MOLECULAR CHARACTERISATION OF CLONAL APPLE ROOTSTOCKS USING ISOZYMES

THESIS

by

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Affectionately
dedicated
to
my parents

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CERTIFICATE-I

This is to certify that the thesis entitled, "**Molecular characterisation of clonal apple rootstocks using isozymes**" submitted in partial fulfilment of the requirements for the award of the degree of **MASTER OF SCIENCE** in **BIOTECHNOLOGY** to Dr. Y.S. Parmar University of Horticulture and Forestry, Solan (H.P.) is a bonafide record of research work carried out by **Ms. Kanika Kaushal** (H-95-35-M) under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigation has been fully acknowledged.

Place : Solan
Dated : 20th December, 1997

Manju Modgil
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Major Advisor

CERTIFICATE-II

This is to certify that the thesis entitled **"Molecular characterisation of clonal apple rootstocks using isozymes"** submitted by **Ms. Kanika Kaushal** (H-95-35-M) to Dr. Y.S. Parmar University of Horticulture and Forestry, Solan (H.P.), in partial fulfilment of the requirements for the award of the degree of **MASTER OF SCIENCE in BIOTECHNOLOGY** has been approved by the Student's Advisory Committee after an oral examination of the same in collaboration with the external examiner.

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(Kanika Kaushal)

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABBREVIATIONS USED

ACP	Acid phosphatase
C	Degree of cross linking $= \dfrac{b \times 100}{a \times b}$ (%)
	where, a is the mass of acrylamide in grams and b is the mass of methylene biscrylamide in grams.
EST	Esterase
GDH	Glutamate dehydrogenase
GOT	Glutamate oxaloacetate transaminase
MDH	Malate dehydrogenase
PER	Peroxidase
6-PGD	6-phosphogluconate dehydrogenase
PPO	Polyphenol oxidase
Rm	Relative mobility
rpm	Rotations per minute
T	Total acrylamide concentration $\dfrac{(a+b) \times 100}{V}$ (%)
	where, a is the mass of acrylamide in grams. b is the methylene biscrylamide in grams V is the volume in ml
TEMED	N, N, N^1, N^1 - tetramethyl ethylene diamine
TRIS	Tris (hydroxymethyl) - aminoethane

INTRODUCTION

1

INTRODUCTION

Apple (*Malus domestica* Borkh.) is the most important temperate fruit of the world (Childers, 1983). It is a highly remunerative deciduous fruit and is the most ubiquitous of all temperate fruits, cultivated in Europe and Asia from antiquity.

It has been found wild in most of the temperate parts of the world and cooler hills of subtropical areas. Apple (*Malus domestica* Borkh.) is believed to have been originated as a result of the hybridization between *Malus sylvestris* and other *Malus* species, with its original home in south of Caucasus (Hulme and Rhodes, 1971). It was probably first domesticated in Caucasus, but fast spread all over Europe, even in prehistorical times. From Europe, apple spread to USA, Australia and South America.

The exact number of *Malus* species is not confirmed, it ranges from 25 (Rehder, 1940) upto 122 (Ponomarenko, 1986). The distinction of primary species of *Malus* from interspecific hybrids has not been well documented primarily due to lack of accurate genetic, biochemical and morphological analysis. The genus *Malus* belongs to family *Rosaceae* and subfamily *Pomoideae* with 17 as its basic chromosome number. Most of the species are diploid (2n = 34), although triploids (2n=51) and tetraploids (2n=68) have also been observed (Rehder, 1990).

Apple is the most important temperate fruit crop of India with regard to acreage, production, economic value and above all popularity. Because of some inherent characteristics of apple such as high productivity, good storage life, attractive appearance and excellent flavour, it has become a favourite fruit of the people.

Apple is being commercially produced in India for nearly half a century. It is cultivated in three north western Himalayan states namely Himachal Pradesh, Jammu and Kashmir and hill districts of Uttar Pradesh. India produces about 1000 MT of apples. The total area under apple cultivation in 1993 in H.P. was 69439 hectares and the total produce was 27905 tonnes.

Worldwide commercial apple production has been revolutionized in the last few decades by the widespread use of clonal rootstocks. Malling and Malling Merton apple rootstocks are a small, specialized group of apple cultivars, which are used today on a worldwide basis for commercial apple growing, being related by origin to Paradise and Doucin apples (Hatton, 1917). A number of biochemical techniques have been used to distinguish and characterise isozymes, PAGE (Polyacrylamide gel electrophoresis) is one of them. The term **isozyme** was proposed by Markert and Moller (1959) for multiple molecular forms of an enzyme sharing a catalytic activity, derived from a tissue of single organism. Electrophoretic analysis is used to obtain **zymograms** which show discrete bands representing isozyme, the product of distinct alleles. Many isozyme polymorphisms have a simple genetic basis with the isozyme bands that denote allelic polymorphism at a single locus often termed as **allozymes** (Prakash *et al.*, 1969).

Torres (1983) predicted that the application of isozyme techniques to fruit trees would produce a quantum leap in the amount of genetic information available for these crops. Allelic or allozyme polymorphism has been identified in virtually every plant species examined and the utility of these simple genetic markers has been firmly established (Tanksley and Orton, 1983). An important aspect of apple industry, particularly to nurserymen and growers is the reliable verification of the cultivar being grown. In recent years, most of the apple trees planted in commercial orchards are on clonal rootstocks. To a large extent, the rootstock determines the relative tree growth rate, ultimate size, anchorage, precocity, productivity and response to hazards of soil environment. Therefore, proper selection and planting of rootstock cultivar is essential for the orchardist.

Identification and genetic purity of various rootstocks based on morphological responses is costly, time consuming and often confounded by the environmental conditions and moreover, visual rootstock identification based on morphological characteristics is difficult because of genetic similarity of many rootstocks and the restriction of observation to the nursery stage.

Isozymes as molecular markers for cultivar and rootstock identification has proven to be reliable, consistent and essentially unaffected by environmental conditions (Bailey, 1983). They show good activity in leaf extracts and can be used as genetic tools for studying pedigree and marking other genes of interest.

So, the present study was undertaken with the following objectives in mind :

i) **To identify isozyme polymorphism in seven clonal apple rootstocks**

ii) **To identify the most characteristic and stable isozyme marker for individual rootstocks.**

REVIEW OF LITERATURE

2

REVIEW OF LITERATURE

Electrophoresis is an investigative tool that has been applied successfully in many botanical disciplines, originating in 1930's (Tiselius, 1937a). Early evidence for isozymes was obtained by Neilands in 1951, when he showed that previously reported protein contaminants of crystalline lactate dehydrogenase constituted a second type of enzyme.

The term **isoenzyme** was coined by Markert and Moller (1959) to describe the different molecular forms of proteins which exhibit the same enzymatic specificity. The high resolution **zymogram** method for displaying enzyme activity on gels (Hunter and Markert, 1957) coupled with electrophoresis, has been the tool of choice of geneticists, systematists and population biologists (Gottleib, 1977; Brown, 1979; Hamrick *et al.*, 1979).

Isozyme analysis offers a rapid and more reliable means for producing genetic profiles and elucidation of genetic relationships within and between different taxa. Isozyme markers have been extensively used to fingerprint cultivars and to classify germplasm in fruit and nut crops (Arulsekar and Parfitt, 1986; Vithanage and Winks, 1992; Aradhya *et al.*, 1995).

The use of marker genes has recently been reviewed by Weeden (1989). Isozyme and allozyme analysis have helped in distinguishing apple cultivars (Weeden and Lamb, 1985), screening of germplasm for new sources of variation (Rick, 1988), detection of hybrids and determination of outcrossing rate (Parfitt *et al.*, 1985), pedigree analysis (Chyi and Weeden, 1984), marking of single gene controlling commercially important traits (Manganaris and Alston, 1987), population genetics and taxonomic studies (Torres *et al.*, 1978).

The literature available is reviewed under the following sub-headings:

2.1 Isozyme markers
2.2 Uses
2.3 Electrophoretic methodologies
2.4 Source tissue
2.5 Extraction of enzyme
2.6 Buffer systems
2.7 Staining procedures
2.8 Genetic interpretation

2.1 ISOZYME MARKERS

Peroxidase polymorphism in scion cultivars was demonstrated by Aoki *et al.*(1974); Misic *et al.* (1980). Cheng and Jia (1981) comparatively studied the peroxidase and esterase isozymes in apple varieties. Wang *et al.* (1981) did the comparative study of the peroxidase isozymes of dwarf and vigorous rootstocks of apple. They examined both leaves and branches and obtained similar zymograms for the dwarfing rootstocks M.9 and M.7 which were different from those obtained for the vigorous rootstock M.16. Xiu *et al.* (1981) also studied the comparison of the peroxidase isozyme of dwarf and vigorous rootstocks of apple.

Cheng (1984) carried out peroxidase isozyme analysis for intervarietal hybrids in apple. Two triploid apple cultivars 'Spigold' and 'Jonagold' and their parental cultivars were analysed for a number of isozymes by starch gel electrophoresis. Banding pattern of one isozyme system 6-phosphogluconate dehydrogenase provided strong evidence that the contribution of the 2n gametes in both 3x cultivars was by the female (Chyi and Weeden, 1984). Chevreau *et al.* (1985) identified seven isozyme loci in five apple progenies and provided biochemical evidence for polyploid nature of this genome.

Weeden and Lamb (1985) characterised fifty four cultivars electrophoretically using 6 isozyme systems. Intra cultivar variation in enzyme phenotype was not observed, whereas intercultivar polymorphism was sufficient to permit reliable and unambiguous identification of nearly every cultivar.

Electrophoretic analysis of genetic variability in apple was done by Boumival and Korban (1987). Eight progenies from controlled crosses and one self progeny of apples were analysed by electrophoresis of six leaf isozymes to explain the pattern of inheritance in apple (Chevreau and Laurens, 1987). Genetics and Linkage analysis of 19 isozyme loci in apple was done by Weeden and Lamb (1987).

Chernat *et al.* (1989) studied the effect of soil moisture level on protein content and isozyme composition of some oxidases in the apple leaves. Extensive genetic analysis of inheritance and linkage analysis of glutamate oxaloacetate transaminase isozymes in apple has revealed 3 genes,GOT-1 (Manganaris and Alston, 1987), GOT-2 and GOT-4 (Manganaris and Alston, 1988) with six, three and two alleles, respectively. The presence of these three allelic bands or variations in the intensity of bands in triploids facilitated the identification of the parent contributing the 2n component (Manganaris and Alston, 1989). Quarta and Arone (1988) studied peroxidase polymorphism in apple cultivars.

Bryne (1990) studied isozyme variability in four diploid stone fruits and compared the level of variability with the published results of studies in apple, avocado and *Camellia japonica*. Isozyme survey of various species of *Prunus* in the subgenus *Amygdalus* was done by Mowrey *et al.* (1990).

Stampar and Smole (1992) identified apple and sweet cherry cultivars, peach hybrids and apricot ecotypes by isozyme phenotyping. The studies on inheritance of enzyme band 9 of peroxidase isoenzyme in apple seedling were carried out by Tang and Zhang (1992a). The recent work of Manganaris and Alston (1992), Manganaris and Alston (1993) further clarified the genetics of peroxidase in *Malus* and the use of peroxidase isoenzyme genes in the identification of apple cultivars and *Malus* species.

Samper *et al.* (1994) studied the inheritance of leaf isozyme of apple (*Malus domestica* Borkh and *Malus floribunda* Van Houtte). In eight enzyme systems twenty eight loci were defined and nine of them displayed polymorphism and the inheritance of each of these loci was consistent with Mendelian principles.

Nong and Aolin (1995) studied genetic variation in 41 *Malus* accessions by polyacrylamide gel electrophoresis. Marquard and Chan (1995) identified crab apple cultivars by isozyme analysis.

2.2 USES

Isozyme analysis has been widely used as molecular marker for different plant species. Application of gel electrophoresis and isoenzyme analysis in horticultural science were outlined more than a decade ago by Peirce and Brewbaker (1973). Isozymes have been intensively used as molecular tags in systemic, genetic and evolutionary studies (Scandalios, 1974).

