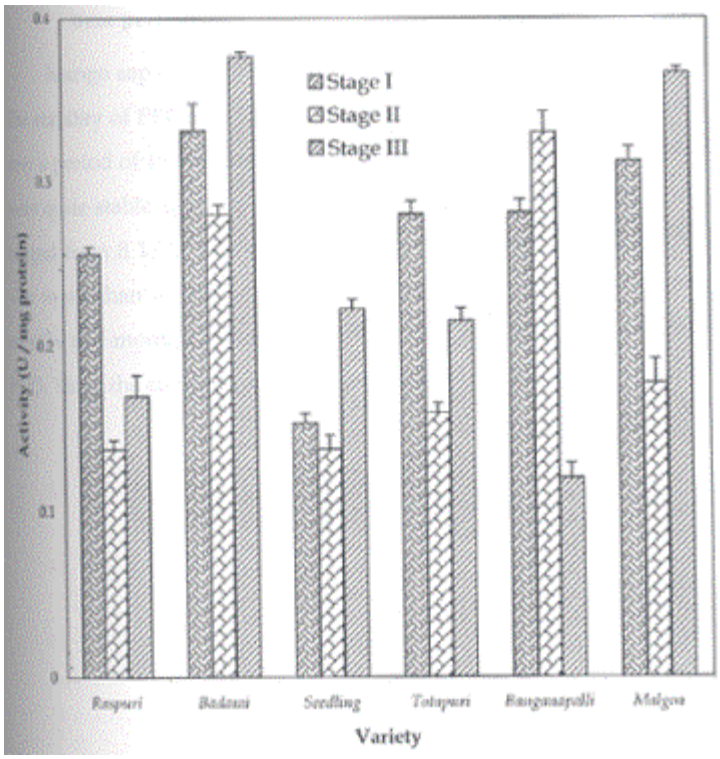



Fig 2.13 Cysteine protease activity in the aqueous phase of mango sap at different stages of fruit development

Mean of four values ± SD

2.4.2.11Stability of polyphenol oxidase and peroxidase at different time periods

Mango sap contains a good amount of oxidative enzyme activities stability of PPO and POD at 4°C and room temperature was studied over period of 15 months (Fig. 2.14. -2.17.). The enzymes PPO and POD quite stable at 4°C. In the case of PPO, the half-life of these enzymes ranged from 8 1/2 months to 13 months, whereas in the case of POD, it was more than a year. At room temperature, its half-life was about 5 months to 8 months in the case of PPO, and 3 to 6 months, in the case of POD. Thus, the enzymes are quite stable when the sap is stored at 4°C.

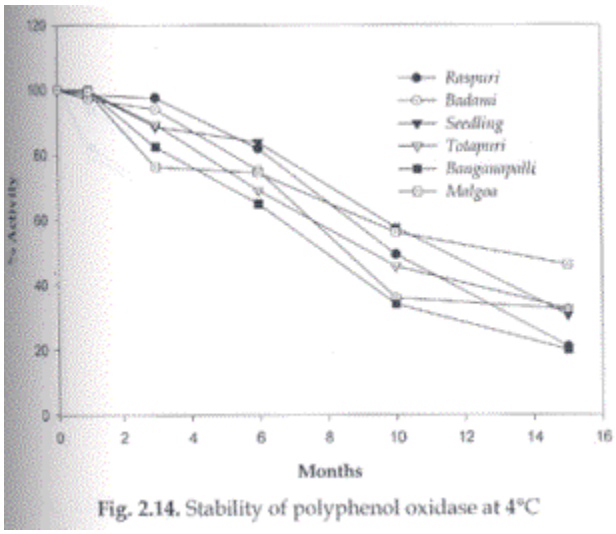


Fig. 2.14. Stability of polyphenol oxidase at 4°C

Fig.1.14. Stability of plyphenol oxidase at 4°C

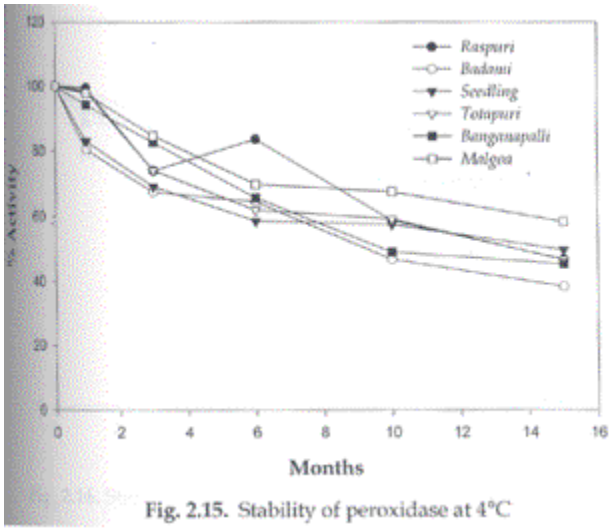


Fig. 2.15. Stability of peroxidase at 4°C

Fig.2.15. Stability of peroxidase at 4°C

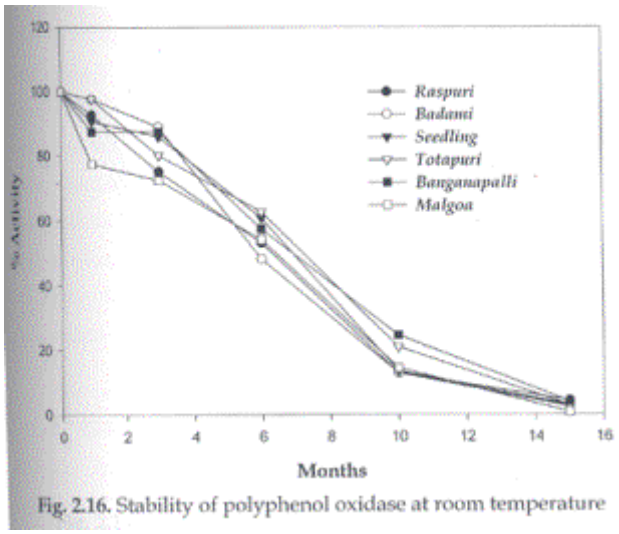


Fig. 2.16. Stability of polyphenol oxidase at room temperature

Fig.2.16 Stability of polyphenol oxidase at room temperature

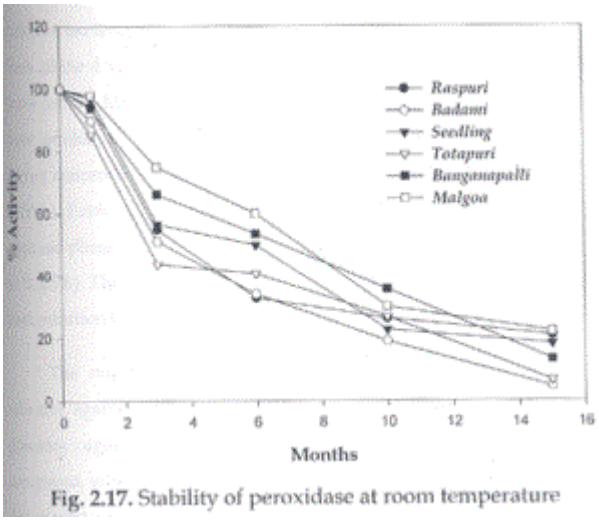


Fig. 2.17. Stability of peroxidase at room temperature

Fig.2.17 Stability of peroxidase at room temperature

2.4.3. SECTION C: ANTI-MICROBIAL ACTIVITY OF MANGO SAP

2.4.3.1. Bacterial Growth Inhibition

The whole sap was separated into aqueous and non-aqueous phases and their effect on bacterial growth was studied. The minimum inhibitory concentration (MIC) values of non-aqueous phase of saps of four mango varieties, viz., Seedling, Raspuri, Totapuri and Mallika against different bacteria are presented in Fig. 2.18. Non-aqueous phases of sap from all the 4 varieties of saps inhibited the growth of different bacteria, however, the MIC for different bacteria varied. B.cereus and P. aenlginosa were inhibited between 0.10 to 0.15% level whereas S.aureus required higher concentration of non-aqueous phase to inhibit its growth (0.175-0.250%). E.coli was the most resistant and higher concentration of non-aqueous phase was necessary for complete inhibition of its growth (0.25 to 0.325%). There was a significant difference in the minimum Inhibitory concentration levels for different bacteria.

The non-aqueous phases of sap of Seedling variety was most effective against all bacteria tested. Raspuri non-aqueous phase was less effective against Bacillus cereus, Eschzerechia coli and Pseudomonas aeruginosa, whereas Mallika non-aqueous phase was less effective against Escherechia coli and Staphylococcus aeruginosa. Bacilliis cereus was the most sensitive bacterium and Escherechia coli was found to be the most resistant of all bacteria tested in the study.

Results of the anti-bacterial activities of the aqueous phase of sap showed that even a high concentration (>7.5%) of the saps were not able to inhibit the growth of any of the bacteria. Therefore, further anti- microbial studies were not carried out with aqueous phase.

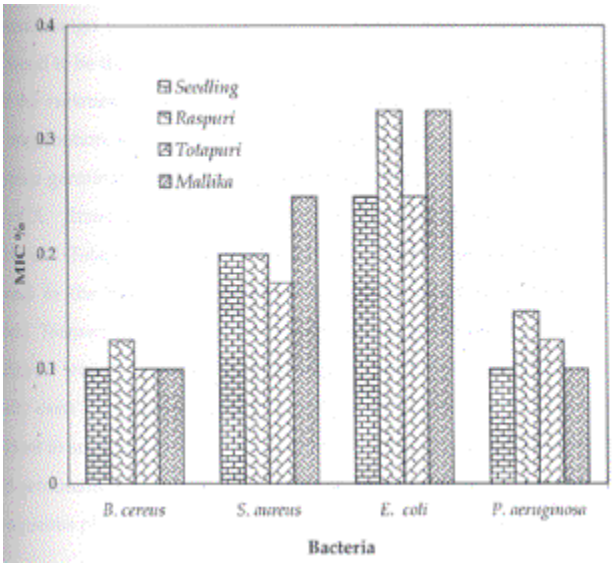


Fig 2.18 Minimum inhibitory concentration(MIC%) of non-aqueous phase of mango sap against bacteria (result of 4 replications)

14.3.2. Anti-fungal activity

The results of the spore germination assay of Aspergillus jlavus, A. 'ficus, Fusarium moniliforme and Penicillium spp. against sap of mango varieties are presented in Figure 2.19. Penicillium spp. found to be the most sensitive among all the fungi tested, and the sap all the varieties suppressed its spore germination up to three days at concentration. In case of Mallika and Totapuri sap, even after five days spore germination was negligible (<6%) at 2% concentration. In the of A, flavus, 2% concentration of non-aqueous phase of Seedling, Mallika and Totapuri sap inhibited spore germination up to three days, as in the case of A. parasiticus a similar effect was shown by Totapuri and Mallika sap at the same concentration. Fusarillm 'liforme was the most resistant among all the fungi tested. Although initially even 1 % concentration of Seedling, Mallika and Totapuri sap was sufficient to suppress spore germination for three days, after three days, rapid germination of spores was observed. However, Mallika and Raspuri non-aqueous phases were able to limit its germination at 2% level.

In general, non-aqueous phases from saps of different mango varieties inhibit the growth of different fungi at different levels. This difference may be due to the variation in terpenoid composition of saps. Non-aqueous phase of Mallika sap was found to be most effective for the amtrol of spore germination. Mallika sap is unique in its terpenoid composition as it had limonene as major terpenoid (61%).

The reports on anti-microbial actions of citrus oils indicated that molds were more resistant than bacteria [Subba Rao et a11963; Subba etal 1965]. Even in the present study similar results were obtained Karapinar [1985] reported that lemon and orange peel oil effectively induced growth of A. parasiticus at 1.6% concentration. Mango sap was observed to be affective at similar concentrations.

The results obtained clearly indicate that the non-aqueous phase of mango sap has potent anti-fungal and significant antibacterial activities substantiates the suggestion that mango sap has a defensive physiological role. It is known that the mango fruits are more susceptible 10 disease and pest infestation at the later stages of fruit development and we have found that the amount of sap and its constituent non-aqueous reduces as the mango matures. The decrease in sap may be responsible for the concurrent increase in fruit susceptibility to disease. It has been reported that a preformed anti-fungal activity present in the sap of unripe mango fruits is responsible for the latency of development of the black spot disease caused by the fungus

Alternaria alternata [Cojocaru et aI, 1986]. Similarly, the terpenoids present in the sap of mango may also exert a potential anti-microbial activity to protect the maturing fruit.

As sap causes sap-injury to the fruits and reduces its acceptability, die mangoes are de-sapped after harvesting. The sap thus obtained is a by-product. The non-aqueous phase of sap has raw mango aroma. As reported here, it also has good anti-microbial activity. Therefore, the mn-aqueous phase of the sap, which is a by-product from mango can be used as a preservative and flavouring agent.

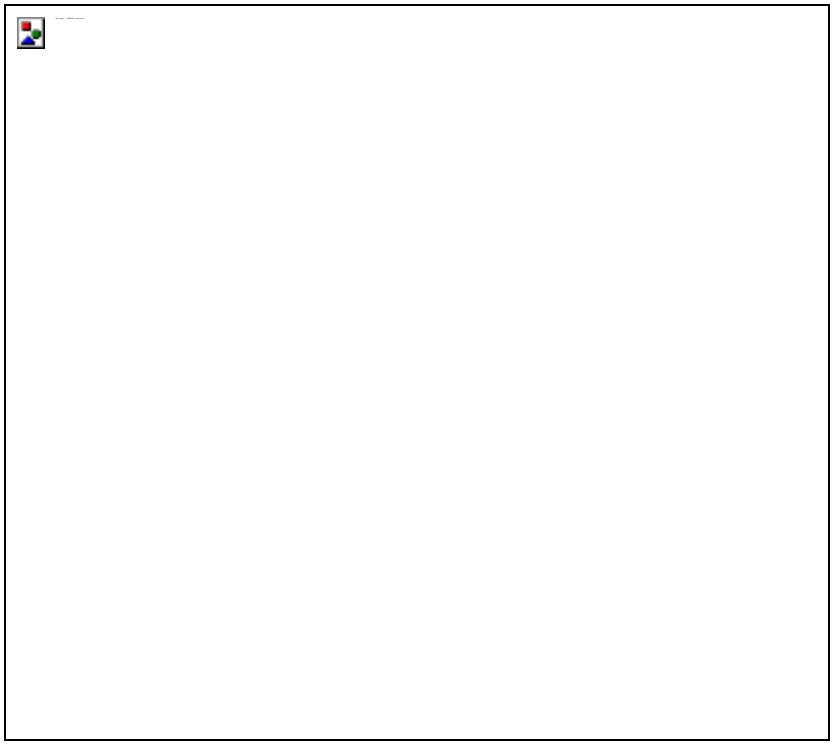


Fig 2.19. Effect of non-aqueous phases of saps of different mango varieties on fungal spore germination (mean ± SD, n=4)

# CHAPTER III

# MECHANISM OF SAP-INJURY AND ITS CONTROL

## 3.1. INTRODUCTION

In India, unripe mature green mangoes are harvested. It takes about 10-15 days to ripen at room temperature under normal conditions. During harvesting, a good amount of sap flow

occurs. When the fruit is severed from the stalk at the abscission zone, sap spurts out from the abscission point ("spurt sap") and subsequently continues to exude out ' from the stalk end ("ooze sap"). Sap-injury is the damage that is caused when mango sap comes in contact with the surface of the fruit during harvesting or post-harvest handling. It is characterized by the formation «brown or black coloured blotches on the peel. It can occur when sap wm the stalk end runs down the side of the fruit or spurt sap from other harvested fruit falls on the surface of the mango. It may also appear as irregular blotches on the peel where fruits have been in contact with one another.

Sap flow after harvest of mango fruit occurs from the abscission point of the fruit stalk. Joel [1981] has shown that the duct system of both the fruit and the stalk penetrate the transition zone, which lies between fruit base and its stalk. Several large fruit ducts continue into the stalk but the end a short distance beneath the abscission zone. In fruit sap is under pressure and when the fruit is picked, the sap is rapidly exuded from the ducts. A fruit harvested with a short stalk (15 mm) will not exude sap, however if this stalk is broken at the abscission point during subsequent post-harvest handling then flow of sap will occur. A fruit that ripens on the tree, will absciss with no sappy exudate. It has been observed that the volume of sap that flowed from the mango after harvest varies significantly in different varieties and stage of maturity at harvest [Chapter 2, p.51].

Brown et al [1986] carried out studies on the incidence of sap bum the Kensington Pride variety of mango and its control. It was found that amount of sap flow on the fruit had a direct correlation with the degree of sap-injury. Early morning harvest yielded more sap than a noon harvest, less mature fruit tended to have more sap flow than more mature fruit and spurt sap caused sap-injury whereas ooze sap did not, under the conditions used. Loveys et al [1992] reported that the sap of Australian Kensington variety was rich in terpenolene, whereas the Irwin variety of Florida was rich in car-3-ene. The Florida variety was found to be less susceptible to sap-injury than the Kensington variety. Sap can be separated into two phases, aqueous and non-aqueous. As described in previous Chapter, the non-aqueous phases of saps from seven Indian mango varieties were isolated and their terpenoid composition was determined. It was found that the terpenoid composition among the Indian mango varieties was different compared to the Australian and Florida varieties. The non-aqueous phase of saps from Indian mango varieties had mainly ocimene (cis- or trans-), p-myrcene or limonene depending on the variety. Terpinolene and car-3-ene were not detected in any of the Indian varieties studied.

Sap-injury not only reduces consumer acceptance of the fruit, but also lowers its shelf life, as the injured regions of the peel tend to be more susceptible to fungal or bacterial infections [Ulmert, Y., 1970; Johnson et aI, 1993; Kalra, et al., 1995]. The problem of sap-injury, thus, necessitates post-harvest procedures to reduce injury in addition to the mandatory post-harvest treatment of fruits.

Different approaches have been used to reduce sap-injury. These include de-sapping of fruit on the ground, on special de-sapping racks, trays, tables, conveyor machines or de-sapping in water, dish washing or clothes detergent, sodium bicarbonate, Chlorsan agral and swimming bleach [Lim and Bowman, 1995]. O'Hare and Prasad [1991] used calcium hydroxide dips to reduce sap-injury. Landrigan et al [1991] attempted to control sap-injury by dabbing the fruits in non-emulsified vegetable oil, waxes or powder. Most of these approaches result in also cause difficulties associated with sap on machinery and equipment. In addition, many of the commonly used dip chemicals are not compatible with the mandatory dimethoate fruit fly dip, as they are alkaline in nature. The insecticidal activity of dimethoate (an be nullified by alkaline chemicals or in alkaline solutions (Worthington, 1987]. Lizada, et al [1986] reported that subjecting freshly ested Carabao mangoes to hot water treatment for disease control (51-55°C) minimised the adverse effects of latex on peel quality. However, subjecting the fruits to hot water treatment after latex has dried as found to aggravate latex injury. Lim and Kuppelweiser [1993] reported the reduction of sap-injury when fruits were dipped in low entrations (0.1-0.2 ml/litre) of DC Tron solution for 30-60 seconds to removal of stalk. More recently, Lim and Bowman [1995] used different post-harvest chemical treatments to control sap-injury. They found that dipping fruits in DC Tron NR at 0.1 ml/litre for 30-60 seconds gave best control compared to similar rates of other highly refined paraffinic oils viz., Caltex Summer Spray Oil, Caltex Lovis Oil and Mobil Mulrex Summer Spray Oil and did not cause blotchiness or off-flavour. Tron and Bioshield oil, too, gave significant control of sap-injury at 0.2ml/litre. The primary drawback of this method is that it requires that fruits be harvested with stalk intact, a process that is time consuming and tedious.

In the present study, aqueous and non-aqueous phases of saps seven Indian mango varieties were separated from sap. As the terpenoid composition of sap was entirely different in Indian mango saps pared to mango varieties from Florida and Australia, the extent of browning caused by these liquids on the different varieties was determined. Also, some of the terpenoids that were identified in the saps of these Indian varieties were obtained commercially in the pure form lid the extent of browning caused by these was studied. In addition, organic solvents were used to determine whether organic compounds other than terpenoids could induce browning.

The role of mango peel components like polyphenol oxidase (PPO), peroxidase (POD) and polyphenol content in causing sap-injury were also investigated.

In India, no precaution is taken while harvesting mangoes to keep intact. Stalks of most of the harvested fruit are usually severed during harvest. Therefore, after harvest, mangoes come in contact with either of the same fruit or from other fruits or both. Hence, efforts were made during this investigation to control sap-injury in mangoes exposed to whole sap by using suitable wash solutions.

## 3.2. MATERIALS

### 12.1. Plant materials

The source of mango varieties used for obtaining sap is described Chapter2p. 36.

### 3.2.2 Chemicals

Limonene, p-myrcene, a-, p-pinene, were obtained from Aldrich mical company Limited, USA. Ocimene was procured from Fluka, Switzerland. Catechol, o-dianisidine, polyvinylpyrrolidone, gallic acid, Triton X-IOO, Tween-20 and Tween-80 were obtained from Sigma mical Company, St. Louis, USA. H202 was obtained from Hi Media (Pvt.) Ltd. Mumbai, India. All other chemicals were analytical grade reagents.

## 3.3. METHODS

### 3.3.1. Collection of mango sap and its separation into constituent phases

As described previously (chapter 2, p.37) mango fruits were harvested with pedicel (stalk) intact. Subsequently, the pedicels were detached from the fruit and the sap was allowed to flow into the glass tube for about one min. Mango sap was separated into an upper non-aqueous and a lower aqueous phase by centrifugation at 3000xg for 5 min at room temperature.

### 3.3.2. Application of sap and its constituent phases on mango surface

The non-aqueous phase (5 µI) and aqueous phase (0.1 ml) of mango sap, were applied, separately, on the surface of mango fruits and spread gently using a smooth glass rod over a circular area of around one cm diameter. Spurt sap, defined as sap that is obtained during the first 10 sec, was applied by breaking the stem and allowing a small amount of sap to spurt out

onto the side of the same fruit. Ooze sap was collected after breaking the stem, discarding the initial spurt that occurs during the first ten sec, and then allowing the remaining sap to ooze into a clean glass tube. This ooze sap (0.1 ml) was immediately applied on to the surface of the test mango fruits and spread using a glass rod. The test fruits were examined for sap-injury immediately after application and each day thereafter, for three to six days. Damage was scored by a panel of five persons using a system of relative grading on a scale ranging from zero (no injury) to five (maximum injury) depending upon the intensity of browning in the applied regions.

3.3.3. Application of non-aqueous phase of sap, pure terpenes and organic solvents on mango surface

The non-aqueous phase (5 μl) of sap from Badami, Totapuri, Raspuri, Malgoa Seedling, Mallika and Banganapalli varieties; and 5 μl each of pure oids viz" ocimene, limonene, β-myrcene, α-pinene and β-pinene; O.l mlof acetone, hexane, methanol, petroleum ether and diethyl were applied on the surface of experimental mango fruits as 'bed above. (As the solvents are more volatile, they tend to rate rapidly during application larger volumes were used compared to terpenes). The examination and scoring of sap-injury were carried out as mentioned above.

3.3.4. Heat treatment of mango peel

The mango peel was removed using a sharp knife and the underlying pulp removed by gently scraping with its blunt edge, without damaging the peel. The peel was divided into three equal portions by weight. One portion was heat treated to a temperature of 95°C, a second to 60°C and the third kept at room temperature (27°C) for 5 min. of the treated peels were then cut into pieces and 5 μl of the aqueous phase was applied on the surface of the peels as described above. The examination of damage and scoring was carried out as described earlier. The remaining part of the heat-treated peels was used determination of enzyme activities and polyphenol content.

3.3.5. Extraction of polyphenol oxidase (PPO) and peroxidase (POD) from peel

Mango peel was cut into small pieces and 0.5 g wet weight of cut were suspended in 15 ml of 0.05 M sodium phosphate buffer, 7.5) containing 0.15 g of insoluble PVP and 0.015 g of ascorbic acid, incubated for 5 min at 4°C. These were then ground with acid washed neutralized sand using a mortar and pestle at 4°C. To the resultant paste, 10 ml of the phosphate buffer was added and this was stirred for 1 h at 4°C. The slurry was centrifuged at 10,000 x g for 10 min 8t4°(, and the supernatant was used for PPO and POD assays.

3.3.6. Assay of PPO and POD activities in mango peel extracts

PPO activity was assayed using catechol as substrate and POD activity was assayed using 1% H202 and o-dianisidine as substrates as described earlier (Chapter 2, pp. 43-44). In both the cases, one unit of activity was defined as that amount of enzyme, which produces an increase of 1 absorbance per minute.

3.3.7. Extraction of total phenols in mango peel

Total phenol content was determined using the method of Swain and Hillis [1959]. The details of the estimation procedure are given in chapter 2, p. 40.

3.3.8. Protein assay

Protein content in different samples was estimated using the Bradford method [Bradford, 1976].

3.3.9. Statistical evaluation

The sap-injury data were subjected to statistical analysis using the method of Steel and Torrie [1980].

3.3.10. Control of sap-injury by different wash solutions

Badami, Banganapalli, and Raspuri varieties of mango were harvested with stalk. The stalk was cut at the abscission zone and, the sap collected. The collected whole sap (0.1 ml) was applied on the marked area and allowed to dry for 60 min unless otherwise mentioned. , treated mangoes were washed for minimum period of 5 min with occasional rolling of mangoes in the following solutions: a) water, b) 1.0% sodium chloride, c) 0.1% Tween-80, d) 0.1% Tween-20, e) 0:1% Triton X-I 00, f) 10% alcohol, g) 0.1 M ascorbic acid, h) 0.1 M sodium metabisulfite, i) 0.1 M sodium pyrophosphate (pH 8.0), j) 1% Teepol@, k) 1% Surr. Washed mangoes were air dried for 30 minutes and stored at room temperature for ripening. The extent of sap-injury was scored by a panel of 5 people scored in the grades of zero (no injury) or five (maximum injury).

3.3.11. Control of sap-injury using different concentrations of detergents

Mango varieties used, harvesting practice, application of sap, were followed as described earlier. The mangoes treated with sap were washed with 0.05,0.1,0.5,1.0 and 1.5% of Tween-20,

or Tween-80, or Triton X-I00 for 5 min with occasional rolling of the mangoes in the solution. Washed mangoes were air dried for 30 min and stored at room temperature for ripening. The extent of sap-injury was scored as mentioned earlier.

3.3.12. Control of sap-injury by washing the mangoes for different time periods

Mango varieties used, harvesting practice, application of sap, were, followed as described earlier. The mangoes treated with sap were washed .0.1% Tween-80 for different time periods- I, 3, 5, 7,10 min. Washed goes were air dried for 30 min and stored at ambient temperature for ripening. The extent of sap-injury was scored as mentioned earlier.

3.3.13. Effect of time between harvest and washing of mangoes on control of sap-injury

Mango varieties used, harvesting practice, application of sap, were wed as described earlier. The collected sap was applied on the pre-marked area. At different time intervals- I, 2, 3, 4, 6, 8 h -the treated -goes were washed in 0.1 % Tween-80 for 5 min. Washed mangoes air dried for 30 min and stored at ambient temperature for ripening extent of sap-injury was scored as described earlier.

3.4. RESULTS AND DISCUSSION

3.4.1. SECTION A: MECHANISM OF SAP-INJURY

3.4.1.1. Sap-injury by various components of sap

Sap from seven varieties of mango namely- Badami, Banganapalli, Malgovll, Raspuri, Seedling, Mallika and Totapuri- was separated into aqueous and non-aqueous phases. Whole sap as well as the aqueous and non-aqueous phases were applied on the surface of the mango. The aqueous phase did not cause injury, whereas whole sap caused injury. Non-aqueous phase of the sap caused maximum injury (Fig. 3.1). In the case of whole sap, the spurt sap caused more injury than ooze sap. When the content of non-aqueous phase in the ooze sap and spurt sap were determined, it was found that the volume of the non-aqueous phase in spurt sap was much higher (nearly 20 times) than that of ooze sap. Thus, the greater extent of injury caused by spurt sap may be attributed to its greater content of non-aqueous phase. Earlier, Brown et al [1986] have also reported that spurt sap caused more injury.

3.4.1.2. Sap-injury by non-aqueous phases of saps on different mango varieties

Non-aqueous phase of sap from different varieties, caused injury on the same as well as other varieties of mango (Fig. 3.2; Table 3.1). The extent of injury varied depending on the mango variety, however, injury on Totapuri variety was minimal compared to that on other varieties. As seen from Fig. 3.2 and Table 3.1, non-aqueous phases of Raspuri, Totapuri, Banganapalli, Mallika, Seedling and Malgoa caused less injury on Totapuri variety. It is commonly observed that Totapuri mangoes are less susceptible for sap-injury. However, Totapuri non-aqueous phase and sap caused injury on other varieties of mangoes.

3.4.1.3. Sap-injury on mangoes by different terpenoids and organic solvents

Earlier, we have showed that the content and composition of different in different Indian mango varieties The major terpenoids in Indian varieties were β-myrcene, cis-/trans-ocimene, limonene. Hence, it is possible that different terpenoids may cause injury to different extents. Therefore, of the terpenoids identified in saps of the Indian mango varieties viz β-myrcene , α-limonene, β-pinene, were applied on fruits of different Indian mango varieties. Limonene caused more injury, followed by ocimene. α-Pinene caused the least injury (Fig. 3.2; Table 3.1). Similar to the observation made with non- aqueous phase, injury caused by terpenoids on Totapltri mango was less.

It is speculated that the organic and liquid nature of the compound may be responsible for initiating the process of sap-injury in the goes. Therefore, attempts were made to see the effect of few organic solvents like petroleum ether, hexane, acetone, diethyl ether, methanol on browning of mangoes. Diethyl ether caused maximum injury compared with other solvents (Fig. 3.2; Table 3.2).

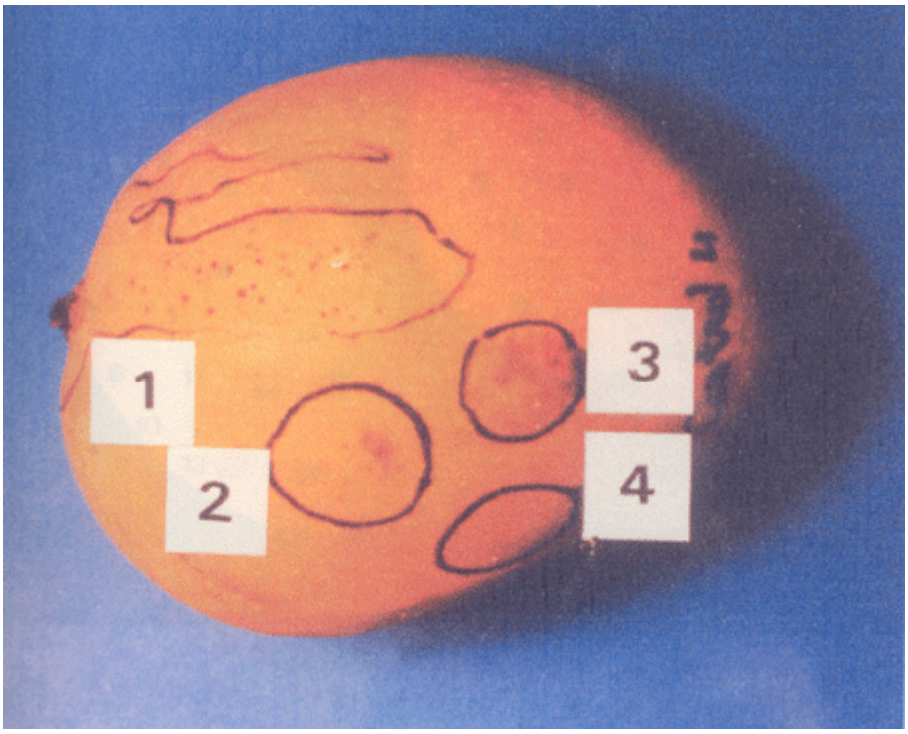



Fig 3.1: Sap-injury on Badami mango caused by mango sap and its components

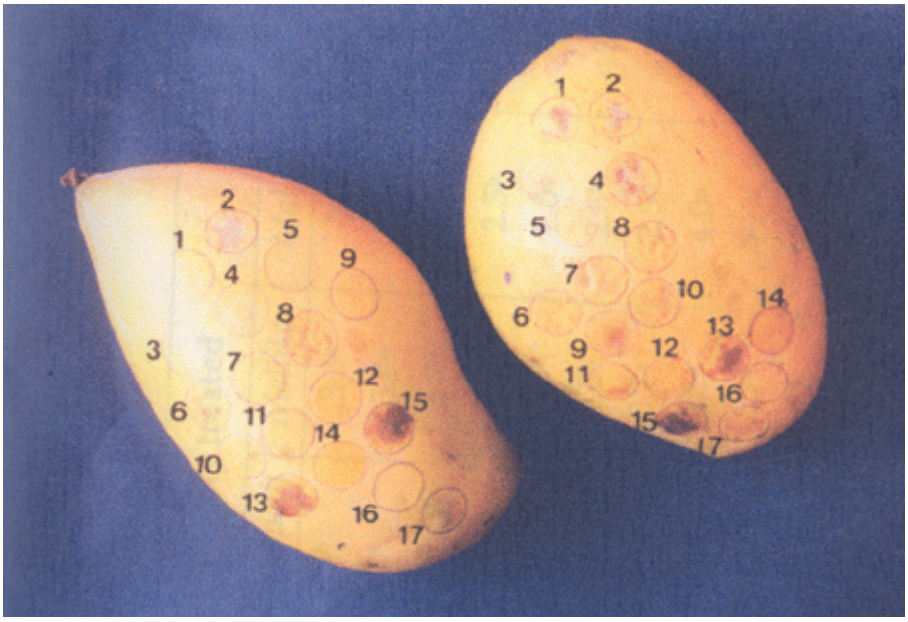



Fig 3.2 Injury caused by organic solvents, non-aqueous phases of sap (NAP) of different varieties of mango, and terpenoids A. Totapuri; B. Banganapalli mangoes