An electrophoretic analysis was made for isozymes of peroxidase, 0-diphenol oxidase, malate dehydrogenase and esterase in the readily soluble proteins of leaves and pollen of different species and varieties. Closely related form had similar isozyme patterns. The genetic specificity of isozyme enables them to be used as protein markers (Veidenberg *et al.*, 1977).

Buttner (1983) concluded that the great variability in banding patterns of peroxidase isozyme precludes their use for determining the degree of relationship or diagnosis of graft incompatibility. Vinterhalter and James (1983) showed the use of the peroxidase polymorphism in the identification of apple scion cultivars. They reported that the isozyme patterns are consistent for the cultivars and independent of season, age or eco-physiological conditions of the plant which permits cultivar identification both throughout the year and during the juvenile phase of growth.

Menendez *et al.* (1986a) used isozyme analysis for the identification of apple rootstock cultivars. Vinterhalter and James (1986) showed that peroxidase polymorphism could be used in the identification of Malling and Malling Merton apple rootstocks. The usefulness of isozyme banding patterns as genetic marker in peach was investigated using starch gel electrophoresis by Durham *et al.* (1987). Bryne and Littleton (1988) used leucine aminopeptidase and 6-phosphogluconate dehydrogenase isoenzyme analysis to confirm the F1 hybrid origin of Hann almond, Pollardi, Rogani Goy and PIII 7679.

Sufficient polymorphism at the MDH locus has been identified to permit its use as a biochemical genetic marker in interspecific hybridizations (Hancock and Iezzoni, 1988). Bryne and Littledon (1989) used isozyme analysis for the verification of interspecific hybrids of plum x apricot hybrids. Friend and Carter (1989) carried out isozyme analysis for using it as a biological marker for identifying *Prunus* species and F1 hybrids.

Samimy and Cummins (1992) distinguished apple rootstocks by isozyme banding patterns. Tang and Zhang (1992b) carried out studies on the preselection of dwarf type apple seedling by starch gel electrophoresis. Zhang *et al.* (1992) showed that utilization of marker bands of isozyme for preselection of dwarf type apple trees is effective, and the inheritance of marker bands of isozyme is reliable.

Martelli *et al.* (1993) used isozyme analysis to investigate genetic variation of 5 apple clonal rootstocks. Isozyme polymorphism was observed among regenerants and based on banding patterns, rootstocks and regenerants could be distinguished.

Chung *et al.* (1995) identified and classified fifty *Pyrus* taxa by isozyme polymorphisms. Isozyme analysis and cluster analysis using isoenzyme band patterns were useful tools for taxon identification and for determining putative taxonomic relationships.

2.3 ELECTROPHORETIC METHODOLOGIES

Following the classical studies of Tiselius (1937b) on the electrophoretic properties of human plasma proteins using the free-boundary procedure, a number of investigators introduced solid support material to the solution medium in an attempt to stabilize the bands of macro-ions observed during electrophoresis (Suber *et al.*, 1965). The most common material used today is polyacrylamide the application of which was originally investigated by Raymond and Weintraub (1959). Horizontal system was given by Beckman and Johnson (1964) and vertical system by Shaw and Koen (1968).

Several books and review papers are now available on the theory and techniques of electrophoresis (e.g. Harris and Hopkinson, 1976; Soltis *et al.*, 1983; Tanksley and Orton, 1983; Werth, 1985).

Horizontal starch gel electrophoresis was employed as a tool in characterising California strawberry cultivars (Bringhurst *et al.*, 1981). Sheilds *et al.* (1983) compiled information regarding the equipment needed, various buffer systems and generalised methodologies for starch and acrylamide gel electrophoresis.

2.4 SOURCE TISSUE

Different types of tissues have been used for the enzyme extraction and the literature available is reviewed as under.

Vinterhalter (1979) separated peroxidase isozymes from callus tissue of Golden Delicious apple by electrophoresis. Electrophoretic analysis of peroxidase isozyme was carried out on leaves and cambial tissue of the Callery pear cultivars by Santamour and Demuth (1980). With the seedlings, leaf peroxidases were distinct in several cases.

A modified method for the polyacrylamide gel electrophoresis of malate dehydrogenase and extracted from pollen of apple and black currant varieties was described by Samorodova *et al.* (1981). Cheng *et al.* (1984) carried out peroxidase isozyme analysis of intervarietal hybrids in apple using cotyledons and leaves of selfed seedlings as the source tissue.

Weller and Costante (1986) analysed shoot bark and root extract of 16 different apple rootstocks for multiple electrophoretic forms of peroxidase using polyacrylamide gel electrophoresis. Seed and leaf tissue derived from controlled crosses in the apple were analysed for catalase, esterase and peroxidase by Korban and Bournival (1987).

Menendez *et al.* (1987) identified *Pyrus* germplasm through combined spectroscopy of intact leaf lamina and isozyme analysis of stem tissue.

Biles and Abeles (1991) collected xylem sap from apple, peach and pear twigs by means of pressure extrusion. The sap contained a number of acidic peroxidases and other proteins.

Jang *et al.* (1991) identified *Pyrus* species by peroxidase isozyme phenotypes of flower buds. Liu-KiuYing *et al.* (1994) carried out quantitative study of peroxidase isozymes in apple leaves.

Murata *et al.* (1995) examined the expression and induction of polyphenol oxidase in apple cultures. The isozymes expressed in cell culture were different from those in fruit and leaf.

2.5 EXTRACTION

In systematic evolutionary, and ecological studies, protocols for the extraction of enzyme from plant tissues are among the least discussed aspects of the experimental procedures with some notable exceptions, as reported by Kelley and Adams (1977) and Soltis *et al.* (1983).

Isozymes have not been exploited fully in tree fruits and nut crops and only limited studies have been reported (Torres, 1983). This limited progress is attributed, in a part, to technical problems associated with the extraction of active enzymes from plant tissue.

The presence of phenolic compounds within the tissue of these woody perennial species interferes with the enzyme extraction and enzyme assay (Loomis, 1969; Loomis, 1974). Due to the binding of natural pigments and unstable compounds, especially phenolics and quinones to proteins, protein denaturation or enzyme inactivation occures and which leads to almost invariably poor electrophoretic separation. Addition of reducing agents, polyphenol oxidase inhibitors and synthetic polymers may over come these problems (Anderson and Rowan, 1967).

The polyphenol oxidase system in Clingstone peach was investigated. Polyacrylamide disc gel electrophoresis indicated four bands with polyphenol oxidase activity in extract from acetone powder of Clingstone peach (Wong *et al.*, 1971).

Lee (1973) stated that peroxidase in tobacco callus tissue differed in extractability depending on the subcellular distribution of enzymes and based on the extractability it consisted of four fractions, freely soluble and less freely soluble in phosphate buffer, KCl soluble and insoluble.

Kuhns and Fretz (1978) developed the procedure to extract proteins and active enzymes from rose leaves. A suitable extraction medium, extraction time and sample application rate was determined for each system in which bands could be separated.

Plants generally require more complex buffers (Werth, 1985) while animal tissue and some edible plants may be extracted by grinding tissues in relatively simpler buffers.

Plant ground in minimal volumes may be insufficiently buffered or protected from phenolics (Wendel and Weeden, 1989) leading to low band intensity and or artifactual shadow or ghost bands. Friend and Carter (1989) ground the leaf samples using liquid nitrogen.

Recently Raman and Dhillon (1994) used cold sucrose solution (12%) containing Beta-mercaptoethanol (0.1%) for making the extracts.

2.6 BUFFER SYSTEM

A buffer system consists of a gel buffer used in preparing the gel and an electrode buffer, which is an ionized solution that conducts current through the gel during a run.

Buffer system may be continuous (Meizel and Markert, 1967) or discontinuous (Scandalios, 1969). Discontinuities of buffer lead to the compactness of the protein bands thereby sharpening resolution (Smith, 1976). Lower molarities of a given buffer yield faster migration rates (Andrews, 1981).

Large number of buffer systems are available in the literature e.g. Orstein, (1964); Davis (1964); Sheilds *et al.* (1983); Soltis *et al.* (1983); Stuber *et al.* (1988); Wendel and Weeden (1989).

2.7 STAINING PROCEDURES

Preparing the stain for each enzyme assay poses logistical problems. Some staining protocols available are from Shaw and Koen (1968); Scandalios (1969); Shaw and Prasad (1970); Lee (1973); Schwennesen *et al.* (1982); Vallejos (1983); Arulsekar and Parfitt (1986) and Cousineau and Donnelly (1992).

Dual staining of isozymes for both peroxidase and polyphenol oxidase on the same gel was carried out by Vellejos (1983).

The chemical bases of the enzymatic reactions have been reviewed in a variety of publications e.g. Harris and Hopkinson (1976) and Pasteur *et al.* (1988).

2.8 GENETIC INTERPRETATION

Stavrakakis and Loukas (1983) reported genetic variation between and within grape cultivars. They obtained the similarity values between cultivars. Wendel and Parks (1985) used starch gel electrophoresis to score allelic variation at 20 loci in seeds collected from 60 populations distributed throughout the species range. In comparison to other plant species, the level of genetic diversity with in *Camellia japonica* populations was very high. In addition, principal component analysis revealed that populations tends to genetically cluster into four regions representing the geographic areas.

Hyun *et al.* (1987) studied the genetic variation on the basis of the isozyme studies conducted on root tips of 200 *Populus tremuloides* clones selected from eight geographic regions among north-south and east-west transects in Ontario. Rajora (1989) obtained the genetic structure and identified *Populus deltoides* clones on the basis of allozymes. Rajora and Zsuffa (1990) used horizontal starch gel electrophoresis to study the genetic divergence and evolutionary relationships among *Populus deltoides, P. nigra and P. maximowiczii.*

Isozyme variation in 13 species of *Cucumis*, including *C. melo and C. sativus* was studied for three enzyme systems viz. peroxidase, GOT and GDH. There was very little similarity among the species for the three isozymes put together (Sujatha *et al.*, 1991). Aradhya and Philips (1993) evaluated the genetic variability in fourteen provenances of *Eucalyptus* species in Hawaii. Raman and Dhillon (1994) studied the isozyme polymorphism and phylogenetic relationship among the species of *Citrus, Fortunella and Poncirus.*

Goncharenko *et al.* (1994) studied the allozyme variation in natural populations of Eurasian pines. Estimated parameters of gene diversity, genetic distance and gene flow showed that *Pinus sylvestris* populations studied shared a common gene pool. Ferguson and Robertson (1996) carried out a survey of allozyme polymorphism at 11 loci to study the genetic diversity and taxonomic relationships within the genus *Lens.*

Lorenzo *et al.* (1996) analyzed two hundred and ninety five trees sampled from seventy five local chestnut cultivars in north western Spain to describe the intracultivar and intercultivar variability by isozyme analysis and to establish a classification of the cultivars. Schiller and Korol (1997) carried out electrophoretic analysis of diversity within *Cupressus sempervirens* L. growing in Israel.

MATERIALS AND METHODS

3

MATERIALS AND METHODS

The present investigation entitled "Molecular characterisation of clonal apple rootstocks using isozymes" was carried out in the Department of Biotechnology, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan. Different rootstocks viz., M.3, M.4, M.7, M.9, M.26, MM.106 and MM.111 were selected on the basis of anchorage, precocity, productivity, relative tree growth rate, ultimate size and response to hazards of soil environment covering dwarfing, semi-dwarfing and vigorous characteristics. On the basis of literature available the following five enzymes were selected to be studied during the present investigation.

a.	Acid phosphatase (ACP)	(EC 3.1.3.2)
b.	Esterase (EST)	(EC 3.1.1.1)
c.	Malate Dehydrogenase (MDH)	(E.C. 1.1.1.37)
d.	Peroxidase (PER)	(EC 1.11.1.7)
e.	Polyphenol oxidase (PPO)	(EC 1.10.3.2)

The following methodologies have been adopted to study isozyme patterns:

3.1 COLLECTION OF MATERIAL

For the analysis of isozymes, fresh and young leaves were collected during summer season from above mentioned seven clonal rootstocks of apple, growing in the field of Pomology and Department of Plant Pathology, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni.