1. Petroleum ether; 2. Diethylether; 3. Acetone; 4. Hexane; 5. Methanol; 6. NAP Raspuri; 7. NAP Badami; 8. NAP Seedling; 9. NAP Totapuri;

10. NAP Banganapalli; 11. NAP Mallika;12. NAP Malgoa; 13. Ocimene;

14. Myrcene; 15. Limonene; 16. $\alpha$–pinene; 17.$\beta$-pinene

Table 3.1. Injury on different mango varieties caused by non-aqueous phase of saps from different mango varieties and different terpenoids.

| Component Applied | Variety treated | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Raspuri | Badami | Totapuri | Banganapalli | Mallika | Mmalgoa | Seedling | SEM |
| Raspuri NAP | 2.5b | 2.0c | 1.0d | 2.5b | 3.3a | 2.5b | 2.4b,c | 0.18 |
| Badami NAP | 2.3b | 2.2b | Nil | 3.2a | 3.0a | 2.3b | 3.3a | 0.15 |
| Totapuri NAP | 1.9c | 2.6b | 0.7d | 3.2a | 3.2a | 2.5b | 2.6b | 0.11 |
| Banganapalli NAP | 2.5b | 2.5b | 0.7c | 3.2a | 3.4a | 2.5b | 3.4a | 0.14 |
| Mallika NAP | 2.5b | 2.6b | 0.9d | 2.5b | 3.5a | 2.0c | 3.5a | 0.14 |
| Malgoa NAP | 1.9c | 1.9c | 0.6d | 2.5b | 2.5b | 2.6b | 3.1a | 0.12 |
| Seedling NAP | 2.5b | 2.5b | 1.3c | 2.5b | 3.7a | 2.5b | 2.5b | 0.15 |
| Ocimene | 4.0a | 4.1a | 3.2b | 4.1a | 4.1a | 4.0a | 4.1a | 0.15 |
| $\beta$-myrcene | 1.6b | 1.8b | 0.7c | 2.6a | 2.7a | 1.6b | ND | 0.11 |
| Limonene | 5.0a | 5.0a | 1.0b | 5.0a | 5.0a | 5.0a | 5.0a | 0.17 |
| $\alpha$ - pinene | 0.8a | 0.7a | Nil | 0.9a | 0.7a | 0.8a | 0.8a | 0.10 |
| $\beta$ - pinene | 1.8 c,b | 2.3b | 0.9d | 2.7a | 1.9c | 2.6a | 2.7a | 0.12 |

Values shown in the table are mean of five values. Different alphabets shown in the rows differ significantly at level $P<0.05$. ND = not determined; NAP = non-aqueous phase

Table 3.2 Injury caused by organic solvents on different varieties of mangoes

| Solvent | Variety treated | | | | | | |
|---|---|---|---|---|---|---|---|
| | Raspuri | Badami | Totapuri | Banganapalli | Mallika | Malgoa | SEM (±) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Petroleum ether | 1.5c | 2.7a | 0.8d | 2.6a | 1.4c | 2.1b | 0.118 |
| Diethyl ether | 2.0c | 2.2c | 2.5b | 2.6b | 3.1a | 3.1a | 0.122 |
| Acetone | Nil | Nil | 1.0b | 1.1b | 2.1a | Nil | 0.820 |
| Hexane | Nil | 1.7b | Nil | 2.1a | nil | 1.6b | 0.108 |
| Methanol | 1.0a | 0.9a | 0.8a | | 0.8a | Nil | 0.108 |

Values shown in the table are mean of five values.

Different alphabets shown in the rows differs significantly at level $P<0.05$

Mango furit has a waxy layer covering the fruit surface to prevent uncontrolled loss of water and hinder attach by pathogens and insects. As the organic solvents, terpenoids, non-aqueous phase of sap induce browning on mango, it is possible that the waxy layer on mango may be removed by these solvents, and this is followed by damage in the cellular structure, leading to darkening or browning of peel.

3.4.1.4 Role of ppo, POD and polyphenol contents of mango peel in sap-injury

The extent of sap-injury on different varieties of mango is different, and it is evident that Totapuri variety is least susceptible to sap-injury although its sap components caused injury on other varieties. It is likely that the enzyme activities and substrate content of the peels may also be different. Therefore, the PPO, POD and plyphenol contents in different mango peels were determined. The specific activities of PPO in seven varieties ranged from 0.7 to 3.2, while those of POD ranged from 0.6 to 4.0 .The total polyphenol content varied from 3.7 to 15 mgj g peel (Table 3.3). It is interesting to note that both Totapuri and Mallika peels had very low PPO activity. The Totapuri peel also had the lowest polyphenol content. This suggested that the low scoring values for sap-injury in Totapuri may be due to both the low PPO activity and low polyphenol contents. Although Mallika also had a very low pro activity, it had a higher value for sap-injury. But it contained maximum activity of POD and the highest content of polyphenols (Table 3.3). Peroxidases were also reported to catalyze the oxidation of certain phenolic compounds to their quinones, which may in turn get polymerized and form brown colored pigments [Danner et ai, 1973; Sawada et ai, 1975; V amos- Vigyazo, 1981; Sawahata et ai, 1982; Kruger and Reed, 1988; Mahanta et ai, 1993; Zapata et ai, 1998]. Earlier, Loveys et al [1992] reported that PPO may be involved in the sap-injury.

The involvement of PPO and POD in sap-injury was further supported by the results of another experiment described below. When the peel of Banganapalli mango was subjected to heat treatment at three different temperatures (27°C, 60°C and 95°C), it was observed that both PPO and POD activities were lost at 95°C (Table 3.3). However, peels treated at 60°C had both the enzyme activities, but the activities were less than those of peels maintained at room temperature. When the non-aqueous phase was applied on these three different peels, no browning was noticed on the 95°C treated peel. Exogeneous application of PPO and POD, in the form of sap aqueous phase, did not induce browning on the peels which were subjected to heat-treatment at 95°C, followed by non-aqueous phase application (Fig. 3.3; Table 3.4). This may be due to the inability of sap PPO to penetrate through the pores of the peel and come in contact with the peel polyphenols. The peels that were treated at 60°C showed significant browning and also had significant enzyme activity, but to a lesser of the control. Prabha and Patwadhan [1982] reported the activity in purified PPO of peel even at 60°C, but it was less than that at room temperature. Thus, there is a good correlation between the extent of browning, and PPO and POD activities in the peels.

Table. 3.3. Polyphenol oxidase, peroxidase activities and polyphenol content of mango peel

| Variety | Specific Activity (U/mg protein) | | Polyphenol Content (mg/g tissue) |
|---|---|---|---|
| | PPO | POD | |
| Badami | $3.2^{d}$ | $2.1^{c}$ | $11.0^{c}$ |
| Raspuri | $2.4^{c}$ | $1.5^{b}$ | $15.0^{d}$ |
| Banganapalli | $2.2^{c}$ | $1.9^{c}$ | $6.4^{b}$ |
| Malgoa | $1.3^{b}$ | $1.5^{b}$ | $15.0^{d}$ |
| Seedling | $1.3^{b}$ | $1.3^{b}$ | $14.4^{d}$ |
| Totapuri | $0.8^{a}$ | $0.6^{a}$ | $3.7^{a}$ |
| Mallika | $0.7^{a}$ | $4.0^{d}$ | $22.0^{e}$ |
| SEM (±) | 0.039 | 0.035 | 0.39 |

Values shown in the table are mean of three values.

Different alphabets shown in the column differ significantly at level $P < 0.05$

Table. 3.4. Effect of heat treatment on sap-injury and on activities of polyphenol oxidase and peroxidase in Banganapalli mango peel

| Temperature (°C) | Specific Activity (U / mg protein) | | Sap-injury (Browning) |
|---|---|---|---|
| | PPO | POD | |
| 27 | 2.2 | 1.9 | 5.0 |
| 60 | 1.5 | 1.4 | 3.0 |
| 95 | 0 | 0 | Nil |

Values shown in the table are mean of three values.

Loveys et al [1992] observed no injury in the boiled peel and, therefore, suggested that this was due to inactivation of the enzyme PPO present in the peel. They did not report any data on enzyme activity. We have carried out a systematic study to correlate the enzyme activities of the peel enzymes with the browning reaction by subjecting the peel to heat treatment at different temperatures (27°C, 60°C, 90°C) and correlating with the activities of these enzymes after such treatment. Our studies by assaying the PPO, POD activities and determining the polyphenol content in the peel have clearly indicated that the extent of sap-injury depends not only on the PPO activity but also on the POD activity and the content of polyphenols in the peel.

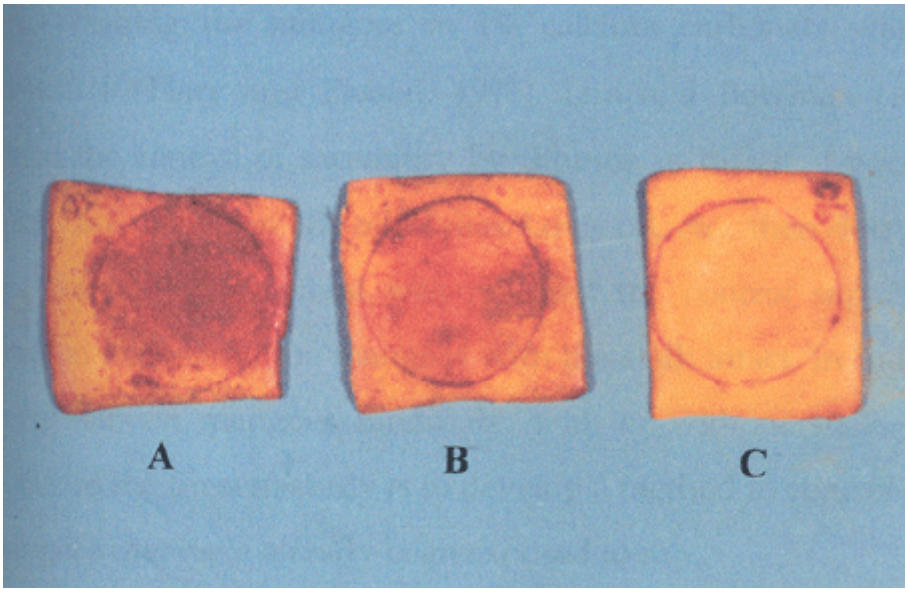



Fig 3.3: Sap-injury caused by non-aqueous phase of mango sap on Banganapalli mango peels treated at different temperatures;

3.4.2. SECTION B: CONTROL OF SAP-INJURY

Sap-injury is one of the major problems in post-harvest handling of mangoes (Fig. 3.4). Several workers used different methods to control sap-injury. Brown et al [1986] used dry powders, alum, or sodium bicarbonate, to stop flow of sap from mangoes harvested with stalk. However, their attempt was not successful. Dipping the mangoes in 1% solution of alum or sodium bicarbonate was not useful in control of sap- injury. Washing the mangoes in 1% calcium carbonate was also not successful [O'Hare and Prasad, 1991]. Lim and Bowman [1995] have reported the control of sap-injury by dipping in different paraffinic oils like Amphol, DC Tron NR oil, Caltex Lovis oil, etc., then de-stalking the fruits. Though this method was reported to control sap-injury, it is difficult to harvest all the mangoes with the stalk intact. In India, mango growers harvest mangoes randomly, with or without stalk. The main objective of the present study is to develop a method to control sap-injury

in mangoes that have already been exposed to sap.

In the previous section, it was stated that terpenoid components of non-aqueous phase of sap initiate the injury, and peel components like ppo, POD and polyphenols cause the darkening or browning reaction. Therefore, wash solutions were formulated based on the nature of the components responsible for sap-injury. As terpenoids are organic in nature, neutral detergents like Tween-20, Tween-80, Triton X-100 and commercial laundry detergents like Teepol@ and Surf, were used as wash solutions.

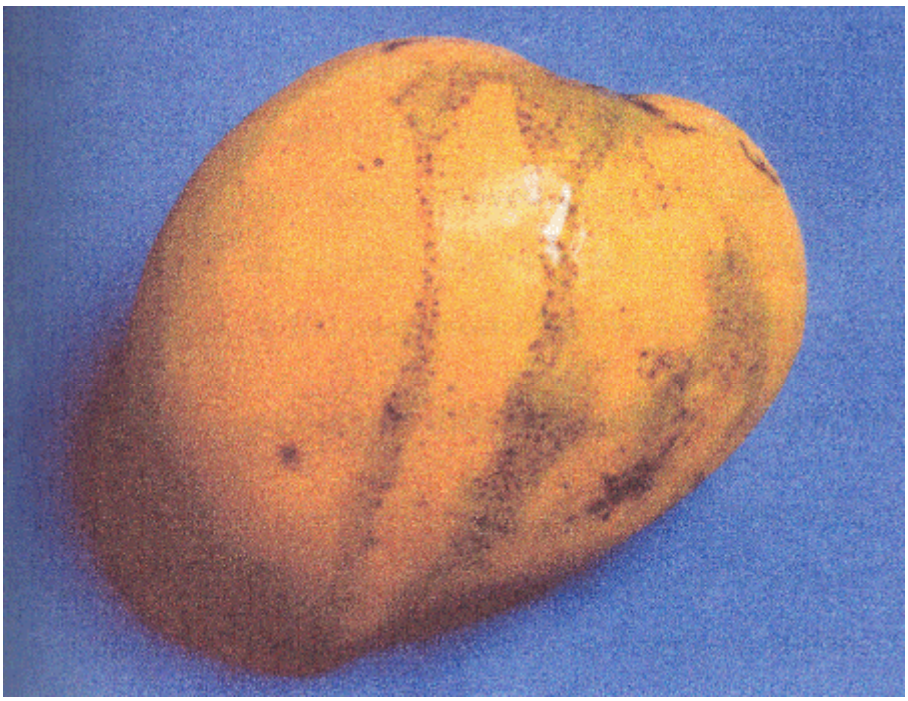


Fig.3.4: Sap-injury on Seedling mango

As enzymes like PPO, POD are also involved in sap-injury, their inhibitors like sodium metabisulphite, sodium chloride, ascorbic acid were also used. Sodium pyrophosphate (pH 8.0) was used, as the enzymes are less active at this pH.

Three varieties of mango viz., Raspuri, Badami, Banganapalli, were used for studies on the control of sap-injury. As can be seen from results presented in Table 3.5, little or no control of sap-injury was observed by washing in water, 1.0% sodium chloride, 0.1 M ascorbic acid, 0.1 M sodium metabisulphite and 0.1 M sodium pyrophosphate (pH 8). All the detergents, on the other hand, showed great promise for use in wash solutions to control sap-injury. Non-ionic detergents like Tween-20 and Tween-80 and Triton X-100 were more effective in controlling sap-injury.

3.4.2.1. Control of sap-injury by different concentrations of detergents

Since the non-ionic detergents Tween-20, Tween-80 and Triton X-lOa yielded best results in controlling sap-injury, attempts were made to determine the minimum concentration of detergent required to control sap-injury. As can be seen from Table 3.6, significant control of sap-injury was observed even at the detergent concentrations as low as 0.05%. However, at concentrations higher than this, there is almost complete control of sap-injury. For practical applications, one could employ 0.1% Tween-20, or Tween-80, or Triton X-100 for controlling sap-injury.

Table. 3.5. Control of sap-injury by washing with different formulations Wash Solution

| Wash solution | Variety | | |
|---|---|---|---|
| | Raspuri | Badami | Banganapalli |
| Control (no wash) | 5.00f | 5.00f | 5.00f |
| Water | 4.00d,e | 3.17c | 3.42e |
| 1.0% Sodium chloride | 3.80d,e | 3.90d,e | 3.9f |
| 0.1% Tween 80 | 0.29a | 0.31a | 0.40a |
| 0.1% Tween 20 | 0.35a | 0.38a | 0.42a |
| 0.1% TritonX-100 | 0.42a | 0.52a | 0.52b |
| 10% Alcohol | 5.00f | 5.00f | 5.00g |
| 100 mM Ascorbic acid | 3.90de | 3.67d,e | 2.12d |
| 100 mM sodium | 3.81d,e | 3.58d,e | 3.42e |

| | | | |
|---|---|---|---|
| metabisulphite | | | |
| 100 mM Sodium pyrophosphate (pH 8.0) | 4.50f | 3.75d,e | 3.33e |
| 1% Teepol | 1.80b | 1.40b | 1.62c |
| 1% Surf | 2.31c | 1.59b | 1.91c,d |
| SEM(±) | 0.064 | 0.102 | 0.158 |

Values shown in the table are mean of twelve values. Different alphabets shown in the columns differ significantly at level P<0.05.

Table 3.6. Control of sap-injury by different concentrations of detergents

| Concentration of Detergent | Variety | | |
|---|---|---|---|
| | Raspuri | Badani | Banganapalli |
| 0.05% Tween-20 | 0.56c | 0.58b | 0.56c |
| 0.1% Tween-20 | 0.35a,b | 0.38a | 0.40a,b |
| 0.5% Tween-20 | 0.29a,b | 0.36a | 0.38a,b |
| 1.0% Tween-20 | 0.25a,b | 0.35a | 0.38a,b |
| 1.5% Tween-20 | 0.21a | 0.27a | 0.33a,b |
| 0.05% Tween-80 | 0.58c | 0.62c | 0.52c |
| 0.1% Tween-80 | 0.38a,b | 0.46a,b | 0.38a,b |
| 0.5% Tween-80 | 0.33a,b | 0.42a,b | 0.38a,b |
| 1.0% Tween-80 | 0.27a,b | 0.33a | 0.37a,b |
| 1.5% Tween-80 | 0.25a,b | 0.31a | 0.25a |
| 0.05% Triton X – 100 | 0.90d | 0.95c | 0.62c |
| 0.1% Triton X – 100 | 0.72C,D | 0.75c | 0.5c |
| 0.05% Triton X – 100 | 0.68C | 0.70c | 0.52c |
| 1% Triton X – 100 | 0.65C | 0.66b,c | 0.52c |
| 1.5% Triton X – 100 | 0.65C | 0.62b | 0.48c |
| SEM (±) | 0.061 | 0.069 | 0.060 |

Values shown in the table are mean of twelve values. Different alphabets shown in the column differ significantly at level P<0.05.

3.4.2.2 Effect of washing time on the control of sap-injury

To determine the minimum washing time required for controlling of sap-injury, the sap treated mangoes were washed in 0.1% Tween-20 for different time intervals. As can be seen from Table 3.7, washing of the mangoes for even short periods of time (1 min) in 0.1% Tween-20, was effective in significantly controlling sap-injury. Washing the mangoes for 5 min or more could control sap-injury almost completely.

Table. 3.7. Effect of washing time on the control of sap-injury Time Variety

| Time (min) | Variety | | |
|---|---|---|---|
| | Raspuri | Badani | Banganapalli |
| 1 | 0.60b | 0.54b | 0.63c |
| 3 | 0.58b | 0.48b | 0.54b |
| 5 | 0.33a | 0.35a | 0.44a |
| 6 | 0.29a | 0.33a | 0.44a |
| 10 | 0.25a | 0.29a | 0.40a |
| SEM (±) | 0.067 | 0.072 | 0.063 |

Values shown in the table are mean of twelve values. Different alphabets shown il1 the mns differ significantly at level $P<0.05$.

3.4.2.3. Effect of time interval between the harvesting and the washing of mangoes to control sap-injury.

Normally, it may not be possible to wash the mangoes immediately after they have been harvested. Therefore, studies were carried out to find out the maximum permissible interval between the harvesting and the washing of mangoes. Sap was applied on the mangoes and left at room temperature for 1 to 8 h. The fruits were then washed after different time intervals. As shown in the Table 3.8, the extent of sap-injury increased with increase in time elapsed between washing of the mangoes and application of the sap. Almost complete control of sap-injury was observed in mangoes washed within 3 h of sap application.

Table. 3.8. Effect of time interval between the harvest and the washing of mangoes to control sap-injury.

| Time (h) | Variety | | |
|---|---|---|---|
| | Raspuri | Badami | Banganapalli |
| 1 | 0.25a | 0.33a | 0.31a |
| 2 | 0.21a | 0.33a | 0.31a |
| 3 | 0.33a | 0.43a | 0.42a |
| 4 | 0.73b | 0.83b | 0.83b |
| 6 | 1.20c | 1.30c | 1.30c |
| 8 | 2.1d | 2.2d | 2.3d |
| SEM (±) | 0.054 | 0.070 | 0.072 |

Values shown in the table are mean of twelve values. Different alphabets shown in the

Columns differ significantly at level $P<0.05$.

Earlier several methods were described to control sap-injury. All these methods have certain disadvantages. Methods very often used by harvesters involve de-stalking of the mango, inverting and dipping the mango in soil. This method is very un-hygenic and causes stem-end rot disease due to the presence of high innoculum of micro-organisms in the soil. Other methods involving plugging of the stalk with alum, sodium bicarbonate, were also not successful. Washing of mangoes with water (cold and hot) is also not effective [Lizada et aI, 1986]. The only method which was reported to be effective in control of sap-injury was pre- coating of the fruits with certain paraffinic oil is not commercially used. We have controlled the occurrence of sap-injury in mangoes that have already come in contact with sap. Therefore, this method may be easily adapted in fields where the harvesting of mangoes is done, without taking much precaution to ensure that the stalk is retained intact. On the other hand, methods to control sap-injury so far reported require harvesting the mango fruit with the stalk intact, then either manually de-stalking it, or giving a protective coating by dipping in paraffinic oil solutions [Brown et al., 1986; Lim and Bowman, 1985]. Also important is the fact that sap-injury can be controlled even after the fruit has been exposed to sap for up to 3 hours, thus allowing sufficient time to the harvester to employ this method of controlling sap-injury by washing in the neutral detergent solution. Since non-ionic, neutral detergents are used in the wash solutions, further washing/ removal of detergent may not be required. This method would not interfere with other post-harvest treatment methods applied to mangoes especially fungicide treatments, which are mandatory prior to the export of mangoes. Furthermore, washing of mangoes with a detergent may also

help to remove various pesticides/insecticides sprayed on to the mango as well as other extraneous materials adhering to the fruit, and thereby result in a cleaner product.

# CHAPTER IV

# PURIFICATION AND CHARACTERIZATION OF POLYPHENOL OXIDASE FROM MANGO SAP AQUEOUS PHASE

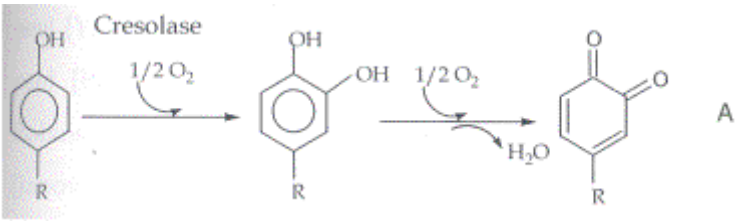
## 4.1. INTRODUCTION

### 4.1.1.Polyphenol Oxidases

Polyphenol oxidases are copper containing enzymes and are widely distributed in living organisms viz., bacteria, fungi, plants and mammals [Mayer and Harel, 1979; Vamos-Vigyazo, 1981; Martinez and Whitaker, 1995; Sherman et aI, 1995]. They oxidize a wide range of substrates into reactive quinones, which further polymerize to brown pigments. The term polyphenol oxidase refers to three enzymes-monophenol monooxygenase, tyrosinase and cresolase (EC 1.14.18.1), o-diphenol oxidase, catechol oxidase, diphenol oxygen oxidoreductase (EC 1.10.3.2) and laccase or p-diphenol oxygen oxidoreductase (EC 1.10.3.1) [Mayer, 1987].

### 4.1.2. Reactions catalyzed by different types of polyphenol oxidases

Tyrosinases (EC 1.14.18.1), which were first discovered by Schoenbein in 1856 in mushrooms, catalyze the oxidation of monophenols to diphenols and subsequently oxidize these to quinones (Fig. 4.1.A). Catechol oxidases (EC 1.10.3.2) catalyze the oxidation of 0- diphenols to quinones (Fig. 4.1.B), whereas laccases (EC 1.10.3.1), which were first discovered in the sap of the Japanese lac tree Rhus vernicifera catalyze the oxidation of 0- and p-dihydroxyphenols (Fig. 4.1.C).



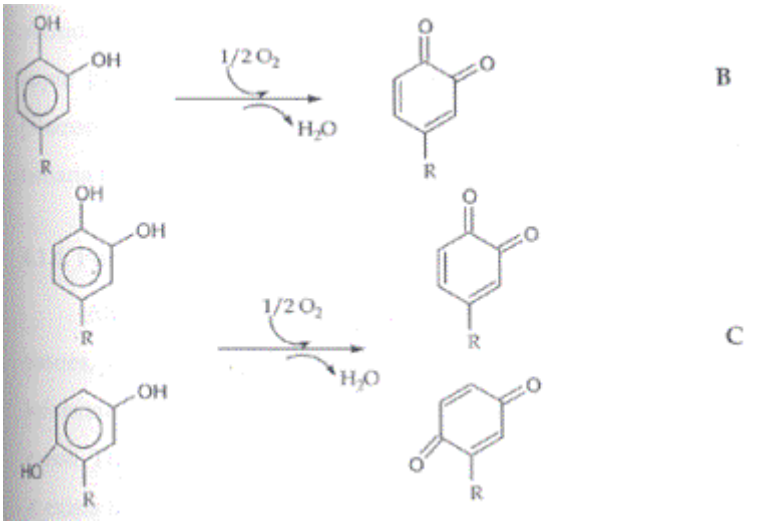



Fig. 4.1. Reactions catalyzed by A. Tyrosinases (E.C.1.14.18.1)

B. Catechol oxidases (EC 1.10.3.2)

C. Laccases (EC 1.10.3.1).

[Source: Whitaker and Lee, 1995]

4.1.3. Distribution and localization of polyphenol oxidase in plants

Polyphenol oxidases are believed to be ubiquitous in the plant kingdom. They were isolated from a number of plants like tomato [Newman et aI, 1993], potato [Steffens et aI, 1994], banana [Griffiths, 1959], apple [Amiot et ai, 1992], mango [Prabha and Patwardhan, 1982] and different parts of plants such as banana pulp [Yang et ai, 2000], banana peel [Yang et ai, 2001], mango pulp [Selvaraj and Kumar, 1989], mango peel [Prabha and Patwardhan, 1982], mango latex [Joel et ai, 1978, Robinson et ai, 1993], green leaves [Tobert, 1973] apple peel and cortex (Janovitz - Klapp et al., 1989]. PPOs are located in a variety of cell fraction. Plant PPOs were reported to be localized in membranes of plastids. PPO activity was also obtained from mitochondria in olive and apple [Shomer et al., 1979; Harel et al 1965], microbodies in avocado '; [Sharon and Kahn, 1979], and partly associated with the cell wall in banana (Jayaraman et aI, 1987].

4.1.4. Purification of polyphenol oxidase

Although PPOs have been partially purified from many plant tissues, reports on purification to homogeneity are few. Most available ..information comes from fungi, particularly mushrooms and

Nellrospora crassa. The reason for this being that PPO is relatively difficult to purify as it exists in different isoform in number of species, and during purification inter conversions between these isoforms may occur [Mayar and Harel 1979].

For purification of the crude PPO preparation, several methods were reported. Most often precipitation with ammonium sulphate, ion - exchange chromatography on DEAE-cellulose or DEAE-Sphadex, or gel filtration, or affinity chromatography, or combination of some of these methods were used [Lee-Kim, 1995; Yang, 2001; Shimoda et aI, 1975; Das et aI, 1997]. Preparative isoelectric focusing was also used in few cases [Dubernet and Riberau-Gayon, 1974].

4.1.5. Properties of polyphenol oxidase

Inactive or latent PPO forms have been frequently reported in plants and require activation. In fruits latency has been reported in mango [Robinson et aI, 1993], avocado [Kahn, 1977], and grape [Marques et aI, 1994]. Very different pH optima have been reported for fruit PPO

activity ranging from acidic pH (3.5) in grape [Valero et aI, 1988] to tral pH in kiwi [Park and Luh, 1985], banana [Yang et aI, 2001]. In lime cases, two different pH optima have been observed in the same ies for example pH 4 and pH 6.5-7.0 in egg plant, [Fujita and Tono, Sherma and Ali, 1980] and in apple [Harel, et aI, 1965]. The molecular weights of PPOs in different species range from 20 kDa to 200 a [Mayer, 1987; Sherman et aI, 1991; Hunt et aI, 1993; Yang et aI, 2001]. The molecular weight of PPO from grape [Rathjen and Robinson 1992], apple [Murata et aI, 1993; Marques et aI, 1994] and broad bean was found to be around 60 kDa [Ganesa et aI, 1992]. PPO exists in isoforms and their molecular weights were also found to vary. For exampl,e in apple, the molecular weight of PPO isoforms varied from 24 to 134 kDa [Hare I and Mayer, 1968], and in potato, from 36 kDa to >540 kDa (Matheis and Belitz, 1975].

The primary structure of PPOs from plants and microorganisms and animals were determined by amino acid sequencing and cDNA sequencing techniques [Lerch, 1978; Wong, 1995]. Between tomato and potato pros, there was nearly 91% sequence homology, but between tomato and fava bean only 40% homology was reported [Wong, 1995]. However, in all the cases the sequence around the active site was found to be highly conserved. The quarternary structure of tyrosinase from Agaricus bispora was found to contain two types of polypeptides chains, Heavy (43 kDa) and Light (13 kDa) chains [Strothkamp, 1976].

4.1.6 Substrates and inhibitors of polyphenol oxidases

a) Substrate Specificity

Polyphenol oxidases have a wide number of substrates. They react with monophenols, 0- and p-diphenols, amines. The relative specificities of PPOs on substrates vary from source to source. Tyrosinase oxidizes monophenols and o-diphenols. Aromatic amines and o-amino phenols, structural analogues of mono-phenols and o-diphenols, respectively were also oxidized to o-quinoneimines by tyrosinase [Toussait and Lerch (1987]. Catechol oxidase catalyzes the oxidation of a number of compounds such as catechin, epicatechin, catechol, chlorogenic acid, caffeic acid, 4-methyl catechol, gallic acid [Vamos- Vigyazao, 1981]. On the other hand, laccase has wide substrate specificity. Laccase oxidizes most of the o-diphenols that are also oxidized by catechol oxidases. In addition, laccases are known to catalyse the oxidation of syringaldazine, p-phenylene diamine, p-hydroxy phenol [Harkin and Obst, 1973; Leonowicz and Grywnowicz, 1981; Walker,1995].

b) Inhibitors of polyphenol oxidases

Browning reactions caused by PPO affect the sensory properties of products with respect to color, flavor, nutritional quality of fruits and vegetables. In order to overcome the problem, various approaches like heat inactivation, or altering of pH from optimal value, or eliminating one of the substrates (oxygen or polyphenol), or addition of various compounds that inhibit PPO activity were used. Compounds that are known to inhibit PPO activity include ascorbic acid, sodium metabisufite, cysteine, glutathione, mercaptoethanol, cynide, azide, fluoride, tropolone,

p-coumaric acid, benzoic acid, PVP, CT A B, 4- hexy I resorcinol, sodium chloride, citric acid, sodium diethyl-dithiocarbamate [Vamos-Vigyazo, 1981; Martinez and Whitaker, 1995; Yang et al, 2001].

c) Differentiation between catechol oxidase and laccase

Differentiation between different pros can be achieved by comparision of substrate specificity and use of selective inhibitors as summarized in Table 4.1. As can be seen from the Table, syringaldazine: a good substrate for differentiation of laccase from catechol oxidase. Laccases are selectively inhibited by CTAB.