3.2 EXTRACTION OF ENZYME

Fresh, young and fully expanded leaves from the rootstocks were excised early in the morning. Leaves were washed thoroughly and were homogenised mechanically in a cold pestle and mortar with a pinch of acid washed sand to assist grinding. Leaf samples were ground in an extraction buffer at a ratio of 5 ml buffer per gram fresh weight of tissue. The extraction buffer used was Tris citrate buffer (0.1 M, pH 8.3) (Appendix-I) with various protectants, to prevent phenol oxidation. 0.1 g of insoluble polyvinyl pyrolidone was added during crushing. The crude extract was filtered through cheese cloth and then this homogenate was centrifuged at 25,000 rpm for 20 min at 4°C. The residue was discarded and the supernatant was stored in the freezer for the enzymatic studies.

3.3 BUFFER SYSTEM

A discontinous buffer system of Shaw and Koen.(1968) with a slight modifications of Scandalios (1969), was used. (Appendix-II)

3.4 GEL PREPARATION

Gels were prepared in 12x25 cm^2 glass slabs horizontally held by clamps with 2.5 cm spacer of 4.0 per cent (T=30.8, C=2.6) and the resolving gels were of different percentage for different enzymes.

	ENZYME	RESOLVING GEL PERCENTAGE
a.	Acid phosphatase	9.2
b.	Esterase	9.2
c.	Malate dehydrogenase	9.2
d.	Peroxidase	7.7
e.	Polyphenol oxidase	10.2

(Appendix-III)

3.5 SAMPLE LOADING

The gels were allowed to cool at 5°C for 20 min before the sample was loaded. One drop of extract was loaded into the wells formed in the gel, with a micropipette and tracking (marker) dye used was bromophenol blue.

3.6 RUN CONDITIONS

The buffer tanks were filled two third with lithium borate buffer (Appendix-II). The gels were placed in the separation chamber and the contact between the gel and the buffer was made with the help of Whatman filter paper-wicks. The resolution was obtained by applying a constant current of 50 mA.

3.7 REMOVAL OF THE GELS

After the completion of the electrophoretic run, the wicks were removed and the gels were taken out and were immediately kept in the staining buffer for incubation, if required, or were directly kept in the staining solution.

3.8 CALCULATION OF Rm VALUES

The Rm values were calculated by measuring the distance travelled by the marker (tracking) dye as well as the bands. The Rm values were calculated as follows:

$$\text{Relative mobility value (Rm)} : \frac{\text{Distance travelled by protein molecule}}{\text{Distance travelled by the dye(solvent)}}$$

3.9 STAINING PROCEDURES

After the electrophoresis was completed, gels were immediately kept for the staining, for the presence of enzymes and the staining procedures were specific for each enzyme as follows:

i) ACID PHOSPHATASE (E.C. 3.1.3.2)

(Arulshekar and Parfitt, 1986)

Reagents

Alpha - Napthyl phosphate	-	100mg (Dissolved in 2 ml of 50% aqueous acetone)
Fast Red TR salt	-	100 mg
1 per cent Magnesium chloride	-	1ml

Staining buffer

0.1 M Sodium acetate	-	pH 5.0

Procedure

The gels were first incubated in the staining buffer for about 10 minutes and then gels were placed in staining solution which was prepared by dissolving 100 mg fast red TR salt in the 0.1M sodium acetate buffer (pH 5.0) to which was added alpha - napthyl phosphate and 1 ml of magnesium chloride just prior to use and the stain was mixed thoroughly by shaking. The gels were incubated in the staining solution at 37°C and kept in dark until the bands developed. The position of bands was noted and the Rm values were calculated.

ii) ESTERASE (E.C. 3.1.1.1)

(Kuhns and Fretz, 1978)

Reagents

Fast blue RR salt	-	70 mg
Alpha - Napthyl acetate	-	2 ml (1.0% in 1 acetone : 1 Water)

Staining buffer

0.1 M Sodium phosphate buffer - pH 6.2

Procedure

After the completion of run, gels were incubated in the staining buffer for about 10 minutes and then the gels were placed in the staining solution prepared by dissolving 70 mg fast blue RR in the 100 ml of 0.1 M sodium phosphate buffer and filtered prior to use, and to the filtrate was added 2 ml of alpha - napthyl acetate. Gels were incubated in the staining solution for about an hour at room temperature. The position of the bands were noted and the Rm values were calculated.

iii) MALATE DEHYDROGENASE (E.C. 1.1.1.37)

(Estilai *et al.*, 1990)

Reagents

DL-malic acid	-	2.68 g
Sodium carbonate	-	2.14 g

DL-malic acid and sodium carbonate were dissolved separately in 5 ml distilled water. Sodium carbonate was slowly added to the malic acid over an ice bath and the pH was adjusted to 8.0 with NaOH.

Nicotinamide Adenine Dinucleotide(NAD)	-	10mg
Nitro Blue Tetrazolium (NBT)	-	10mg
		(Dissolved in 1 ml methanol)
Phenazine Methosulphate (PMS)	-	5 mg

Staining buffer

0.1 M Tris Cl - pH 8.0

Procedure

The staining solution contained 10 ml of solution (a) and 90 ml of solution (b) and to which was added 10 mg NBT, 5 mg PMS and 1 ml NBT. The gels were incubated in the staining solution in dark at 37°C until the bands were developed. The position of the bands were noted and the Rm values were calculated.

iv) PEROXIDASE (E.C. 1.11.1.7)

(Lee, 1973)

Reagents

Benzidine	-	50 mg (Dissolved in 1 acetic acid : 1 H_2O)
H_2O_2 30 %	-	0.1 ml
Staining buffer		
0.1 M Sodium acetate	-	pH 4.5

Procedure

The gels were first incubated at room temperature in the staining buffer for about 10 minutes and then the gels were placed in the staining solution. The staining solution contained 50 mg of benzidine and 0.1 ml of 30 per cent hydrogen peroxide which was added just prior to use. The bands were developed immediately. The position of bands were noted and the Rm values were calculated.

v) POLYPHENOL OXIDASE (E.C. 1.10.3.2.)

(Vallejos, 1983)

Reagents

Catechol	-	0.5 g
Sulphanilic acid	-	0.5 g

Staining buffer

0.1M Sodium acetate - pH 5.0

Procedure

After the electrophoresis was over the gels were incubated first in the staining buffer for about 10 minutes and then they were placed in the staining solution. The staining solution contained 0.5 g of catechol and 0.5 g of sulphanilic acid dissolved in 100 ml of the 0.1M sodium acetate buffer (pH 5.0). The gels were incubated in dark at room temperature for about one and a half hour. The position of bands were noted and the Rm values were calculated.

In case of all the enzymes, after staining when bands were developed gels were taken out, washed and photographs were taken.

N.B. : All the stains were prepared in amber coloured bottles to avoid exposure to light because of the sensitivity of the stains to the light.

3.10 NOMENCLATURE OF LOCI AND ALLELES

The zymograms were divided into different zones of activity which were termed as loci and the bands present in zones were termed as **allozymes or alleles**. The fastest moving zone was termed as Locus I and the slower moving zone were labelled accordingly as I, II, III. Similarly the bands or alleles present in a locus were numbered in the descending order i.e. the faster moving anodal band was numbered 1 and the rest accordingly.

3.11 GENETIC INTERPRETATIONS

The genetic interpretations were carried out on the basis of following parameters:

3.11.1 ALLELE FREQUENCY

The probable frequency of alleles for a particular locus was the proportion of a particular allele present in that locus.

3.11.2 GENETIC VARIATION

Genetic variation was determined on the basis of the following six parameters :

3.11.2.1 PERCENTAGE OF POLYMORPHIC LOCI

A locus was said to be polymorphic if the frequency of the most common allele was not more than 99 per cent.

3.11.2.2 AVERAGE NUMBER OF ALLELES PER LOCUS

It was computed as :

$$A = \frac{\text{Total number of alleles}}{\text{Total number of loci}}$$

3.11.2.3 EFFECTIVE NUMBER OF ALLELES PER LOCUS

It was calculated according to Crow and Kimura (1970):

$$n_e = \frac{1}{\sum_i p_i^2}$$

where, p_i is the frequency of ith allele.

3.11.2.4 HETEROZYGOSITIES

It was calculated according to Nei (1975).

a) Observed heterozygosity (h_o) is the proportion of all the genotypes that were heterozygotes.

Mean observed heterozygosity (H_o) is the arithmetic mean of all the observed heterozygosities for a rootstock.

b) Expected heterozygosity (h_e) is as under

$$h_e = (1-\sum_i p_i^2)$$

where, p_i is the frequency of ith allele

$$\text{Mean expected heterozygosity } (H_e) = \frac{\sum(H_e)}{n}$$

where, n = Number of loci studied in a rootstock

3.11.2.5 POLYMORPHIC INDEX

It was calculated according to Allard *et al.* (1978):

$$PI = \frac{1}{m\sum_{ij} P_{ij}(1-P_{ij})}$$

where, P_{ij} is the frequency of ith allele at jth locus and m is the total number of loci.

3.11.2.6 WRIGHT'S FIXATION INDEX (F)

It was given by Wright (1965) and it measures the net deviation of genotype frequencies from Hardy - Weinberg expectations. It is calculated as :

$$F = 1 - H_o / H_e$$

3.11.3 DIVERSITY INDEX

Diversity index was calculated according to Hunter and Kannenberg (1971) with slight modifications. The enzyme systems of two rootstocks were compared both qualitatively and quantitatively. Presence and absence of bands were taken as qualitative differences and the quantity parameter considered were thickness and darkness of the band. The individuals with identical bands in a particular system were rated as zero. The summation of qualitative and quantitative scores was taken as the total diversity index.

3.11.4 GENETIC DISTANCE

It is the measure of genetic differentiation based on gene identity between rootstocks. It is calculated according to the formula given by Nei (1975). Genetic distance between two rootstocks is:

$$D = -\ln\left(J_{xy} / \sqrt{J_x . J_y}\right)$$

where, J_x, J_y and J_{xy} are the arthmetic mean of j_x, j_y and j_{xy}, respectively. For this, all loci including monomorphic loci were taken, and

$$j_x = x_i^2$$
$$j_y = y_i^2$$
$$j_{xy} = x_i y_i$$

where, x_i and y_i are the sample gene frequency of the ith allele in the rootstocks x and y, respectively.

3.11.5 SIMILARITY COEFFICIENT

The similarity coefficient (I), between any pair of rootstocks x and y was calculated by using the following formula (Stavrakakis and Loukas, 1983):

$$I = \frac{\sum_i C_i}{\sum_i (C_i + U_i)}$$

where, C_i is the number of common (homologous) bands between the rootstocks x and y for enzyme i, and U_i is the number of uncommon (non-homologous) bands. I ranges from 0 (no band in common) to 1 (all bands in common).

3.11.6 STATISTICAL ANALYSIS

Principal component analysis (a multivariate statistical analysis) was carried out to find correlation between different rootstocks on the basis of the various genetic variation parameters and the first two principal components were plotted on the graph.

EXPERIMENTAL RESULTS

4

EXPERIMENTAL RESULTS

The results obtained during this investigation are as follows:

4.1 PHENOTYPE

4.1.1 Acid phosphatase

Three different zones of activity were observed for acid phosphatase in the leaf extracts of apple rootstocks (Plate-I). These were designated as loci ACP-I, ACP-II and ACP-III in descending order with respect to their relative mobilities.

The first two zones showed only single band i.e. ACP-I(1) and ACP-II(1) with Rm values 0.74 and 0.65. Both these zones were light and lacked clarity. Both ACP-I and ACP-II were present in all the seven rootstocks. The third zone of enzyme activity showed two bands which were sharp and distinct. ACP-III(1) and ACP-III(2) were having Rm values of 0.34 and 0.29, respectively. ACP-III(1) with Rm value 0.34 was observed in all the rootstocks, the band was sharp and distinct. Isozyme band with Rm value 0.29, was absent in M.7, M.9 and MM.111.