Table 4.1. Differential Test for Catecholase and Laccases

| Test | Catecholase (o-DPO) | |
|---|---|---|

| Substrate specificity: | | Laccase (ρ-DPO) |
|---|---|---|
| o-Dihydroxyphenols | Oxidized | - |
| p- Dihydroxyphenols | Nil or slow | Oxidized |
| p-Cresol | Oxidized (orange-red) | - |
| Guiacol | - | Oxidized |
| 1-Naphthol | - | Oxidized |
| p-Phenylene-diamine | - | Oxidized |
| Syringaldazine | - | Oxidized |
| Inhibitor specificity: | | |
| Cinnamic, p-coumaric and ferulic acids | Inhibition | Nil |
| PVP | Inhibition | Nil |
| SHAM | Inhibition | Nil |
| 4-Hexyl-resorcinol | Inhibition | Nil |
| CTAB (and other QACs) | Nil | Inhibition |

[Source: Walker, 1995]

4.l.7. Action of polyphenol oxidase in plants

The action of PPO (Fig. 4.2) leads to major economic losses in some fresh fruits such as apples, bananas, grapes, peaches and strawberries and vegetables like Irish potatoes, lettuce and some other leafy vegetables 100uga and Whitaker, 1994]. In some tropical fresh fruits up to 50% can be lost due to enzyme-caused browning. Browning also leads to off-flavours and losses in nutritional quality. PPO activity in plants is II desirable in processing of prunes, black raisins, black figs, zapote, tea, coffee and cocoa [Lee, 1991]. Various physiological roles have been proposed for PPO. Because of its location in plastids, it was postulated to play a role in photosynthetic reactions of chloroplasts [Vaughn et ai, 1988]. But it is more widely accepted that PPO has a possible role in protection of plants against stress conditions like attack by insects and micro-organisms, as well as adverse climatic conditions [Bonner, 1957;Farkas and Kiraly, 1952; Rubin and Artsikovskaya, 1960; Joel, 1981; Mayer, 1987; Steffens et ai, 1994]. The appearance of browning is thus one of the first signs of a response to wounding or fungal attack and the quinones formed appear to possess more effective anti-microbial properties than the original compounds that participate in resistance mechanisms [Bell, 1981]. It has been suggested that the quinones formed upon the action of the enzyme undergo secondary polymerization reactions yielding dark, insoluble, polymers. The tissues impregnated with these polymers act as barriers in

the way of spreading infection [Rubin and Artsikovskaya, 1960]. Hydroxylation reaction of PPO plays a part in phenol biosynthesis [Walker, 1975]. PPO also participates in auxin biosynthesis, thus it may playa role 'in plant growth regulation together with auxin degrading enzyme POD [Gordon and Paleg, 1961]. Laccases along with lignin peroxidases and manganese peroxidases are the main oxidative enzymes that have been implicated in lignin modification [Gold and Alic, 1993].

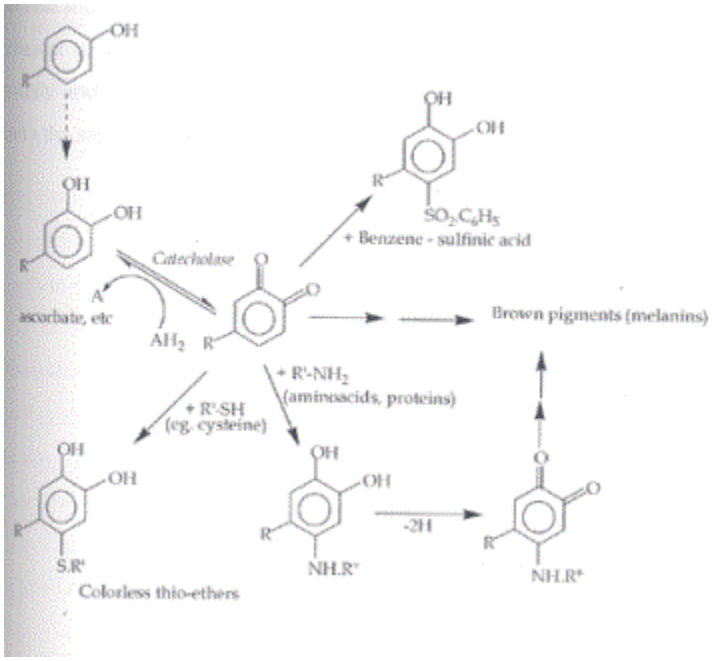



Fig. 4.2. Reactions catalysed by polyphenol oxidase

[Source: Walker, 1995J

Very little information is available on the purification of PPO from mango. Earlier, Prabha and Patwardhan [1982] have purified PPO from mango peel from an Indian mango variety (Badami) and reported some of its properties. Selvaraj and Kumar [1989] reported the content of PPO mango pulp of different Indian varieties. Joel, et al [1978] demonstrated the presence of PPO (laccase) in mango sap and Robinson et al [1993] studied some of the biochemical properties of partially purified PPO (laccase) from Kensington mango. As we discussed in chapter 2, mango sap showed a very good PPO activity. Among the several Indian mango varieties studied, RaSptlri sap showed maximum amount of protein and PPO activity. Moreover, this variety is grown widely and therefore, efforts were made to purify and characterize PPO from the sap of this mango variety.

## 4.2. MATERIALS

### 4.2.1. Plant material

Mango sap of the Raspuri variety which was obtained from CFTRI campus; was used in these studies.

### 4.2.2. Chemicals

DEAE-Sephacel and Sephadex G-200 were procured from Pharmacia Fine Chemicals, Uppsala, Sweden; carbonic anhydrase, ovalbumin, bovine plasma albumin, rabbit muscle phosphorylase B, E coli p-galactosidase, rabbit muscle myosin, bovine serum albumin '(monomer and dimer), ovalbumin, trypsin, cytochrome C, blue dextran, catechol, 0- dianisidine, diaminobenzidine, p-phenylenediamine, guaiacol, p-quinol, syringaldazine, SDS, APS, TEMED, Coomassie brilliant blue R-250, Trizma base, glycine, -mercaptoethanol, were obtained from Sigma Chemical Company, St. Louis, USA. H202 was procured from Qualigens Fine Chemicals, Mumbai, India. CT AB was procured from Fluka, Switzerland. All other chemicals used were of analytical grade.

## 4.3. METHODS

### 4.3.1. Polyphenol oxidase assay

PPO activity was assayed using catechol as substrate, as described earlier (chapter 2, p. 43).

### 4.3.2. Peroxidase assay

Activity of POD was measured using H202 and o-dianisidine, as described earlier (chapter 2, p. 44).

### 4.3.3. Protein estimation

Protein was estimated by the dye binding method described by Bradford [1976].

### 4.3.4. Electrophoresis

a) Polyacrylamide gel electrophoresis (Native-PAGE)

Native PAGE followed by staining for protein, polyphenol oxidase and peroxidase was performed as described earlier (Chapter 2, p. 47).

b) SDS-Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed on 10% gel in the presence of 0.1% sodium dodecyl sulphate according to the method of Laemlli [1970]. A mixture of molecular weight markers containing carbonic anhydrase (29,000), ovalbumin (45,000), bovine plasma albumin (66,000), rabbit muscle phosphorylase B (97,400), E coli p-galactosidase (116,000) and rabbit muscle myosin (205,000), as well as the enzyme sample prepared in 1% SDS and 5% mercaptoethanol, were boiled for 5 min. The pH of the running buffer was 8.5 and a constant voltage of 50 V was employed. Following the run, the proteins were stained with

0.25% Coomassie brilliant blue for 5 h. Molecular weight was determined using the calibration of curve shown in Fig. 4.3.

4.3.5. Ion-exchange chromatography on DEAE-Sephacel

Mango sap proteins were diluted with equal volume of 50 mM Tris-HCI buffer, pH 8.0 and loaded onto DEAE-Sephacel column (2.5 x 15 cm), which was pre-equilibrated with the same buffer.

The bound protein was eluted using 0.025 M, 0.050 M 0.075 M and 0.10 M sodium chloride in 0.05 M Tris-HCI buffer, pH 8.0, in a step-wise manner: The flow rate was maintained at 10 ml/h and 2.0 ml fractions were collected, monitored for protein by determining the absorbance at 280 nm and assayed for PPO and POD. The active fractions viz., POD (peak I), PPO (peak II), PPO and POD (peak III), were pooled and used as enzymes for further studies.

4.3.6. Determination of Molecular weight of the purified enzymes using Sephadex G-200 gel filtration chromatography

In order to estimate the molecular weights of the pure enzymes obtained on DEAE-SephaceI ion-exchange chromatography using Sephadex G-200 gel filtration chromatography, a calibration run was made. Sephadex G-200 was calibrated with bovine serum albumin (monomer and dimer), ovalbumin, trypsin and lysozyme. The void volume (Vo) was determined using blue dextran. The log molecular weight of each standard protein was plotted against its Ve/Vo (Ve-elution volume of particular standard) as shown in Fig. 4.4.

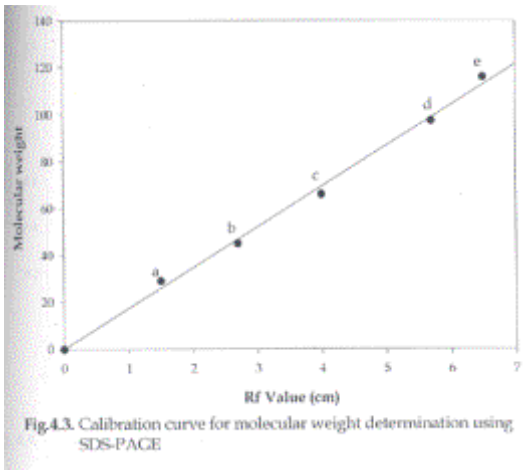


Fig.4.3. Calibration curve for molecular weight determination using SDS-PAGE

Fig.4.3 Calibration curve for molecular weight determinination using SDS-PAGE

a. Carbonic anhydrase (29,000) b. Ovalbumin (45,000)

c. Plasma albumin (66,000) d. Rabbit muscle phosphorylase B (97,400);

e. E.coli β-galctosidase (116,000)

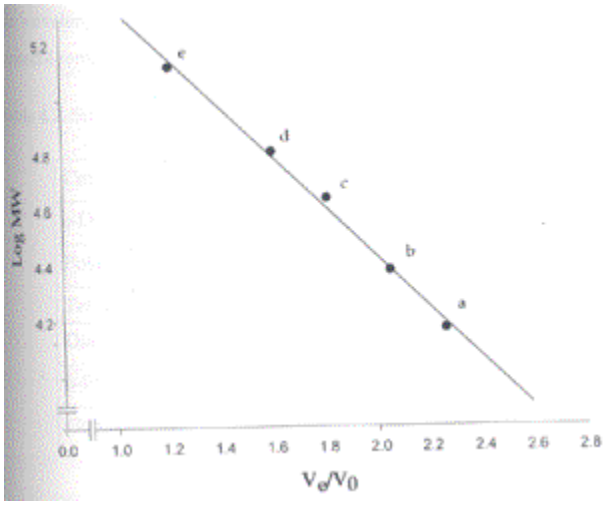


Fig. 4.4. Calibration curve for molecular weight determination using Sephadex G-200 gel filtration chromatography

a. Lysozyme (14,700); b. Trypsin (24,000); c. Ovalbumin (44,000);

d. BSA monomer (66,000); e. BSA dimmer (132,000).

4.3.7. Characterization of the peaks obtained on ion-exchange chromatography

4.3.7.1. Substrate specificity for peak I (peroxidase)

Six different compounds were tested as substrates for peak I. The compounds tested as substrates, the solvents used to dissolve them and the wavelengths used in the assays, are indicated in Table 4.2. The assay protocol remained otherwise unchanged.

Table 4.2. Substrate used for substrate specificity study of Peak I

| Compound | Solvent | Wavelength (nm) |
|---|---|---|
| o-Dianisidine | Water | 460 |
| Potassium cyanide | Water | 350 |
| Tetramethylbenzidine | Water | 655 |
| Diaminobenzidine | Water | 460 |
| p-Phenylenediamine | Water | 460 |
| Guaiacol | Alcohol | 470 |

4.3.7.2. Substrate specificity for peak II (polyp he no 1 oxidase) and peak III (Laccase)

Different compounds were tested as possible substrates for peak II and peak III. The compounds tested as substrates, the solvents used to dissolve, them and the wavelengths used in the assays, are indicated in Table 4.3. The assay protocol remained otherwise unchanged.

Table 4.3. Substrates used for substrate specificity study of Peak II and III

4.3.7.3. Effect of inhibitors on peak I (peroxidase)

| Compound | Solvent | Wavelength (nm) |
|---|---|---|
| Catechol | Water | 420 |
| Pyrogallol | Ethanol | 440 |
| β-Naphthol | Ethanol | 460 |
| Resorcinol | Water | 420 |
| 4-Methyl catechol | Water | 420 |
| p-Quinol | Water | 420 |
| o-Dianisidine | Water | 420 |
| p-Phenylenediamine | Water | 410 |
| Syringaldazine | Ethanol | 525 |
| Tyrosine | NaOH | 280 |

The effect of varying concentrations of inhibitors on the activity of enzyme in peak I was determined. The compounds tested were sodium azide, potassium cyanide, hydrazine, DTT, EDT A and CT AB. With the exception of hydrazine, which is alcohol-soluble, all the compounds are water-soluble. 0.1 ml of appropriately diluted enzyme was incubated with O.1 ml of varying concentrations of inhibitor in 0.6 ml of 0.05 M sodium acetate buffer, pH 6.0 at room temperature for five minutes. To the reaction mixture, 0.1 ml of 1% H202 and 0.1 ml of 0.25% o-dianisidine were then added, and the absorbance was recorded at 460 nm for three minutes.

4.3.7.4. Effect of inhibitors on peak II (polyphenol oxidase) and peak III (Laccase)

The effect of varying concentrations of inhibitors on the activities of enzymes in peak II and peak III was determined. The compounds tested were ascorbic acid, cinnamic acid, citric acid, sodium chloride, sodium thiosulphate, potassium cyanide, cysteine, CT AB and PVP. With the exception of cinnamic acid, which was soluble in methanol, all the other compounds are water-soluble. Appropriately diluted enzyme (0.1 ml) was incubated with (0.1 ml) of varying concentrations of inhibitors in 0.05 M sodium acetate buffer, pH 6.0 at room temperature for 5 min. To this reaction mixture 0.1 ml of catechol was then added and the absorbance was rec6rded at 420 nm for 5 min.

4.3.7.5. Effect of pH

The effect of pH on the enzymes activities Peak I, peak II and peak III was studied at pH values ranging from 4.0 to 6.0 (0.05 M sodium acetate buffer); 6.0 to 8.0 (0.05 M sodium phosphate buffer) and 8.0 to 9.0 (0.05 M Tris-HCI buffer). The enzyme assays were carried out as described earlier.

4.7.3.6. Effect of substrate concentration on Peaks I, II and III

The enzyme activities, at varying concentrations of catechol, of the enzymes PPO (11 mM to 100 mM) and laccase (6 mM to 100 mM) were determired and that of POD was detemined at varying concentrations of H202 (3 mM to 50 mM) and o-dianisidine (0.2 mM to 0.4 mM) in the 1 ml reaction mixture. All other assay conditions were maintained the same as described earlier.

4.3.7.7. Temperature stability

The purified enzymes present in Tris-HCI buffer (0.05 M, pH 8.0) were incubated at room temperature (27°C), 40, 50, 60, 70, 80 and 90°C for 10 min, and assayed for enzyme activity under standard conditions as described earlier.

The results presented are average of minimum three independent values

## 4.4. RESULTS AND DISCUSSION

### 4.4.1. SECTION A: PURIFICATION

As mentioned earlier, (Chapter 2, p. 65), the aqueous phase of sap contained very little protein and most of the protein was associated with 0 and POD enzyme activities. Moreover, the specific activities of these enzymes were fairly high. On native polyacrylamide gel electrophoresis,, go sap from all the varieties studied (with the exception of Totapllri) gave a major band and a few faint bands (chapter 2, p. 65).