4.1.2 Esterase

This enzyme system showed the least variation in all the rootstocks. Plate-II showed two distinct zones of activity for the esterase, which were present in all the rootstocks.

The locus-I showed three bands EST-I(1), EST-II(2) and EST-III(3) with Rm values 0.78, 0.73 and 0.66, respectively. These bands were not very distinct. The locus EST-II showed a single band EST-II(1) with a Rm value of 0.35. This band was conspicuous and common for all the rootstocks.

4.1.3 Peroxidase

This enzyme system showed maximum variation among the rootstocks. Five zones of activity were observed for peroxidase which are PER-I, PER-II, PER-III, PER-IV and PER-V. The 1st zone of activity showed three bands i.e. PER-I(1), PER-I(2), and PER-I(3) with Rm values 0.87, 0.83 and 0.8, respectively. PER-I(1) was absent in rootstocks M.26, MM.106 and MM.111. The 2nd zone of activity showed three bands i.e. PER-II(1), PER-II(2), PER-II(3) with Rm values 0.73, 0.71 and 0.67, respectively. PER-II(1) was present in rootstocks M.9, M.26 and MM.111. PER-II(2) was present in rootstocks M.3, M.4 and MM.106 and PER-II (3) was present in M.7, M.9, M.26 and MM.111. The 3rd zone of activity showed two bands, PER-III(1) and PER-III(2) with Rm values 0.6 and 0.56. Rootstock M.7 lacked the band at Rm value 0.6 and the band at Rm value 0.56 was present only in M.7. The 4th zone of enzyme activity was designated at PER-IV and this showed a single band PER-IV(1) with Rm value 0.38 and was present in all the rootstocks. The 5th zone of enzyme activity showed two bands PER-V(1) and PER-V(2) with Rm values 0.27 and 0.21, respectively. PER-V(1) was present only in rootstock M.3 but PER-V(2) was present in all the rootstocks.

4.1.4 Polyphenol oxidase

Plate-IV showed two zones of activity for polyphenol oxidase namely PPO-I and PPO-II. 1st zone showed a single band PPO-I(1) with Rm value 0.48. PPO-I is present only in rootstocks M.3 and M.4. The 2nd zone of activity showed two bands PPO-II(1) and PPO-II(2) having Rm values 0.43 and 0.36. PPO-II(1) was absent in M.3, M.4 and M.7. PPO-II(2) was a dark and distinct band, present in all the rootstocks.

4.1.5 Malate dehydrogenase

Four different zones of enzyme activity were observed in malate dehydrogenase. These zones were designated as MDH-I, MDH-II, MDH-III and MDH-IV. Each zone of activity showed a single band MDH-I(1), MDH-II(1), MDH-III(1) and MDH-IV(1) with Rm values

4.4 MONOMORPHIC LOCI

A total of 9 loci were monomorphic i.e. showed only single band. These were ACP-I, ACP-II, EST-II, PER-IV, PPO-I, MDH-I, MDH-II, MDH-III and MDH-IV.

4.5 POLYMORPHIC LOCI

Out of the 16 loci studied, seven loci were found to be polymorphic. They showed two or more bands (alleles). These were ACP-III, EST-I, PER-I, PER-II, PER-III, PER-V and PPO-II.

4.6 GENETIC VARIATION

Genetic variation among the various apple rootstocks was explained on the basis of various parameters. The percentage of polymorphic loci varied from 13.33 per cent in M.7 to 35.71 per cent in M.26. Polymorphic index ranged from 0.028 to 0.066, it was maximum in M.7. M.26 showed the highest value for the average and effective number of alleles per locus which was 1.357 and 0.09 respectively. The value of fixation index (F) was -1 for all the rootstocks. In each case, the observed heterozygosity (H_o) was 1 and the expected heterozygosity (H_e) was 0.5 (Table 8).

The pairwise comparison of diversity index on the basis of isozyme pattern showed a range from 7 to 14 (Table 9). The minimum diversity was found between M.7 and M.9, M.9 and M.26, M.26 and MM.111, and the maximum diversity was obtained in M.3 and M.9, M.7 and MM.106.

4.7 SIMILARITY INDEX AND GENETIC DISTANCE

It was observed that similarity index on the basis of five enzyme systems varied from 0.583 between M.3 and M.7 to 0.9 between M.26 and MM.111 (Table 10). MM.111 and M.9, M.26 and M.9 also showed higher values for similarity index.

On the basis of pairwise comparison the genetic distance between various rootstocks showed that it ranged from 0.0 to 0.257. M.9 and MM.106 showed less genetic distance (0.014) while M.3 and M.9 showed more genetic distance (0.257). The genetic distance between M.7 and M.9, M.26 and MM.106 was observed to have negative values i.e. -0.017 and -0.039, respectively (Table 11).

4.8 PRINCIPAL COMPONENT ANALYSIS

Principal component analysis was carried out on the basis of genetic variations parameters. The first two components explained 100 per cent variation therefore only first two components were plotted on the graph (Figure 6). On the basis of the PCA analysis, the rootstocks seems to be divided into two groups. The first group contains rootstocks M.4 and M.7 and second group contains M.3, M.9, M.26, MM.106 and MM.111. M.3 and MM.111 lie at the same point.

Plate 1. Photograph showing isozyme pattern of acid phosphatase

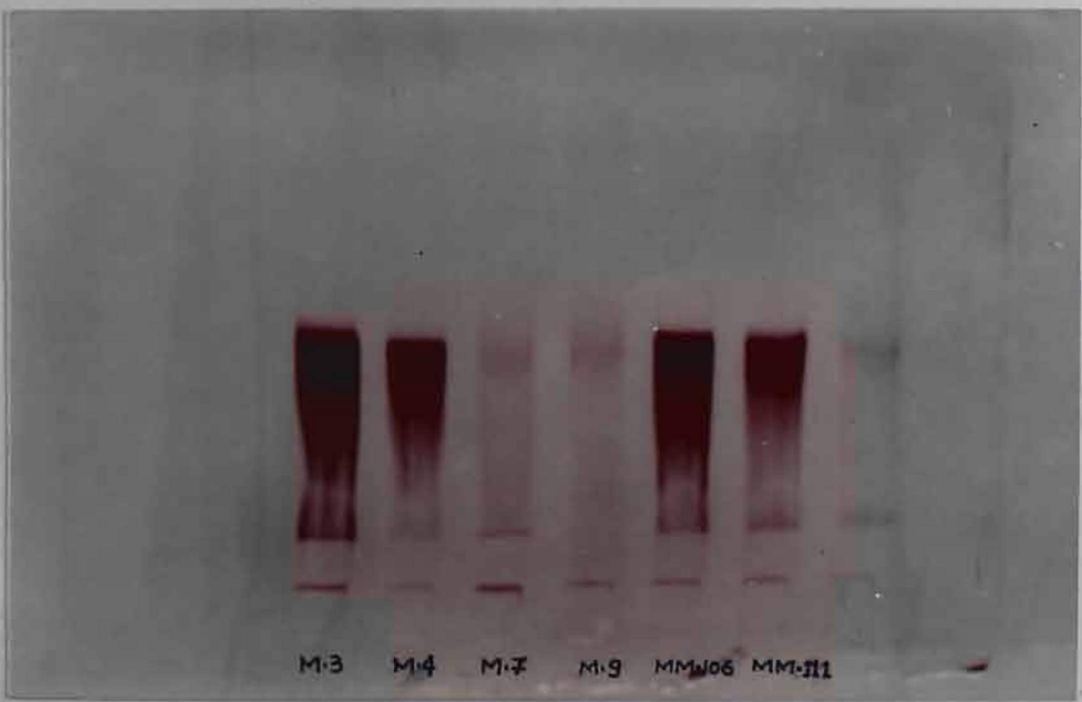

PLATE 1.