The sap of the Raspuri variety was selected for isolation and chacterization of PPO and POD, since the protein content and enzyme activities were found to be higher compared to other varieties, in additions to it greater availability. The initial step attempted to purify 0 and POD was ammonium sulphate precipitation. Although 70% of protein was precipitated by 60-80% ammonium sulphate concentration, the total activity recovered was only 8 %. Activity was not recovered even after removal of ammonium sulphate by dialysis, dicating that ammonium sulphate irreversibly inactivated the enzymes. Other, attempts were made to purify these enzymes by chromatographic methods. Hydrophobic chromatography using Phenyl Sepharose CL-4B ere also unsuccessful, as about 90% of the protein remained unbound and nearly 85% of the enzyme activity loaded was eluted out in the wash solution. Phenyl Sepharose CL-4B has been used for the purification of pro from different sources by different workers. PPO from peaches ~Iurkey and Jen, 1980], Da Chauna grapes [Lee, 1983], strawberries esche-Ebeling and Montgomer, 1990], pineapple [Das et al. 1.997] were using Phenyl Sepharose CL-4B. However, hydrophobic interaction chromatography using Phenyl Sepharose, could not be used kJ purify the mango sap PPO perhaps due to differences in its biochemical nature. Purification was then attempted by gel filtration chromatography on Sephadex G-200. When the aqueous phase was subjected to gel filtration, only one peak was obtained after the void volume. This peak showed both PPO and POD activities.

#### 4.4.1.1 Ion-exchange Chromatography on DEAE-Sephacel

Ion-exchange chromatography on DEAE-Sephacel was carried out. Of the total activity of PPO and POD loaded on the column, 30% PPO and 50% POD were found in the initial column wash (Fig. 4.5). The bound proteins were eluted using a stepwise sodium chloride gradient (0.025, 0.05, 0.075, 0.1 M NaCI) in 0.05 M Tris-HCI buffer (pH 8.0). Washing of column with 0.025 M NaCI in 0.05 M Tris-HCI (pH 8.0) eluted a protein peak, which showed only POD activity but not PPO activity (Peak I). Further, elution with 0.05 M NaCI in Tris-HCI buffer resulted in the elution of another protein peak, which showed only PPO activity but not POD activity (Peak II). Elution with 0.075 M NaCI in Tris-HCI buffer eluted a protein peak, which showed both PPO and POD activities coinciding with the protein peak (Peak III). Eluate of 0.1 M NaCI in Tris-HCl buffer did not show any enzyme activity.

Native gel electrophoresis of these three peaks showed a single band indicating the homogeniety of each peak (Fig. 4.6. B). Further when these peaks were subjected to SDS-P AGE, each peak again showed a single oand (Fig. 4.7.). This further confirmed the purity of protein in these peaks.

Following native PAGE, peak I stained only for POD (Fig. 4.6. C, Pk3), peak II stained only for PPO (Fig. 4.6 A, Pk2) and peak III stained for both PPO and POD (Fig 4.6 A, Pkl and Fig. 4.6 C, Pk2) in agreement with the enzyme activity observed in the chromatography. Thus, the results indicated that peak I is a POD and peak II is a PPO. Although peak III is pure, it showed both PPO and POD activities. This peak was shown to be a laccase which exhibits PPO activity and pseudo-peroxidase activity as described later in this Chapter (p.146).

The specific activity of PPO in peak II was increased by 6 fold with a yield of 50% (Table. 4.4). Where as peak III 2.3 fold purification was achieved with a yield of 19.8%. Peak I yielded 26% activity of POD and the specific activity of the POD was increased by 3.4 fold. Peak III was also associated with POD activity. The POD specific activity associated with this peak was found be 2177 with a 2.7 fold increase. It is to be noted that in the case of all the enzymes the fold purification is only between 2-6. This may be because these enzymes may as such be present in the relatively pure form in sap. Earlier in Chapter 2, (p. 65) it was shown that on native PAGE very few protein bands are seen and all the major band stained for both PPO and POD. About 30% of PPO and 50% of POD activities loaded were not bound to the column and found eluted in the wash solution. This may not be due to over loading since only 6.2 mg of protein were loaded onto a 70 ml Sephacel column. The unbound PPO and POD activity may be different isozymes. The presence of several isozyme of PPO and POD as been reported by different workers [Kay, et aI, 1967; Vamos-Vigyazo, 1981; Lee and Kleim, 1990; Pressy, 1990].

Earlier, Joel et al [1978] reported that laccase was present in mango sap but not in mango peel. Our studies showed that mango sap of Indian varieties studied here, contain both PPO (catechol oxidase and laccase) and POD which could be separated by ion-exchange chromatography. As already discussed in Chapter 2, the presence of POD as one of the major enzyme component in the sap of Indian mango varieties was clearly demonstrated. Joel (et al [1978] has not reported the presence of POD in sap. Prabha and Patwardhan [1982] reported the presence of PPO and POD in the peel of Indian mango varieties.

The mobilities of these three peaks on SDS-PAGE were similar, indicati~g the similarity in their molecular weights. The estimated molecular weight of these enzymes were 105,000 (Fig. 4.7). The molecular weight of the three peaks as determined by gel filtration on Sephadex G- 200 was 100,000 $\pm$ 5,000. Joel et aI, [1978] and Robinson et al, [1993] have reported some of the properties of laccase from mango sap. However, they have not reported the molecular weight of this enzyme, whereas Bar-nun et aI, [1981] reported that the laccase of Schinus molle, a member of the Anacardiaceae, consisted of a single sub-unit with an apparent molecular weight of 96,000. Sherman [1991] reported that the molecular weight of PPO from four species of higher plants ranging from 33,000 to >200,000. The generally accepted molecular weight of mushroom PPO is 128,000 [Mason, 1965].

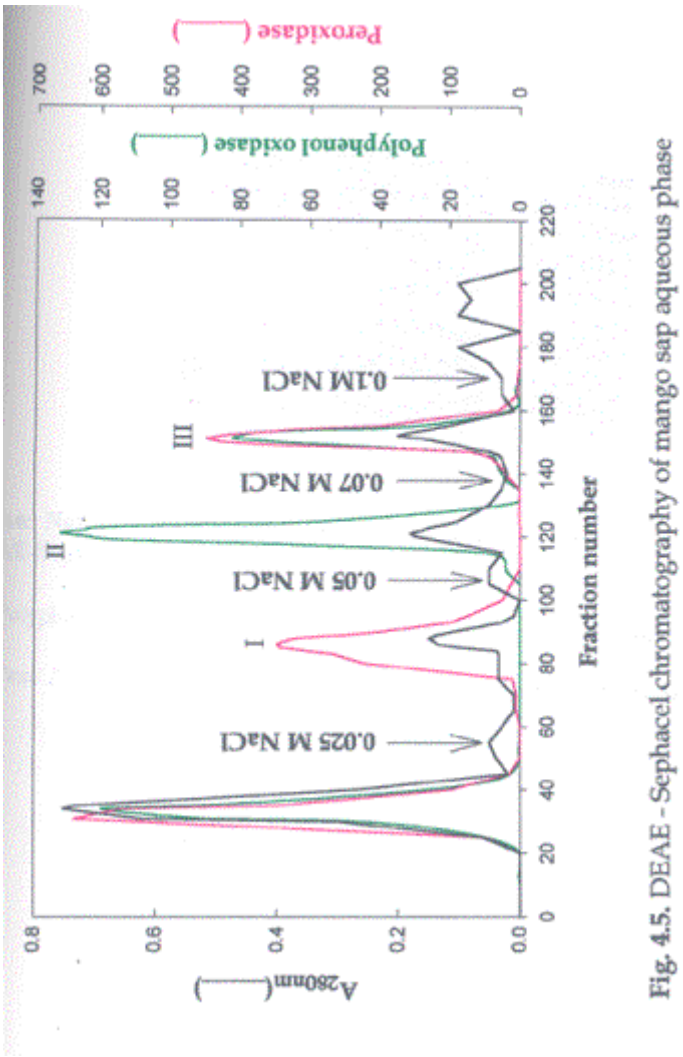



Fig. 4.5. DEAE - Sephacel chromatography of mango sap aqueous phase

Fig.4.5 DEAE – Sephacel chromatography of mango sap aqueous phase

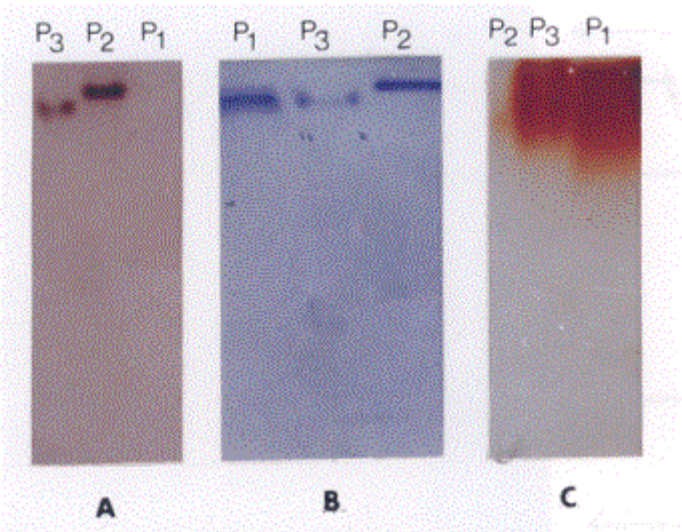


Fig 4.6: Polyacrylamide gel electrophoresis of the peaks obtained on DEAE-Sephacel chromatography

A. Polyphenol oxidase stain: B. Protein stain: C. Peroxidase stain

$P_1$. Peak 1 (Peroxidase) $P_2$. Peak 2 (Polyphenol oxidase) $P_3$. Peak 3 (Laccase)

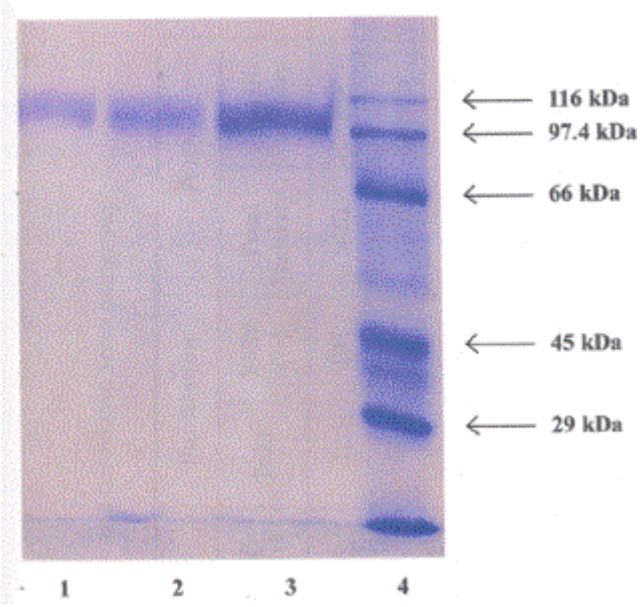



Fig 4.7: SDS- Polyacrylamide gel electrophoresis of the peaks obtained on DEAE-Sephacel Chromatography

1. Peak 3 (laccase); 2. Peak 2 (polyphenol oxidase);

2. 3. Peak 1 (Peroxidase); 4. Standard molecular weight markers.

Table 4.4. Purification of polyphenol oxidase and peroxidase from mango sap

| | Protein (mg) | PPO | | Purification | | POD | | Purification | |
|---|---|---|---|---|---|---|---|---|---|
| Crude DEAE-Sephacel | 6.2 | 1350 | 218 | 1 | 100 | 5010 | 808 | 1 | 100 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Peak (1) | 0.48 | - | - | - | - | 1320 | 2750 | 3.4 | 26 |
| Peak (2) | 0.52 | 680 | 1308 | 6.0 | 50 | - | - | - | - |
| Peak (3) | 0.53 | 268 | 506 | 2.3 | 19.8 | 1154 | 2177 | 2.7 | 23 |

4.4.2. SECTION B: CHARACTERIZATION OF THE PEAKS OBTAINED ON DEAE-SEPHACEL CHROMATOGRAPHY

4.4.2.1. Properties of polyphenol oxidase (peak II)

4.4.2.1.1. pH optimum

In order to determine the optimum pH for activity of the enzyme, the assay was carried out using different buffers as indicated in the materials section. The maximum PPO activity was obtained at pH 6.0 (Fig. 4.8) The enzyme activity was higher when the sodium acetate buffer was used as compared with the sodium phosphate buffer at pH 6.0. The pH optimum of PPO from mango peel was reported to be 5.4 [Prabha and Patwardhan, 1982], whereas that of the enzyme from pineapple ranged from 6-7 [Das et aI, 1997] while in DaChauna grapes it was 6.0 [Lee et al,1983].

4.4.2.1.2. Substrate specificity

As can be seen from Table 4.5, catechol at 50 mM concentration showed maximum activity followed by 4-methyl catechol. The substrate of laccase like syringaldazine, p-quinol, tyrosine, p-phenylenediamine and o-dianisidine did not show any activity. Sap PPO of Indian variety Raspuri, has given better activity with catechol, whereas Robinson .et al [1993] reported higher activity in the case of 4-methyl catechol compared to catechol in Kensington using partially purified enzyme.

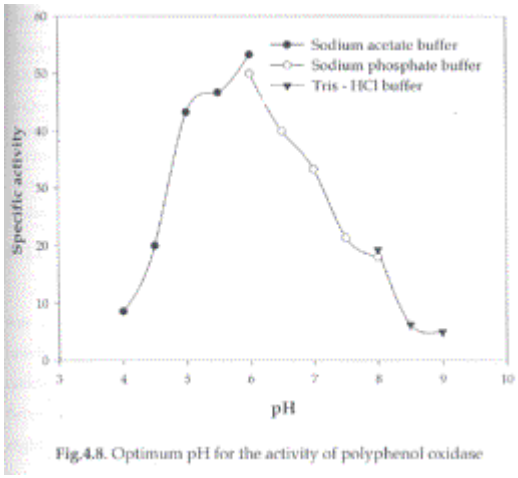



Fig 4.8. Optimum pH for the activity of polyphenol oxidase

Table 4.5. Substrate specificity of Peak I and Peak III

| Substrate | Concentration (mM) | Peak 2 | | Peak 3 | |
|---|---|---|---|---|---|
| | | Activity (U) | % Activity | Activity (U) | % Activity |
| Catechol | 50 | 81 | 100 | 75 | 100 |
| β-Naphthol | 50 | 15 | 18.5 | 68 | 90.7 |
| Potassium ferrocyanide | 50 | 30 | 37 | 15 | 20 |
| Resorcinol | 50 | 27 | 33.3 | 0 | 0 |
| 4-Methyl catechol | 50 | 30 | 48.1 | 27 | 52 |
| p-Quinol | 20 | 0 | 0 | 5.4 | 7.2 |
| p-Phenylene diamine | 0.5 | 0 | 0 | 6.0 | 8 |
| Syringaldazine | 0.1 | 0 | 0 | 153 | 204 |
| o-dianisidine | 2 | 0 | 0 | 120 | 96 |

4.4.2.1.3. Effect of substrate concentration

The effect of substrate (catechol) concentration on purified PPO stl1died by assaying its activity at different concentrations of catechol. Increase in PPO activity with increasing concentration of mM catechol concentration. Beyond this substrate concentration the enzyme activity decreased

slightly' (Fig. 4.9 Inset). The Km calculated by Lineweaver-Burk plot based on initial velocity for PPO was 12.5 x $10^{-3}$ M (Fig. 4.9).

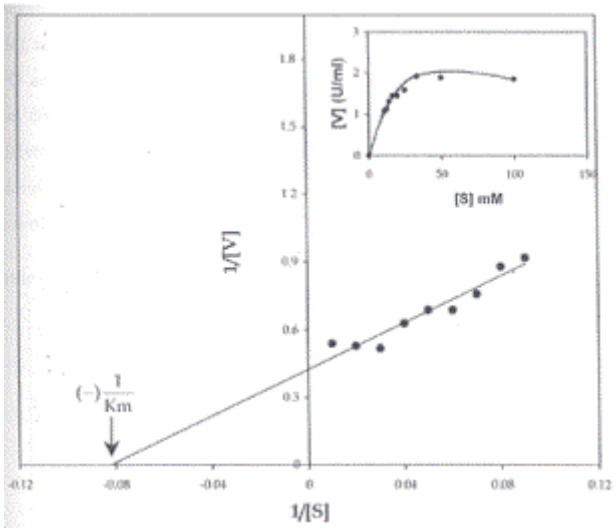



Fig 4.9. Double reciprocal plot of the effect of concentration of catechol on the initial velocity of polyphenol oxidase from mango sap.

4.4.2.1.4. Effect of inhibitors

The PPO activity was almost completely inhibited by sodium thiosulphate at 0.5 mM concentration. Citric acid (100 mM), NaCI (200 mM), ascorbic acid (5 mM), cysteine (5 mM), CT AB (20 mM) inhibited the pro activity upto 75-80%. Potassium cyanide (0.08 M) inhibited more than 80% of the activity, whereas cinnamic acid at 5 mM concentration inhibited only 30% of PPO activity (Table 4.6). Ascorbic acid, sodium chloride, and cysteine are generally used to inhibit the PPO mediated browning reaction. These reagents also inhibited sap PPO.

Table 4.6. Effect of inhibitor on peak II and peak III

| Inhibitor | Concentration 9mM) | Residual activity (%) | |
|---|---|---|---|
| | | Peak 2 | Peak 3 |
| Ascorbic acid | 5 | 19.6 | 0 |
| Citric acid | 100 | 22.4 | 18 |
| Sodium chloride | 200 | 25.2 | 22.5 |
| Sodium thiosulphate | 0.5 | 5.6 | 60.1 |
| Potassium cyanide | 0.08 | 14.0 | 13.5 |

| | | | |
|---|---|---|---|
| Cysteine | 5 | 22.4 | 4.5 |
| PVP | 30 | 47.7 | 54.1 |
| CTAB | 20 | 22.4 | 22.5 |
| Cinnamic acid | 5 | 70.0 | 0 |

4.4.2.1.5. Temperature stability

In order to determine the temperature stability of the enzyme, aliquots' of the enzyme were incubated at temperatures ranging from 27°C (room temperature) to 90°C, for 10 min as described in methods. The temperature stability study showed interesting results (Fig. 4.10). The enzyme activity increased with increasing temperature upto 70°C. Above C a drastic decrease in activity was observed which may be due to temperature meditative inactivation of the enzymes. Increase in enzyme activity pre-incubated at lower temperatures, may be due to temperature mediated activation of PPO. Presence of inactive or latent form of PPO in plants has been reported. Activations were achieved by pH change to III acidic/basic region or protease treatment [Vamos-Vigyazo, 1981]. Our temperature stability studies revealed that incubation of the enzyme at higher temperature also activates the enzymes. Prabha and Patwardhan 1982 reported temperature stability for PPO from mango peel upto 5()°C. Thus, mango sap PPO has a greater thermal stability than the peel enzyme.

4.4.2.2. Properties of Laccase (peak III)

4.4.2.2.1. pH optimum

This peak showed the activities of both PPO and POD. Both the activities were higher at pH 6.0. As in the case of PPO (peak II), the enzyme activity was slightly higher when the sodium acetate buffer stem was used as compared with the sodium phosphate buffer system at pH 6.0 (Fig. 4.11) (only PPO activity was presented). The pH optimum of pro activity of peak II and peak III were similar. Joel et al [1978] have reported the presence of a laccase in the crude mango sap, exhibiting optimum activity at pH 6.0. Bar-Nun et al [1981] reported a pH optimum of 6.2 for the laccase from Schinus molle.

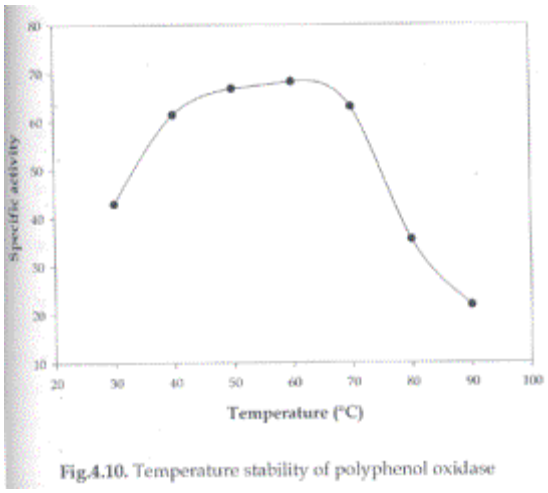



Fig. 4.10 Temperature stability of polyphenol oxidase

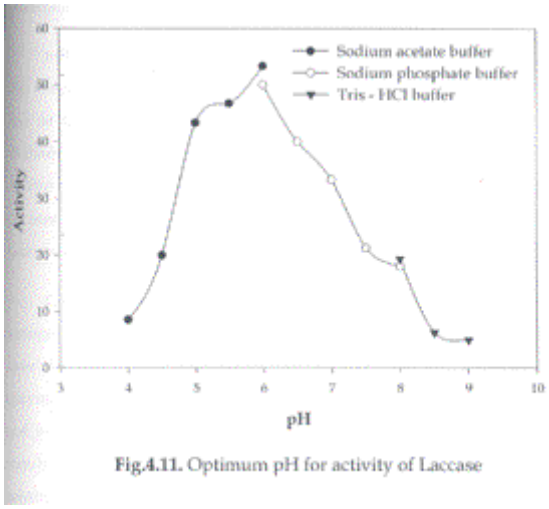



Fig 4.11. Optimum pH for activity of Laccase

4.2.2.2. Substrate specificity

As can be seen from Table 4.5, this peak was active on a number of substrates compared to PPO (peak II). Catechol was found to be a good substrate as observed in the case of peak II. In addition, β-naphthol, and o-dianisidine were found to be good substrates for this enzyme.

ρ-Phenylenediamine, ρ-quinol, also were found to be substrates for this enzyme. Syringaldazine gave very high activity at a concentration as low as 0.1 mM. Laccases are generally differentiated

from catechol oxidases based on their substrate specificities, particularly their ability to oxidize ortho-and para-hydroxy phenols and aromatic amines. Most Jaccases utilize syringaldazine as substrate [Harkin and Obst, 1973; Leonowicz and Grzywnowicz, 1981; Flurkey et aI, 1995].

Catechol oxidases do not oxidize p-hydroxy phenols and syringaldazine [Wood, 1980; Hewitt and Smith, 1975]. Joel et al [1978] have also reported the presence of a laccase in mango sap. Although the enzyme in peak III (laccase) showed less activity on p-phenylenediamine and p-quinol compared to catechol, the enzyme in peak II, which is a catechol oxidase type PPO, did not exhibit any activity with the same substrates. On the other hand resorcinol was oxidized by PPO of peak II but not by the enzyme in peak III whereas tyrosine was not oxidized by either peak. An interesting observation made with respect to the enzyme in peak-III when o-dianisidine was used as a substrate was that the activity increased 8.1 fold on addition of H202 to the reaction mixture, compared to the activity obtained in the absence of H202.

The enzyme exhibited comparable activity with o-dianisidine and Mason [1974] reported that mushroom activity and described this as 'pseudo- peroxidase' activity.

Similar results can also be seen in the purification table where the specific activity of PPO in peak III was only 268 whereas that of POD was 2177. The activity expressed by peak III (laccase) in the presence of o-dianisidine alone is a reflection of PPO activity. When H202 and o-dianisidine are present, it reflects both PPO and POD activities. These two activities may be taking place at the same active site.

4.4.2.2.3. Effect of substrate concentration

The effect of substrate (catechol) concentration on purified laccase was studied by assaying its activity at different concentrations of catechol. Laccase activity increased with increase in concentration of catechol and reached a maximum at 33 mM catechol concentration beyond this substrate concentration the enzyme activity decreased gradually (Fig. 4.12 Inset). The apparent Km value for catechol calculated by Linweaver-Burk plot was 8.33 x 10-3 M (Fig. 4.12).

4.4.2.2.4. Effect of inhibitors

The PPO activity of laccase was completely inhibited by both ascorbic acid and cinnamic acid at 5 mM concentration level. Cysteine (5 mM) also almost completely inhibited its activity. Complete inhibition of activity by these compounds was not seen in the case of PPO of peak II.

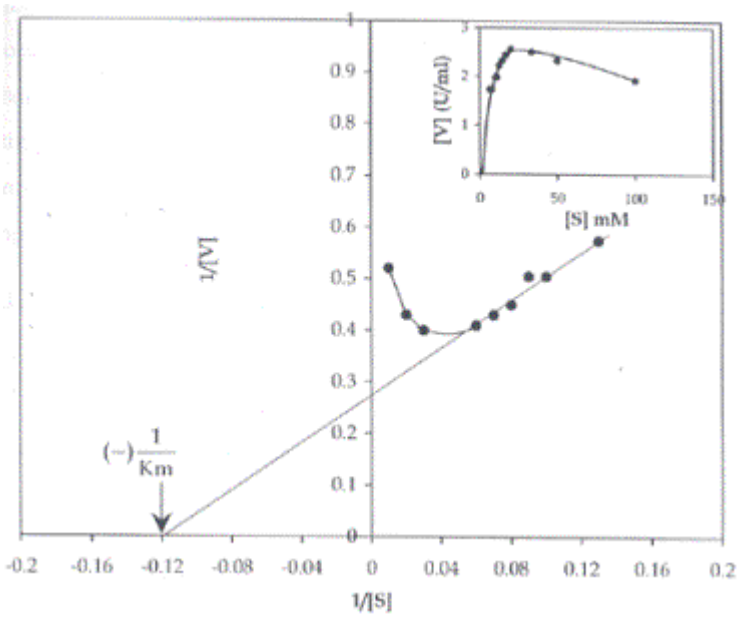



Fig 4.12. Double reciprocal plot of the effect of concentration of catechol on the initial velocity of laccase from mango sap.

Thus, a difference between PPO of peak II and laccase (peak III), with respect to not only substrate specificity but also with the pattern of inhibition were observed (Table 4.6). Walker and McCallion [1980] reported that all the p-diphenol oxidases tested showed inhibition by cinnamic acids and polyvinylpyrrolidone. They were, however, distinguished by their ability to oxidize p-quinol and p-phenylene diamine and by the inhibition by CT AB and other quartenary ammonium

4.4.3.2.5. Temperature stability

Similar to PPO of peak II laccase activity of peak III also showed almost identical temperature stability profile. The enzyme was slightly activated at higher temperatures (Fig. 4.13).

4.4.2.3. Properties of peroxidase (Peak I)

4.4.2.3.1 pH optimum

The enzyme POD showed a pH optimum at 6.0. The enzyme activity was higher when sodium acetate buffer was used as compared with sodium phosphate buffer (Fig. 4.14). The pH optimum of POD activity is known to vary with the source of enzyme, the donor substrate and the buffer used [Vamos-Vigyazo, 1981]. Loss of activity on acidification is attributed to the change in protein resulting from heme detachment. In general, the pH optimum of POD from grape was

5.4, banana, 4.5-5.0, pineapple 4.2, horse radish peroxidase, 3.0, potato, 5.0-5.4 [Vamos-Vigyazo, 1981] are all in the acidic range.

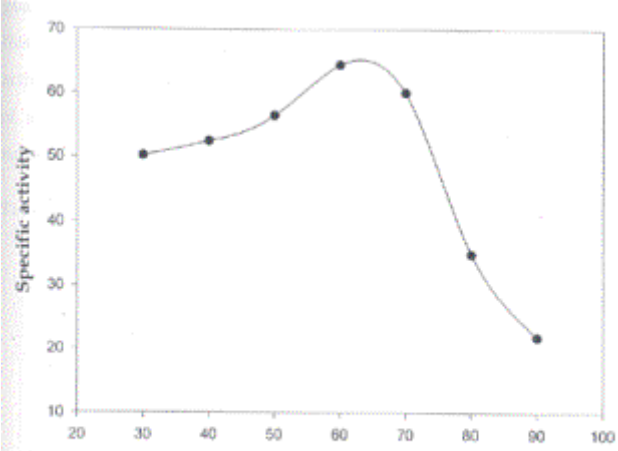



Fig.4.13. Temperature stability of Laccase

4.4.2.3.2. Substrate Specificity

A range of compounds were tested as hydrogen donor substrates purified POD showed activity with o-dianisidine, p-phenylenediamine, tetramethyl benzidine, diaminobenzidine and guaiacol. However, o-dianisidine was found to be the best substrate for this enzyme. p-Phenylenediamine also showed a good amount of enzyme activity..Tetramethylbenzidine, diaminobenzidine, gave only 12.5 to 21% of activity obtained with o-dianisidine, indicating that these are poor substrates for POD (Table 4.7).

Table 4.7. Substrate specificity of peak 1

| Substrate | Concentration (mM) | Activity | % Activity |
|---|---|---|---|
| o-Dianisidine | 1.0 | 140 | 100 |
| Tetramethul benzidine | 1.0 | 20 | 12.5 |
| Diaminobenzidine | 1.0 | 30 | 20.8 |
| p-Phenylendediamine | 1.0 | 100 | 70.8 |
| Guaiacol | 50 | | 16 |

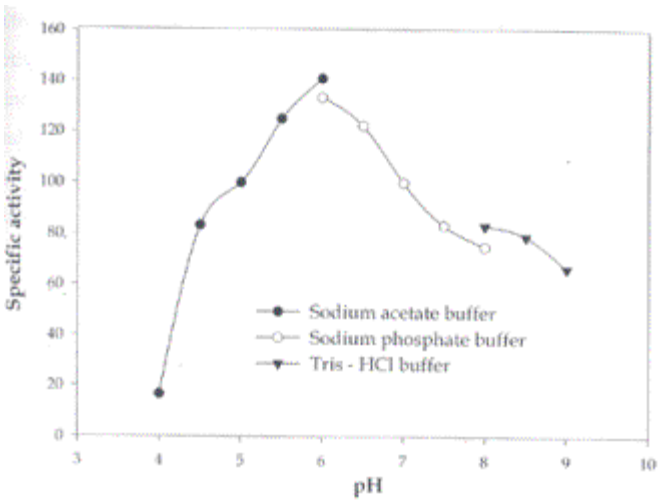



Fig. 4.14. Optimum pH for activity of peroxidase

Fig. 4.14. Optimum pH for activity of peroxidase

4.4.2.3.3 Substrate Concentration

The effect of different concentrations of H202 and o-dianisidine were determined. POD activity increased with increasing concentration of substrates and reached maximum between 12.5-16.7 mM of H202 and 1.25 mM concentration of o-dianisidine (Fig. 4.15., Fig. 4.16. Insets). Beyond this substrate concentration, in the case of H202, the enzyme activity gradually decreased, whereas in the case of o-dianisidine, the activity decreased sharply. The Km value was calculated from the Lineweaver-Burk plot. It was found to be 2.22 x 10-3 M for H202 (Fig. 4.15) and 0.9 x 10-3 M for o-dianisidine (Fig. 4.16). POD has been reported to have a high specificity for H202 and a low specificity for the hydrogen donor substrate [Vamos-Vigyazo, 1981].

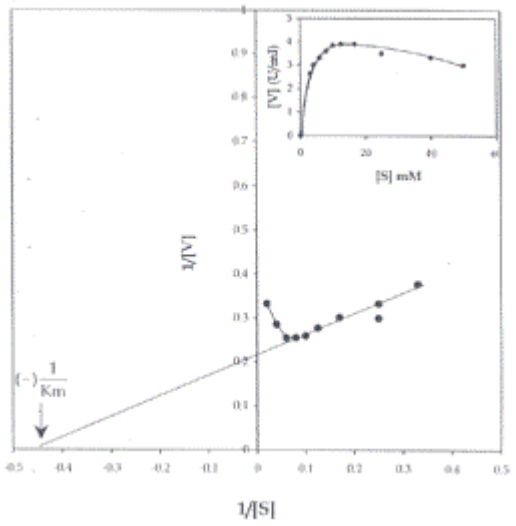



Fig 4.15 Double reciprocal plot of the effect of concentration of H2o2 on the initial velocity of peroxidasae from mango sap.

Inset: Michaelis – Menten Curve

Fig 4.16. Double reciprocal plot of the effect of concentration of o-dianisidine on the initial velocity of peroxidasae from mango sap.

14.4.2.3.4. Effect of Inhibitors

The effect of sodium azide and potassium cyanide, potent " inhibitors of heme containing enzymes, was studied on POD (Table 4.8). At low concentrations of potassium cyanide (0.02 mM), about 85% POD activity was inhibited. Sodium azide and hydrazine also inhibited the POD activity to a considerable extent (Table 4.8).