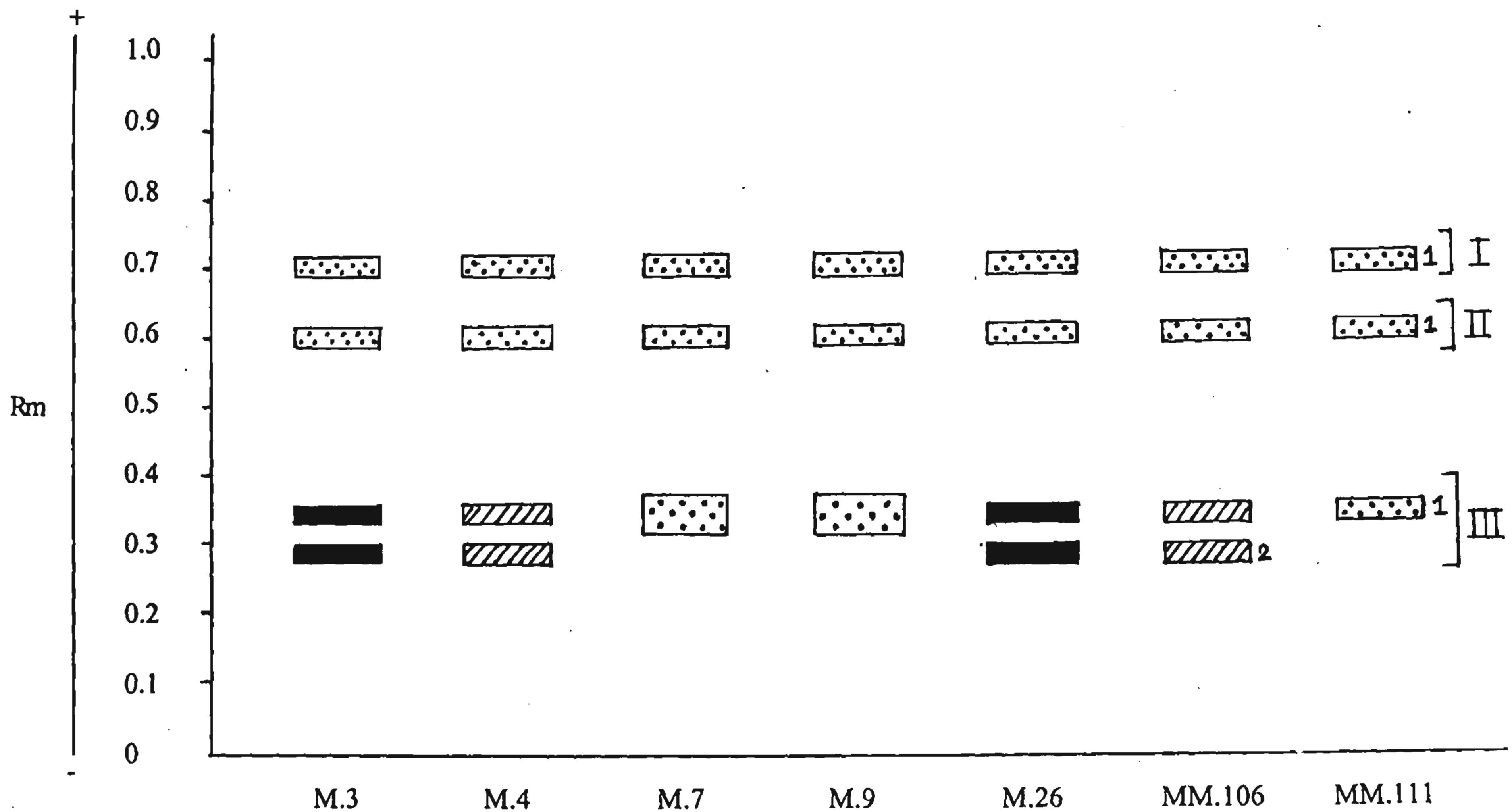

Fig. 1. Zymogram pattern of acid phosphatase isozymes

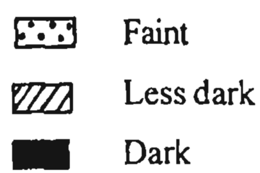

Plate 2. Photograph showing isozyme pattern of esterase

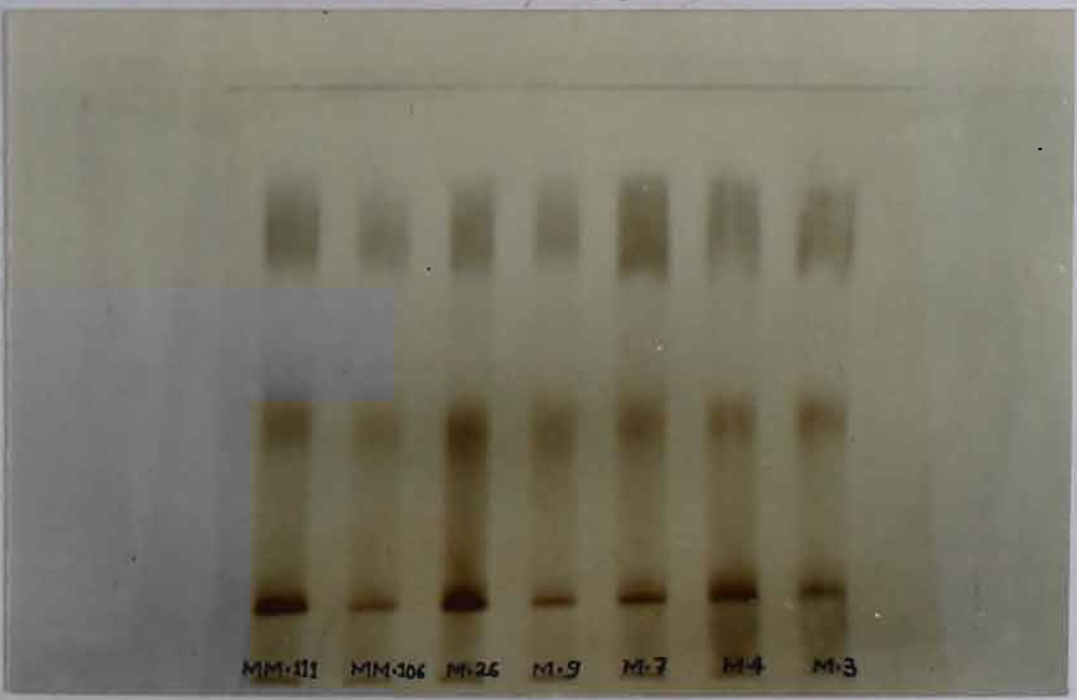

PLATE 2.

Plate 3. Photograph showing isozyme pattern of peroxidase

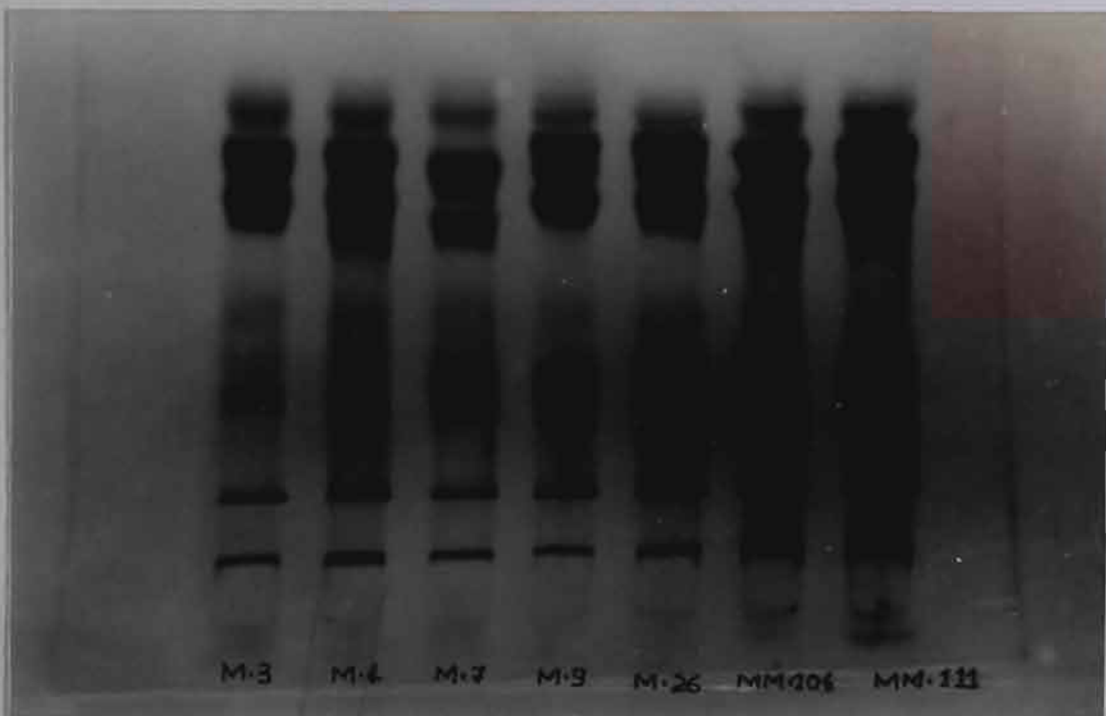

PLATE 3.

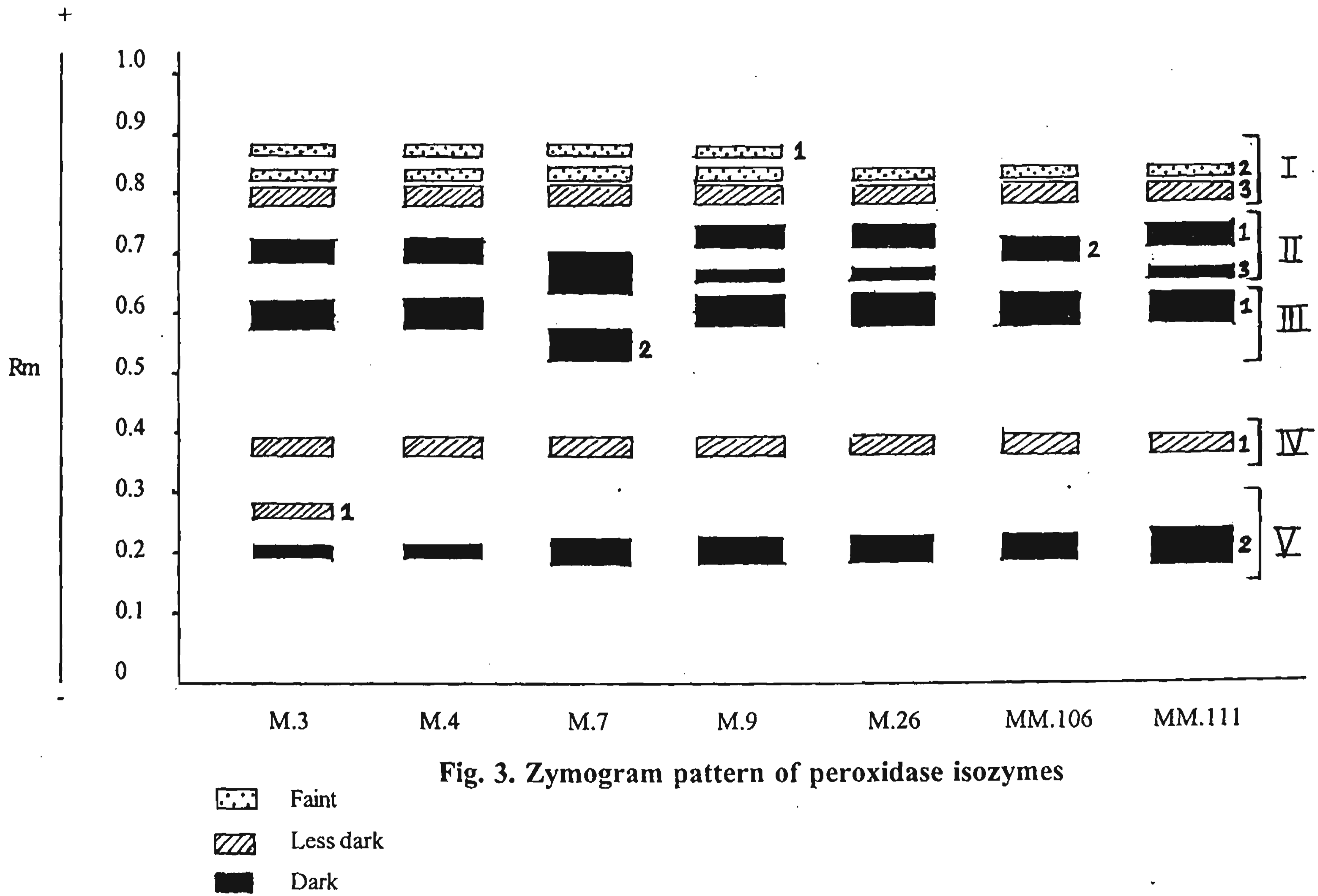

Fig. 3. Zymogram pattern of peroxidase isozymes

Plate 4. Photograph showing isozyme pattern of polyphenol oxidase

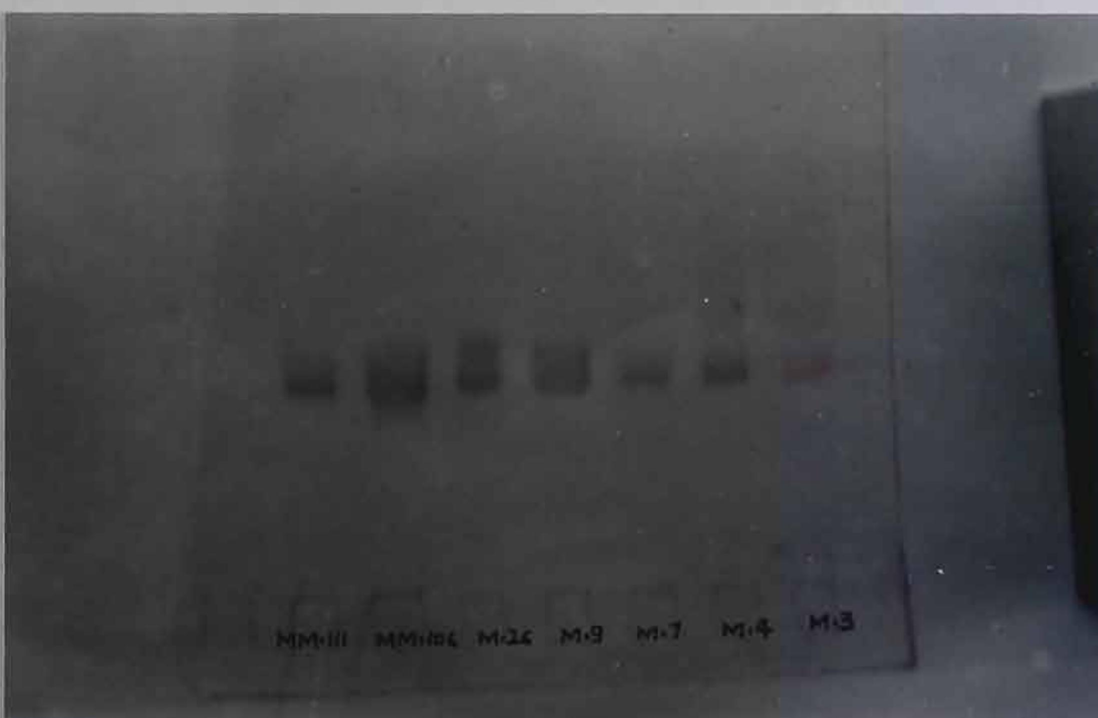

PLATE 4.