Table. 4.8. Effect of inhibitors on peroxidase (Peak I)

| Inhibitor | Concentration (mM) | Residual Activity (%) |
|---|---|---|
| Sodium azide | 10 | 27.19 |
| Potassium cyanide | 0.02 | 14.3 |
| Hydrazine | 10 | 12.31 |
| Dithiothrietol | 10 | 43.42 |
| EDTA | 10 | 61.63 |
| CTAB | 10 | 33.4 |

4.4.2.3.5. Temperature stability

In order to determine the temperature stability of the enzyme, aliquots of the enzyme were incubated at temperatures ranging from 27°C (room temperature) to 90°C, as described in section. POD was found to be quite stable up to 60°C (Fig. 4.17). Above this temperature a drastic loss of, activity was seen. 16.4% of residual enzyme activity was observed on heat treatment at 90°C for 10 min. Potato and cauliflower POD could be completely and irreversibly inactivated by heat treatment at 95°C.

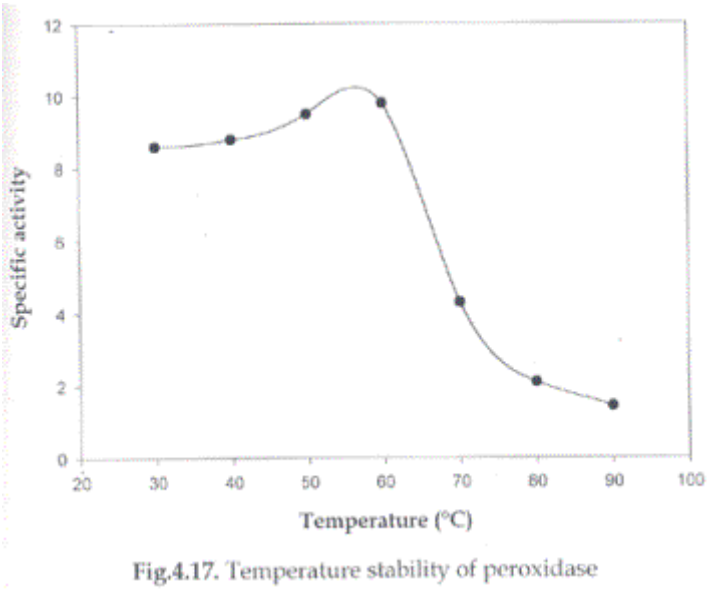



Fig.4.17. Temperature stability of peroxidase

Fig.4.17. Temperature stability of peroxidase

4.4.3. Conclusion

The present study revealed that mango sap contains both PPO and POD activities. These enzymes exist in isoforms, which have been separated on DEAE-Sephacel. However, enzymes activities that are bound had not only similar molecular weight, but also similar pH optima, temperature stability, and similar mobility on gel electrophoresis, even though they exhibited different enzyme activities (PPO and POD).

## CHAPTER - 5

## SUMMARY

Mango is one of the most delicious of fruits, relished by people all over the world. India accounts for over 50% of the world mango production, but most of this is consumed locally. A very small part of this production goes to the export market, primarily due to the low shelf-life of the fruit and other post-harvest problems. Sap-injury is a major post- harvest problem of mango. During harvesting of mangoes, a considerable amount of sap falls on the fruit when the stalk is broken at the abscission causes sap-injury, which is characterized by browning or of the peel. Harvesting of fruit with the stalk intact and subsequent de-sapping, a tedious process, has been recommended as a preventive measure to minimize this problem.

In order to develop methods to control sap-injury a detailed investigation on biochemical characterization of sap, identification of the sap components responsible for sap-injury, possible mechanism of sap-injury and development of suitable methods for the control of sap-injury have been undertaken in the present investigation. The results of this investigation are summarized below.

1 Saps from different Indian mango varieties, viz., Raspurii, Badam, Banganapalli, Totapuri, Mallika, Malgoa, Seedling were collected. The sap is a viscous liquid. It is acidic in nature and has an aroma of raw mango. The volume of sap varies from variety to variety, ranging from 10 to 25 ml/10 kg mango.

2. The mango sap can be easily separated into two phases: an upper, non-aqueous phase, and a lower, aqueous phase. The ratio between non-aqueous phase and aqueous phase varied with different varieties. The Totapuri and Seedling varieties of mango which are generally consumed raw, or used for pickling purposes, had a ratio of around 1:2. RaspLlri, Badami, Banganapalli and Malgoa varieties, which are consumed as ripe fruits had 1:7 to 1:13. Seedling variety yielded maximum (6.8 ml), whereas Raspuri yielded the least (1.4 ml) of non-aqueous phase per 10 kg of mango fruit. The aqueous phase content was the highest in Malgoa (23.4 ml/l0 kg fruit) and the lowest in Totapuri variety (7.3 ml/l0kg mango fruit).

3. GC-MS analysis of the non-aqueous phase of mango sap revealed that it consisted mainly of mono-terpenes -lliz, p-myrcene, trans-/ cis-ocimene and limonene. There were, however, differences in the composition and concentrations of these terpenoid compounds. Mallika is the only variety, which contains limonene as the major terpenoid. The terpenoid composition observed in Indian mango varieties is entirely different from the lnvin variety of Florida, and the Kensington varietiy of Australia. The saps of these

varieties contained, respectively terpinolene and car-3-ene as their primary terpenoid components.

4. The aqueous phase of sap was found to be rich in carbohydrate (302-348 mg/ ml). It also had small amount of protein (2.2 to 4.2 mg/ml). It was found to be rich in potassium (588 to 1588 ppm), sodium (143 to 240 ppm) and magnesium (418 to 1078 ppm). It had significant amounts of iron and copper. The total polyphenol content ranged from 0.045 to 0.16 mg/ ml.

5. Mango sap was found to be rich in polyphenol oxidase and peroxidase. The specific activity of polyphenol oxidase ranged from 147 to 214, and that of peroxidase from 401 to 787 in different varieties. Significant activities of both serine and cysteine (thiol) proteases were found in mango sap. The specific activity of serine proteases varied from 2.1 to 5.2 and that of cysteine protease varied from 0.12 to 0.38 depending on the variety. The sap of the Raspuri variety was found to be richer in protease than that of the other varieties. Lipoxygenase, amylase and polygalacturonase were present with very low specific activities of 0.04 to 0.05, 2.5 to 4.3 and around 1.1 to 1.3 respectively. Catalase and pectinmethyl esterase were absent.

6. When sap proteins were subjected to native PAGE, only one major and a few faint protein bands were observed in each variety with little difference in their mobilities. All the major protein bands were stained for PPO and POD, indicating that PPO and POD are the major proteins present in sap.

7. The carbohydrate content, protein content and enzyme activities in general, decreased from tender stage (Stage I) to mature stage (Stage III). The decrease in protein content from stage I to stage III was 35 to 40%, and the decrease in carbohydrate content was about 20 to 25%. The PPO and POD were higher in sap collected from tender mangoes than in the sap of mature mangoes. With respect to PPO, the decrease in specific activity in sap from stage I to stage III ranged between 12 and 57%, whereas POD activity decreased from 19% to 54%. However, in the case of serine proteases, the specific activity increased in sap as the fruit matured. No definite trend was observed in the case of cysteine protease activity.

8. The aqueous phase of mango sap was found to be rich in carbohydrate content. Total carbohydrate content ranged from 302 to 348 mg/ ml in different varieties. The content of free sugars was found to be less (0.45 mg/ml sap). Most of the carbohydrate was

found to be non-starch in nature and non-dialysable from a 12,000 cut off dialysis bag indicating that its molecular weight is more than 12,000. The viscous nature of sap may be due to the high content of the carbohydrate. The carbohydrate content was found to be the highest in Malgoa sap (348 mg/ ml), which also yielded more volume of sap (23.4 ml/10 kg mango fruit). Carbohydrate may have specific role in maintaining the sap at high pressure in the lactiferous duct system.

9. When different components of mango sap were tested for anti-microbial activity, the non-aqueous phase showed both anti-bacterial and anti-fungal activities. Fusarizlm monilifor1ne was found to be the most resistant and Penicillillm spp. was the most sensitive to the non-aqueous phase of sap. Likewise, in the case of bacteria, E. coli was found to be the least sensitive and Bacilllis cereus was the most sensitive.

10. The aqueous phase of mango sap did not cause sap-injury, whereas whole sap did cause injury. The non-aqueous phase caused maximum injury. In the case of whole sap, spurt sap caused more injury than ooze sap.

11. The non-aqueous phases from different varieties caused injury on the same as well as other varieties of mango. The extent of injury varied depending on the variety of mango.

12. The major terpenoids identified in mango varieties were either p-myrcene or cis- or trans-ocimene or limonene. When these terpenoids were applied on the mango, limonene caused maximum injury followed, by ocimene. Diethyl ether, a non-terpenoid organic solvent, also caused the injury, similar to that caused by the terpenoid component.

13. The PPO and POD activities and polyphenol content of mango peel of different varieties were analyzed. The extent of injury correlated well with the total enzyme activities of PPO and POD, and also the polyphenol content of the peel. Heat inactivation of the enzyme in the peel alleviated browning reaction. Involvement of the enzyme PPO in sap-injury was reported earlier. However, our systematic study indicated that not only PPO but also POD was found to be involved in this reaction. We also showed that the polyphenol content in the peel determines the extent of sap-injury. Even though both PPO and POD are present in the sap, the sap enzymes may not be involved in causing the injury.

14. In our efforts to control sap-injury in a mango, which was already exposed to the sap, wash solutions containing different formulations were employed. Washing of the mango with the neutral detergents viz., Tween-20, Tween-80 and Triton X-I 00 proved to be very effective in controlling sap-injury.

15. Though it was shown that enzymes PPO and POD are causing reactions (sap-injury), washing the mangoes with solutions containing inhibitors such as ascorbic acid, sodium metabisulphite and alkaline buffer did not control sap injury.

16. Almost complete control of sap-injury was observed on washing the mangoes with O.1 % detergent for 5 min.

17. The extent of sap-injury increased with increase in the time elapsed between washing of the mangoes and the time of exposure of mango to sap (harvest of mangoes). Almost complete control of sap-injury was observed in mangoes washed within 3 h of sap-exposure of the mangoes.

18. Efforts were made to isolate and purify PPO and POD from the sap of Raspuri variety. On DEAE-Sephacel column chromatography, about 70% of PPO and 50% of POD activities were bound to the column. The bound enzymes were eluted into three distinct protein peaks by stepwise elution of increasing NaCI concentration (0.025 M, 0.05 M and 0.075 M NaCI in 0.05 M Tris-HCI buffer, pH 8.0).

19. The protein eluted with 0.025 M NaCI in buffer (peak I) exhibited only POD activity, whereas the protein that eluted with 0.05 M NaCI in buffer (peak II) showed only PPO activity. The protein peak eluted with 0.075 M NaCI in buffer (peak III) showed both PPO and POD activities.

20. The substrate specificity studies of the above three peaks indicated that peak I is a peroxidase, peak II is a general catechol oxidase type PPO. Unlike PPO of peak II, the enzyme in peak III oxidized syringaldazine, p-phenylenediamine and p-quinol, the specific substrates of laccase, therefore this could be a laccase type PPO.

21. All the three protein peaks showed single protein band as well as a corresponding enzyme activity band on native P AGE. On SDS-P AGE all the three peaks gave a single band with an approximate molecular weight of 105,000. The molecular weight, as

determined by gel filtration chromatography was 100,000 ±5,000 for all the peaks. These results indicate that these enzymes consist of a single sub-unit.

22. The purified PPO showed optimum activity at pH 6.0 and temperature stability upto 70°C. The Km value of this enzyme was 12.5 mM for catechol.

23. The purified laccase showed optimum activity at pH 6.0 and temperature stability upto 70°C. The Km value of this enzyme was 8.33 mM for catechol.

24. The purified POD showed optimum activity at pH 6.0, and temperature stability upto 60°C. The Km value of this enzyme for H202 was 2.22 mM, and that for o-dianisidine was 2 mM.

CONCLUSION

The study clearly demonstrates that the non-aqueous phase of sap causes sap-injury. The non-aqueous phase of the sap of the Indian mango varieties contains mainly p-myrcene, cis- and trans-ocimene, limonene, as major terpenoid compounds; these compounds have not been reported in the Kensington and lnvin varieties of Australia and US respectively. The extent of sap-injury.correlated well with the contents of PPO, POD and polyphenols in the mango peel. Peel PPO and POD, and not the enzymes of the sap, are responsible for the browning reaction. Even non-terpenoid lorganic solvents, like diethyl ether, caused sap-injury. Therefore, it is concluded that the non-aqueous phase of sap, mainly terpenoids, may dissolve the waxy layer covering the mango fruit, and further disintegrate the sub-cellular structure of the peel, resulting in a browning reaction.

Based on the analysis of aqueous and non-aqueous phases, we.conclude that the mango sap could be a valuable agri-horticultural by-product. Although it is difficult and inconvenient to harvest the mango fruit with the stalk and to de-sap it subsequently, this is one of most effective methods to control sap-injury. During this process, significant quantities of sap can be collected and the non-aqueous and aqueous phases can be easily separated by simple centrifugation. The non-aqueous phase, which is rich in terpenoids having raw mango aroma, also exhibited good anti-microbial properties. Therefore, this component could serve as a potent preservative and flavouring agent. At the same time, the aqueous phase of mango sap is rich in enzymes like PPO and POD, which are present in a relatively pure form, and having high specific activity. Mango sap may hence be used as a Teady source of these enzymes even without any further purification. Both PPO and POD have a number of biotechnological applications, e.g., as protein ross-liking agents, in degradation of xenobiotics, and also as biochemical reagents. This value addition to the mango sap could be an incentive for growers to harvest the mangoes with stalk, even though it is tedious, and de-sap them carefully to collect the sap. This will provide fruit free of sap contamination (and hence no sap-injury) and also yield sap as a valuable

However in the Indian scenario, mangoes are generally harvested without taking the precaution of keeping the stem intact. As a result, mangoes are usually contaminated to the sap of the same fruit and also of other fruits of the same or different varieties. In order to control sap-injury in these fruits, a method has been developed which involves washing of the mangoes within three hours (from the time of harvesting) with a neutral detergent solution (0.1 % Tween-20 / 0.1 % Tween-80 0.1% Triton X-100 for 5 min). This method is also compatible with other post-harvest treatments, especially fungicidal treatments. Further, this method may also help to remove various pesticides/ insecticides sprayeJ on to the mangoes, as well as other extraneous materials adhering to the fruit, thereby resulting in a cleaner product.

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APPENDIX

PAPERS

(Papers Published/Communicated So Far based On The Work Reported In This Thesis)

1) K. Saby John, L. Jagan Mohan Rao, S. G. Bhat, U: J. S. Prasada Rao: 'Characterisation

of aroma components of sap from different Indian mango varieties'; Phytochemistry: 1999, 52: 891-894.

2) K. Saby John, S. G. Bhat and V. J. S. Prasada Rao: 'Chemical and biochemical composition of mango sap at different stages of fruit maturity' (communicated).

3) K. Saby John, S. G. Bhat and V.J. S. Prasada Rao: 'Mango sap-injury: A possible mechanism involving different constituents of sap and peel' (communicated).

PATENT

(Applied In India)

1) A formulation useful for controlling sap-injury in the fruits of harvested mangoes (Mangifera indica). Prasada Rao, U. J. S., Saby, J. K.,, Bhat, S. G., Ramana, K. V. R. and Aravrnda Prasad, B.

**POSTERS**

1. K. Saby John, S.G. Bhat, K.V.R. Ramana, V.J.S. Prasada Rao. ‘Identification of certain enzymes present in saps of different mango varieties': 66th Annual Meeting of the Society of Biological Chemists .1dia), Vishakapatnam, 22-24 December 1997.

2. K. Saby John, L. Jagan Mohan Rao, S.G. Bhat, V.J.S. Prasada Rao. Characterization of volatile components present in mango sap': '4th International Food Convention (IFCON 98), Mysore, 23-27 November 1998.

3. K. Saby John, S.G. Bhat, V.J.S. Prasada Rao. 'Identification of mango components responsible for sap-injury': 67th Annual Meeting of the society of Biological Chemists (India), New Delhi, 19-21 December 1998.

4.Saby John., S.G. Bhat., V.J.S. Prasada Rao. 'Studies on mango sap at different developmental stages': International Conference on life Sciences in the Next Millenium, Hyderabad, 11-14 December, 1999.