Plate 5. Photograph showing isozyme pattern of malate dehydrogenase

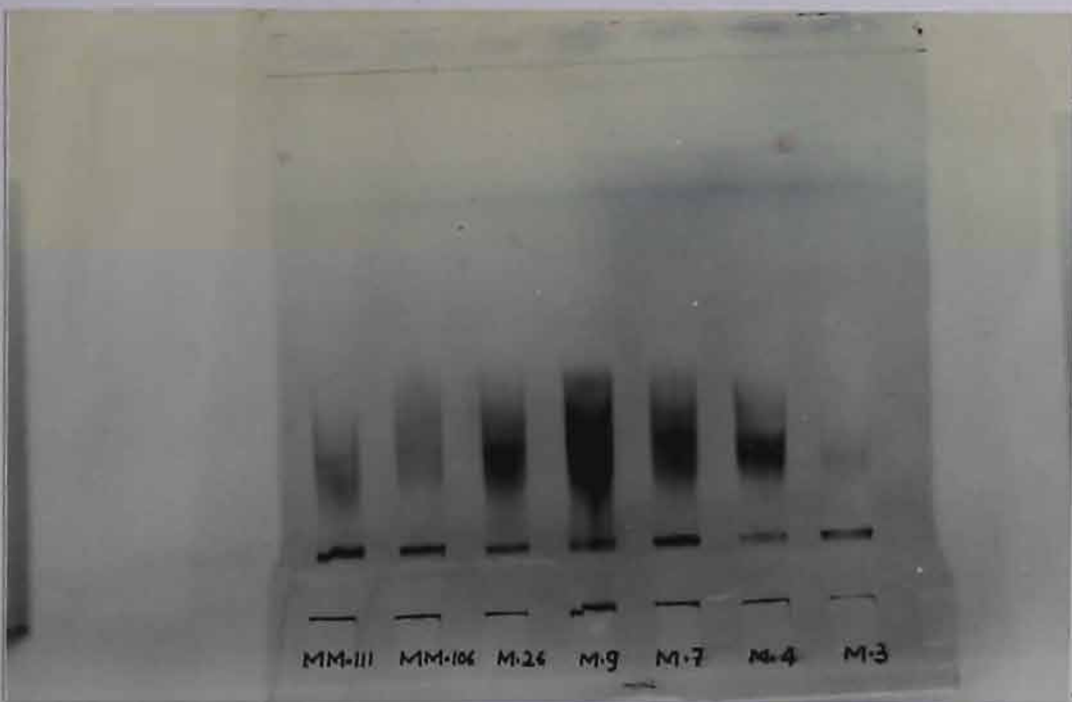

PLATE 5.

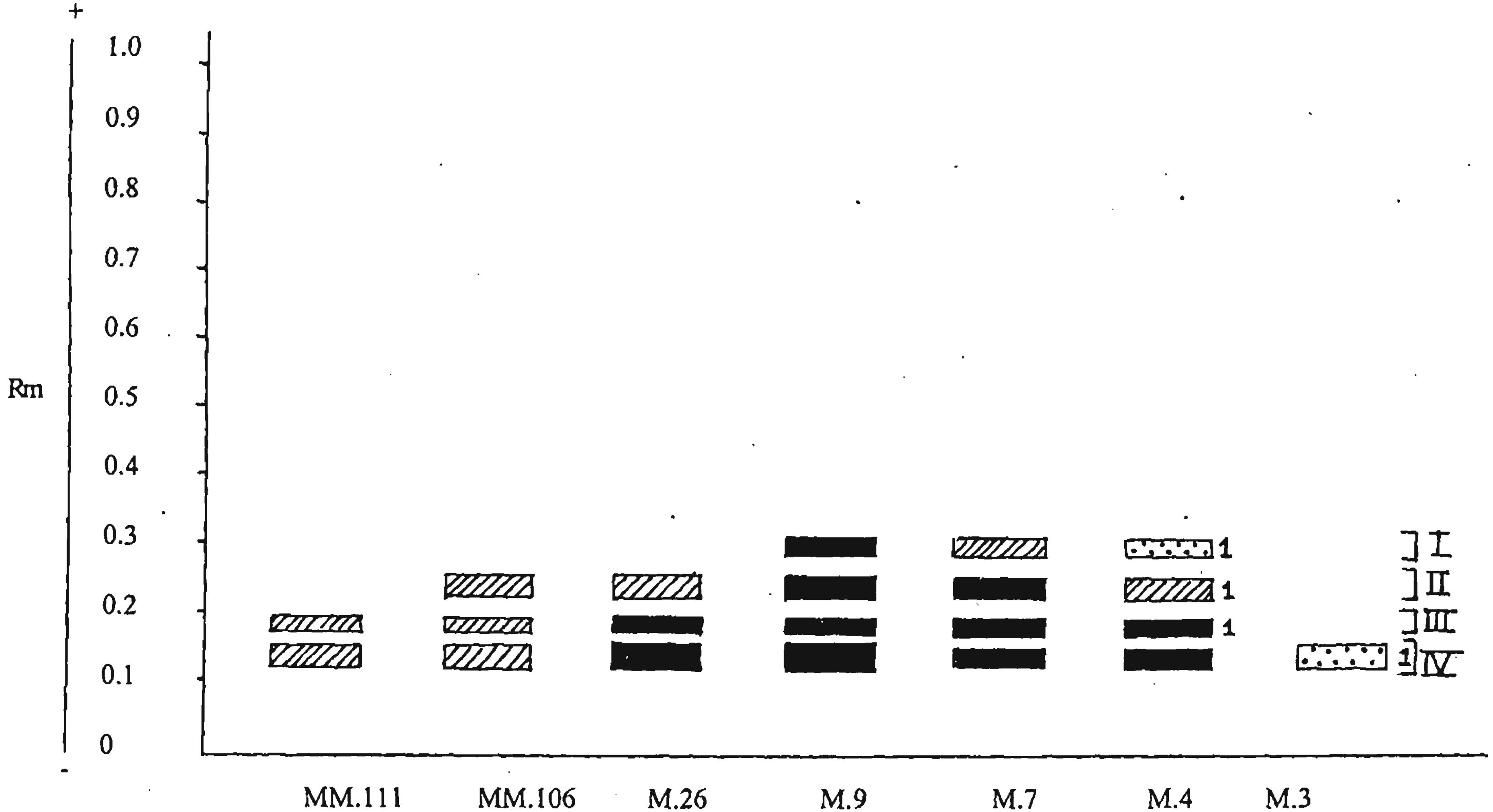

Fig. 5. Zymogram pattern of malate dehydrogenase isozymes

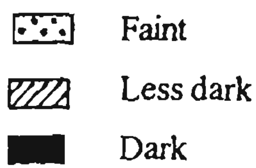

Table 1. Probable genotype, genotype frequency, allele(s) and allele frequencies at various loci in M.3

Locus	Genotype	genotype frequency	Allele(s)	Allele frequency	h_o	h_e
ACP-I	11	1	1	1	-	-
ACP-II	11	1	1	1	-	-
ACP-III	12	1	1&2	0.5&0.5	1	0.5
EST-I	13	1	1&3	0.5&0.5	1	0.5
EST-II	11	1	1	1	-	-
PER-I	13	1	1&3	0.5&0.5	1	0.5
PER-II	22	1	2	1	-	-
PER-III	11	1	1	1	-	-
PER-IV	11	1	1	1	-	-
PER-V	12	1	1&2	0.5&0.5	1	0.5
PPO-I	11	1	1	1	-	-
PPO-II	22	1	2	1	-	-
MDH-I	Absent					
MDH-II	Absent					
MDH-III	Absent					
MDH-IV	11	1	1	1	-	-

Table 1. **Probable genotype, genotype frequency, allele(s) and allele frequencies at various loci in M.3**

Locus	Genotype	genotype frequency	Allele(s)	Allele frequency	h_o	h_e
ACP-I	11	1	1	1	-	-
ACP-II	11	1	1	1	-	-
ACP-III	12	1	1&2	0.5&0.5	1	0.5
EST-I	13	1	1&3	0.5&0.5	1	0.5
EST-II	11	1	1	1	-	-
PER-I	13	1	1&3	0.5&0.5	1	0.5
PER-II	22	1	2	1	-	-
PER-III	11	1	1	1	-	-
PER-IV	11	1	1	1	-	-
PER-V	12	1	1&2	0.5&0.5	1	0.5
PPO-I	11	1	1	1	-	-
PPO-II	22	1	2	1	-	-
MDH-I	Absent					
MDH-II	Absent					
MDH-III	Absent					
MDH-IV	11	1	1	1	-	-

Table 2. **Probable genotype, genotype frequency, allele(s) and allele frequencies at various loci in M.4**

Locus	Genotype	genotype frequency	Allele(s)	Allele frequency	h_o	h_e
ACP-I	11	1	1	1	-	-
ACP-II	11	1	1	1	-	-
ACP-III	12	1	1&2	0.5&0.5	1	0.5
EST-I	13	1	1&3	0.5&0.5	1	0.5
EST-II	11	1	1	1	-	-
PER-I	13	1	1&3	0.5&0.5	1	0.5
PER-II	22	1	2	1	-	-
PER-III	11	1	1	1	-	-
PER-IV	11	1	1	1	-	-
PER-V	22	1	2	1	-	-
PPO-I	11	1	1	1	-	-
PPO-II	22	1	2	1	-	-
MDH-I	11	1	1	1	-	-
MDH-II	11	1	1	1	-	-
MDH-III	11	1	1	1	-	-
MDH-IV	11	1	1	1	-	-

Table 3. **Probable genotype, genotype frequency, allele(s) and allele frequencies at various loci in M.7**

Locus	Genotype	genotype frequency	Allele(s)	Allele frequency	h_o	h_e
ACP-I	11	1	1	1	-	-
ACP-II	11	1	1	1	-	-
ACP-III	11	1	1	1	-	-
EST-I	13	1	1&3	0.5&0.5	1	0.5
EST-II	11	1	1	1	-	-
PER-I	13	1	1&3	0.5&0.5	1	0.5
PER-II	33	1	3	1	-	-
PER-III	22	1	2	1	-	-
PER-IV	11	1	1	1	-	-
PER-V	22	1	2	1	-	-
PPO-I	Absent					
PPO-II	22	1	2	1	-	-
MDH-I	11	1	1	1	-	-
MDH-II	11	1	1	1	-	-
MDH-III	11	1	1	1	-	-
MDH-IV	11	1	1	1	-	-

Table 4. Probable genotype, genotype frequency, allele(s) and allele frequencies at various loci in M.9

Locus	Genotype	genotype frequency	Allele(s)	Allele frequency	h_o	h_e
ACP-I	11	1	1	1	-	-
ACP-II	11	1	1	1	-	-
ACP-III	11	1	1	1	-	-
EST-I	13	1	1&3	0.5&0.5	1	0.5
EST-II	11	1	1	1	-	-
PER-I	13	1	1&3	0.5&0.5	1	0.5
PER-II	13	1	1&3	0.5&0.5	1	0.5
PER-III	11	1	1	1	-	-
PER-IV	11	1	1	1	-	-
PER-V	22	1	2	1	-	-
PPO-I	Absent					
PPO-II	12	1	1&2	0.5&0.5	1	0.5
MDH-I	11	1	1	1	-	-
MDH-II	11	1	1	1	-	-
MDH-III	11	1	1	1	-	-
MDH-IV	11	1	1	1	-	-

Table 5. Probable genotype, genotype frequency, allele(s) and allele frequencies at various loci in M.26

Locus	Genotype	genotype frequency	Allele(s)	Allele frequency	h_o	h_e
ACP-I	11	1	1	1	-	-
ACP-II	11	1	1	1	-	-
ACP-III	12	1	1&2	0.5&0.5	1	0.50
EST-I	13	1	1&3	0.5&0.5	1	0.5
EST-II	11	1	1	1	-	-
PER-I	23	1	2&3	0.5&0.5	1	0.5
PER-II	13	1	1&3	0.5&0.5	1	0.5
PER-III	11	1	1	1	-	-
PER-IV	11	1	1	1	-	-
PER-V	22	1	2	1	-	-
PPO-I	Absent					
PPO-II	12	1	1&2	0.5&0.5	1	0.5
MDH-I	Absent					
MDH-II	11	1	1	1	-	-
MDH-III	11	1	1	1	-	-
MDH-IV	11	1	1	1	-	-

Table 6. **Probable genotype, genotype frequency, allele(s) and allele frequencies at various loci in MM.106**

Locus	Genotype	genotype frequency	Allele(s)	Allele frequency	h_o	h_e
ACP-I	11	1	1	1	-	-
ACP-II	11	1	1	1	-	-
ACP-III	12	1	1&2	0.5&0.5	1	0.5
EST-I	13	1	1&3	0.5&0.5	1	0.5
EST-II	11	1	1	1	-	-
PER-I	23	1	2&3	0.5&0.5	1	0.5
PER-II	22	1	2	1	-	-
PER-III	11	1	1	1	-	-
PER-IV	11	1	1	1	-	-
PER-V	22	1	2	1	-	-
PPO-I	Absent					
PPO-II	12	1	1&2	0.5&0.5	1	0.5
MDH-I	Absent					
MDH-II	11	1	1	1	-	-
MDH-III	11	1	1	1	-	-
MDH-IV	11	1	1	1	-	-

Table 7. **Probable genotype, genotype frequency, allele(s) and allele frequencies at various loci in MM.111**

Locus	Genotype	genotype frequency	Allele(s)	Allele frequency	h_o	h_e
ACP-I	11	1	1	1	-	-
ACP-II	11	1	1	1	-	-
ACP-III	11	1	1	1	-	-
EST-I	13	1	1&3	0.5&0.5	1	0.5
EST-II	11	1	1	1	-	-
PER-I	23	1	2&3	0.5&0.5	1	0.5
PER-II	13	1	1&3	0.5&0.5	1	0.5
PER-III	11	1	1	1	-	-
PER-IV	11	1	1	1	-	-
PER-V	22	1	2	1	-	-
PPO-I	Absent					
PPO-II	12	1	1&2	0.5&0.5	1	0.5
MDH-I	Absent					
MDH-II	Absent					
MDH-III	11	1	1	1	-	-
MDH-IV	11	1	1	1	-	-

Table 8. Genetic variation parameters in various clonal apple rootstocks

	M.3	M.4	M.7	M.9	M.26	MM.106	MM.111
Total No. of loci	13	16	15	15	14	14	13
Total No. of alleles in all the loci	17	19	17	19	19	18	17
Total No. of polymorphic loci	4	3	2	4	5	4	4
% of polymorphic loci	30.76	18.75	13.33	26.66	35.71	28.57	30.76
Polymorphic index	0.038	0.041	0.066	0.033	0.028	0.035	0.038
Average No. of alleles per locus	1.307	1.187	1.133	1.266	1.357	1.285	1.307
Effective No. of alleles per locus	0.090	0.068	0.071	0.076	0.086	0.083	0.090
Ho (Observed heterozygosity)	1	1	1	1	1	1	1
He (Expected heterozygosity)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Wright's fixation index	-1	-1	-1	-1	-1	-1	-1

Table 9. Pairwise comparison of diversity index based on isozymic pattern

	M.3	M.4	M.7	M.9	M.26	MM.106	MM.111
M.3	-						
M.4	8	-					
M.7	13	11	-				
M.9	14	11	7	-			
M.26	12	10	11	7	-		
MM.106	12	9	14	11	10	-	
MM.111	13	13	13	9	7	10	-

Table 10. Pairwise comparison of similarity index in clonal apple rootstocks

	M.3	M.4	M.7	M.9	M.26	MM.106	MM.111
M.3	-						
M.4	0.818	-					
M.7	0.583	0.739	-				
M.9	0.600	0.750	0.772	-			
M.26	0.625	0.708	0.695	0.863	-		
MM.106	0.727	0.818	0.652	0.739	0.857	-	
MM.111	0.608	0.625	0.681	0.857	0.900	0.761	-

Table 11. Pairwise comparison of genetic distance in clonal apple rootstocks

	M.3	M.4	M.7	M.9	M.26	MM.106	MM.111
M.3	-						
M.4	0.198	-					
M.7	0.257	0.049	-				
M.9	0.239	0.030	0*	-			
M.26	0.196	0.121	0.032	0.053	-		
MM.106	0.196	0.121	0.032	0.014	0**	-	
MM.111	0.075	0.154	0.105	0.130	0.036	0.036	-

Negative values replaced with zero

* = -0.017
** = -0.039

Table 12. R-Matrix (species correlation)

	M.3	M.4	M.7	M.9	M.26	MM.106	MM.111
M.3	-						
M.4	0.996**	-					
M.7	0.990**	0.999**	-				
M.9	1.000**	0.998**	0.994**	-			
M.26	0.999**	0.992**	0.984**	0.998**	-		
MM.106	1.000**	0.997**	0.992**	1.000**	0.999**	-	
MM.111	1.000**	0.996**	0.990**	1.000**	0.999**	1.000**	-

*** Significant at 1% level of significance*

Table 13. Summary of eigen analysis

Eigen value	Percentage of trace	Accumulated percentage of trace
1 = 6.977	99.70 %	99.70 %
2 = 0.023	0.30 %	100.00 %
3 = 0.000	0.00 %	100.00 %
4 = 0.000	0.00 %	100.00 %
5 = 0.000	0.00 %	100.00 %
6 = 0.000	0.00 %	100.00 %
7 = -0.000	-0.00 %	100.00 %

Table 14. Species co-ordinates (correlations) on the first 3 principal components

Rootstock	I	II	III
M.3	0.999	-0.035	-0.004
M.4	0.998	0.059	0.003
M.7	0.994	0.106	-0.002
M.9	1.000	-0.005	0.003
M.26	0.997	-0.072	0.003
MM.106	1.000	-0.019	0.000
MM.111	0.999	-0.035	-0.004

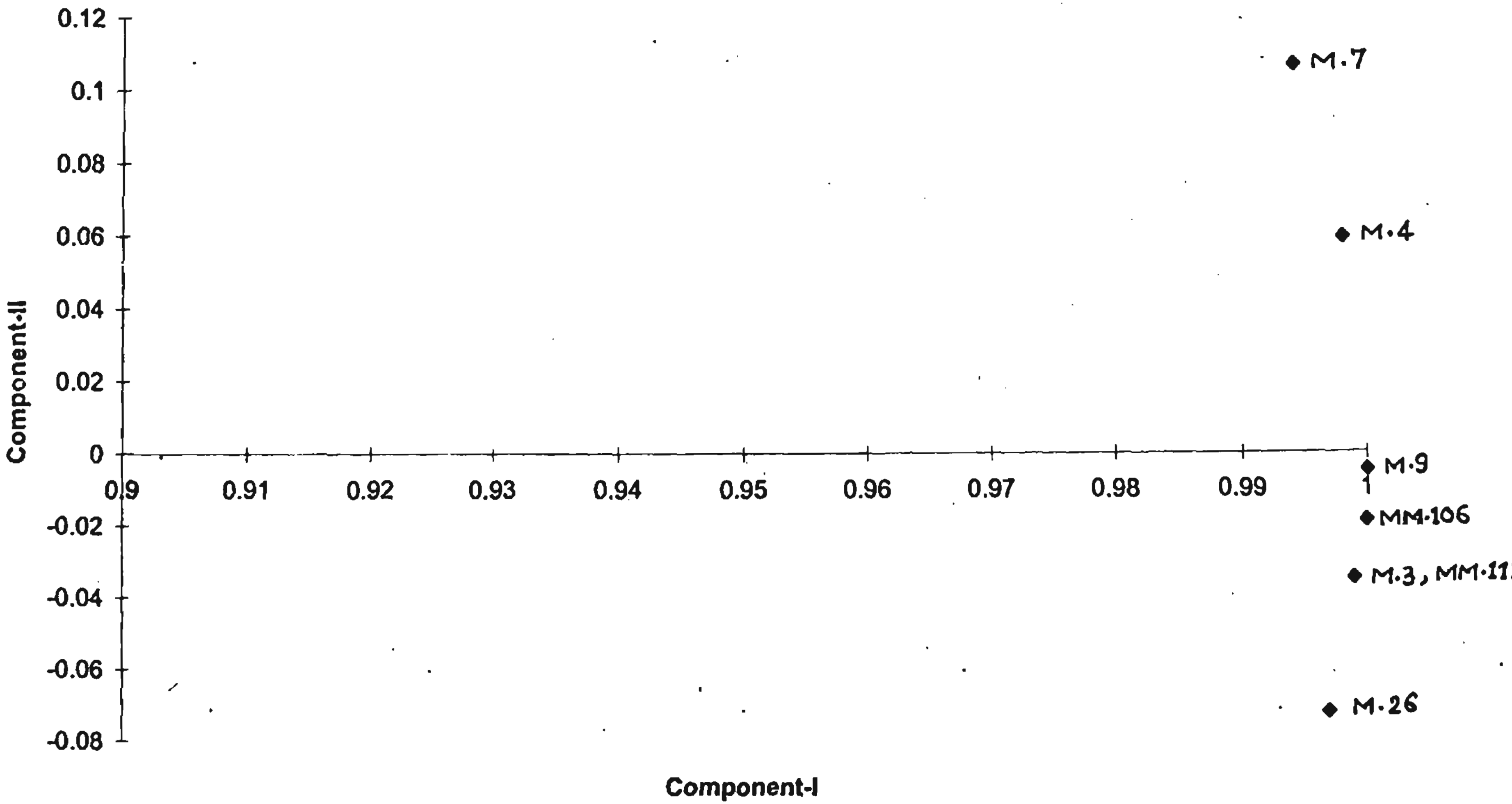

Fig. 6. Clustering of rootstocks through principal component analysis

Table 15. Classification of Malling and Malling Merton rootstocks into ACP, PPO, MDH and PER phenotypic zones

Group No.	Banding pattern	Rootstock(s)
	Acid phosphatase	
Group I	ACP-II(1), ACP-II(2)	M.3, M.4, M.26 and MM.106
Group II	ACP-II(1)	M.7, M.9 and MM.111
	Polyphenol oxidase	
Group I	PPO-I(1), PPO-II(2)	M.3 and M.4
Group II	PPO-II(1), PPO-II(2)	M.9, M.26 and MM.106 and MM.111
Group III	PPO-II(1)	M.7
	Malate dehydrogenase	
Group I	MDH-I(1), MDH-II(1), MDH-III(1), MDH-IV(1)	M.4, M.7 and M.9
Group II	MDH-II(1), MDH-III(1), MDH-IV(1)	M.26 and MM.106
Group III	MDH-III(1), MDH-IV(1)	MM.111
Group IV	MDH-IV(1)	M.3
	Peroxidase	
Group I	PER-II(1), PER-II(3), PER-III(1), PER-IV(1), PER-V(2)	M.9, M.26 and MM.111
Group II	PER-II(2), PER-III(1), PER-IV(1), PER-V(2)	M.4 and MM.106
Group III	PER-II(3), PER-III(2), PER-IV(1), PER-V(2)	M.7
Group IV	PER-II(2), PER-III(1), PER-IV(1), PER-V(1), PER-V(2)	M.3

Table 16. List of Malling and Malling Merton rootstocks with their parentage

Sr.No.	Rootstock	Parentage
1.	M.3	Unknown
2.	M.4	Unknown
3.	M.7	Unknown
4.	M.9	Unknown
5.	M.26	M.16 x M.9
6.	MM.106	Northern spy x M.1
7.	MM.111	Northern spy x Merton 793

DISCUSSION

5

DISCUSSION

Isozyme markers have been found to be appropriate for the identification of various horticultural plants (Kuhns and Fretz, 1978; Weeden and Lamb, 1985; Stampar and Smole, 1992 etc.). The results obtained in the present investigation have been discussed below:

5.1 PHENOTYPE

5.1.1 Acid phosphatase

Among the three zones of enzyme activity, first two zones, ACP-I and ACP-II were found to be monomorphic and the third zone, ACP-III showed two bands.

Menendez *et al.* (1986b) found that acid phosphatase from shoot bark tissue of apple showed isozymic differences useful for identification. This enzyme showed consistent isozyme pattern at different times of the year and from trees growing under different conditions, and the pattern was independent of wood age and rootstock influence. Battistini and Sansavini (1991) also reported that the apricot cultivars can be distinguished on the basis of isozyme pattern of acid phosphatase.

5.1.2 Esterase

In this enzyme system, all the three bands of locus EST-I were not conspicous, but all rootstocks exhibited a distinct band at locus EST-II. Due to lack of variation in esterase, in the present studies, we did not find it much useful for the identification of apple rootstocks. There are conflicting reports about the usage of esterase enzymes in identification of different cultivars of apple. Chyi and Weeden (1984) observed polymorphism in this enzyme system. The reason for this may be the difference in the genotypes used for their study and moreover they used 4-methylumbellyferyl acetate as substrate but we used alpha-napthyl acetate as a substrate. However, Bourriival and Korban (1987) found esterase to be monomorphic for 11 apple cultivars and selections tested. Chevreau and Laurens (1987) used esterase enzyme to study the pattern of inheritance in apple. Menendez *et al.* (1986a) found that the growing conditions and the sample timings did not effect esterase banding patterns therefore, this can be used in the identification of apple rootstocks.

5.1.3 Peroxidase

While studying peroxidase enyzme pattern it was noticed that its zymograms exhibited a little complicated picture having five zones of activity. Large number of isozymes showed varying mobility in each rootstock. Our results are in line with Bournival and Korban (1987) who found the peroxidase enzyme system to be polymorphic among apple cultivars and showing a total of five zones of activity. Polyacrylamide gel electrophoretic separation of peroxidase isozyme from shoot bark tissue of apple showed patterns that were consistent for cultivar and independent of season, age or ecophysiological conditions of the plant (Vinterhalter and James, 1983).

Weller and Costante (1986) also found five zones of activity in apple rootstocks. In the present study, the maximum variability of banding pattern was observed in peroxidase zymograms and such types of phenotype comparison of banding pattern have been widely used for identification of apple cultivars and rootstocks (Misic *et al.*, 1980; Mendendez *et al.*, 1986a; Quarta and Arone, 1987).

5.1.4 Polyphenol oxidase

In the two loci of polyphenol oxidase, the first locus showed only a single band which was present only in M.3 and M.4, but the second locus *PPO-II* showed polymorphism. PPO-II(2) was seen in all the rootstocks. There are very less reports showing the use of this enzyme system in apple cultivar identification. Wong *et al.* (1971) found four bands with polyphenol activity in extracts from acetone powder of Clingston peach. Kahn (1976) detected six active polyphenol isozymes with six different substrates in avocado.

5.1.5 Malate dehydrogenase

All the four different loci observed in MDH were monomorphic, but variable among the rootstocks. Locus IV was present in all the rootstocks which showed a distinct band. Our results differ from Samimy and Cummins (1992) who found MDH to be the least variable enzyme among the apple rootstocks studied, but agrees with Durham *et al.* (1987) who considered MDH to be a useful genetic marker for distinguishing different cultivars of peach. According to Weeden and Lamb (1987) MDH is a dimeric enzyme with five loci coding its synthesis. Bournival and Korban (1987) found MDH to be showing polymorphism for all the apple cultivars and selections tested. Stampar and Smole (1992) classified malate dehydrogenase polymorphism in apple into five groups (MDH-1, MDH-2, MDH-3, MDH-4 and MDH-5).

With respect to all the five enzyme systems studied, all the seven clonal rootstocks showed an identical band at EST-II(1), MDH-IV (1) and PER-IV(1) which was quite distinct. Thus, these isozymes could be characteristic of the apple rootstocks. Sujatha *et al.* (1991) found that in case of GOT, all the *Cucumis* species showed an identical band at GOT_4. This isozyme was absent in other genera like *Praecitrullus* and *Citrullus*.

5.2 DISTINCTION AMONGST THE ROOTSTOCKS

Of the five enzyme systems examined, due to the highly polymorphic nature of the four enzymes i.e. ACP, PPO, PER and MDH, rootstocks exhibited the specific electrophoretic patterns and therefore could be placed in different groups. This grouping uniquely distinguishes the three rootstocks M3, M7, MM111. However, no such sharp distinction could be made on the basis of esterase, due to lack of polymorphism.

Seven rootstocks were divided into two groups, Group I and Group II contained 4 & 3 rootstocks, respectively on the basis of ACP. Three groups identified on the basis of PPO showed that Group III contained a single rootstock M.7, therefore, PPO can be used to distinguish M.7, which showed PPO-II (2) and PPO-I(1) was absent. This type of banding pattern was not shown by any other rootstock.

The phenotypic grouping on the basis of MDH showed that Group-III and IV contained single rootstock M.3 and MM.111. Hence, M.3 and MM.111 can be distinguished on the basis of this enzyme system. M.3 have different banding pattern, only MDH-IV(1) is present. Similarly, MM.111 also showed entirely different banding pattern with MDH-III(1) and MDH-IV(1)

Unique banding pattern was observed for each group on the basis of PER. M.9, M.26 and MM.111 showed PER-II(1) and PER-II (3) bands with genotype (13) not present in other rootstocks. M.4 and MM.106 had genotype (22) for locus PER-II, not present in other rootstocks except M.3. M.3 had PER-V with a genotype (12) and M.7 had PER-II and PER-III with a genotype (33) and (22), respectively. Therefore, peroxidase enzyme system can be used to identify M.3 and M.7 rootstocks.

Menendez *et al.* (1986a) classified 33 rootstocks into 10 groups on the basis of PER banding patterns M.2, M.4, M.7, M.9 and MM.111 which fell in the same phenotypic class in their studies, were classified into 3 different groups in the present work. Manganaris and Alston (1993) also classified the rootstocks we studied, into 6 different classes on the basis of PER. According to him, as the peroxidase system is prone to seasonal and development variation, a more reliable grouping has been obtained on the genetic basis of major stable bands in PER-II and PER-III zones. Peroxidase polymorphism has been widely used to identify apple rootstocks and cultivars (Vinterhalter and James, 1986; Weller and Costante, 1986; Vinterhalter and James, 1983).

Samimy and Cummins (1992) reported that each rootstock could be clearly distinguished by using only two enzyme systems phosphoglucomutase and 6-phosphogluconate dehydrogenase, both of which showed considerable enzyme polymorphism. Weeden and Lamb (1985) found that the most useful enzyme systems for distinguishing among the cultivars were 6-phosphogluconate dehydrogenase and aspartate aminotransferase. PER and 6-PGD enzyme systems were found most useful for distinguishing the cultivars (Stampar and Smole, 1992) and they classified 20 apple cultivars into 17 PER and 8, 6-PGD groups.

In the present studies, large number of banding patterns obtained for peroxidase indicates the possibility of great genetic diversity among clonal apple rootstocks and Menendez *et al.* (1986a) suggested that several genes may be involved in the coding of so many variants.

5.3 PROBABLE GENOTYPE

In the present investigation, five enzyme systems, 16 loci were studied having a total of 26 alleles, and seven of which were polymorphic. Of the five enzyme systems analysed, four showed polymorphism i.e. EST, ACP, PER and PPO.

Weeden and Lamb (1987) studied polymorphism in nine enzyme systems in apple. The product of 27 loci could be distinguished in these enzyme systems, 19 of which displayed polymorphism Bournival and Korban (1987) analysed 9 enzyme systems and found only 3 to have polymorphic loci, peroxidase was one of them. Esterase and malate dehydrogenase were among the enzymes which were monomorphic for all the cultivars and selections examined. Our results are similar with the above observation, with polymorphic PER and monomorphic MDH.

5.4 GENETIC VARIATION

Genetic variation among the various apple rootstocks was explained on the basis of various parameters. The percentage of polymorphic loci which provides a rough guide to the level of genetic variation (Brown and Weir, 1993) varies from 13.33 per cent in M7 to 35.71 per cent in M.26. In *Populus tremuloides* the trend of percentage of polymorphic loci was different it was from 66.7 per cent to 93.3 per cent (Hyun *et al.*, 1987), but in *Populus deltoides* it was 32.4 per cent (Rajora, 1989).

Polymorphic index in apple ranged from 0.028 to 0.066 and M.7 showed the maximum polymorphic index. Rootstock M.26 showed the maximum number of average and effective number of alleles per locus which was 1.357 and 0.09, respectively.

The negative value for fixation index (F) indicates the excess of heterozygotes for all the rootstocks, as the observed heterozygosity was higher than the expected heterozygosity. Lorenzo *et al.* (1996) also reported negative values for fixation index in north western Spanish chestnut cultivars. Positive values for fixation index have been reported in *Camellia japonica* (Wendel and Parks, 1981) and *Populus tremuloides* (Hyun *et al.*, 1987).

Diversity index was calculated by comparing the enzyme systems of any two rootstocks, both qualitatively and quantitatively. Diversity index for apple roostocks varied from 7 to 14.

5.5 SIMILARITY INDEX AND GENETIC DISTANCE

The high or a low value of similarity index indicates a high or low degree of electrophoretic similarity. M.26 and MM.111 showed maximum similarity. The percentage of similarity among the *Cucumis* species varied from 8 to 36 (Sujatha *et al.*, 1991).

On the basis of genetic distance, M.9 seems to be closely related to MM.106 whereas M.3 and M.7 are distantly related as evident from their greater genetic distances. The negative values for M.7 and M.9, M.26 and MM.106 were replaced with 0 on the basis of Nei (1978) who noted that genetic distance can be negative and this negative value is caused by sampling error.

5.6 PRINCIPAL COMPONENT ANALYSIS

According to PCA, M.4 and M.7 of the first group showed, positive correlation values and the second group which contained M.3, M.9, M.26, MM.106 and MM.111 showed negative correlation. In the 2nd group, M.3 and MM.111 lie at same point which suggests that both may have same parentage. As the parentage of M.3 is unknown (Brooks and Olmo, 1960; Brooks and Olmo, 1962), so it might have same parentage as that of MM.111.

Rajora (1989) showed that in *Populus deltoides* principal component analysis of clonal genotypes at 12 polymorphic loci indicated 8 loci to be most differentiating for the clones. The first three principal components accounted for 47.6 per cent of the total variation in 12 polymorphic loci.

In case, enzyme polymorphism is to be used for rootstock identification, there should be only a few rootstocks in each phenotypic group. On the basis of PER, PPO, and MDH there are 3 phenotypic groups with a single rootstock and 2 phenotypic groups with an average of 2 rootstocks. Therefore, unique identification (one rootstock to one group) of apple rootstocks based on PER, PPO and MDH is possible. The similarities in banding pattern of M.9, M.26, MM.106 and MM.111, M.4 and MM.106 may be due to the common origin of Malling and Malling Merton rootstocks which are believed to be related to Paradise and Doucin apples (Hatton, 1917). Similarities between PPO patterns of MM.106 and MM.111 may be due to one common parent, Northern spy. Similarities between M.9 and M.26 on the basis of PPO and PER banding patterns may be because M.9 is one of the parent of M.26 (Table 16) which is contrary to Vinterhalter and James (1986) who classified M.26 and M.9 in different classes on the basis of PER phenotypic regions. However, we have found these two rootstocks in separate groups on the basis of ACP and MDH.

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SUMMARY AND CONCLUSION

6

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Molecular characterisation of clonal apple rootstocks using isozymes was carried out to identify isozyme polymorphism in seven clonal apple rootstocks and to identify the most characteristic and stable isozyme marker for individual rootstocks. The summary of this investigation is as under:

1. Three zones of activity were observed for the enzyme acid phosphatase. First two zones showed only single bands with Rm values 0.74 and 0.65. The third zone of enzyme activity showed two bands with Rm values 0.34 and 0.29. ACP-III(1) is a dark band present in all the seven clonal apple rootstocks.

2. Esterase enzyme system showed the least variation for all the rootstocks. Two distinct zones of enzyme activity were observed for esterase. Locus-I showed three bands with Rm values 0.78, 0.73 and 0.66, respectively. The locus EST-II showed a single band with Rm value 0.35 which is a distinct band present in all the rootstocks.

3. Peroxidase enzyme system showed maximum variation among the rootstocks. Five zones of activity were observed with four polymorphic loci. Ist zone of activity showed three bands with Rm values 0.87, 0.83 and 0.8. The 2nd zone of activity also showed three bands with Rm values 0.73, 0.71 and 0.67. 3rd zone showed two bands with Rm values 0.6 and 0.56. 4th zone showed only single band with Rm value 0.38, 5th zone of enzyme activity showed two bands with Rm values 0.27 and 0.21. PER V(2) is present in all the rootstocks.

4. Polyphenol oxidase showed two zones of activity. Ist zone showed a single band with Rm value 0.48. The 2nd zone of activity showed two bands with Rm values 0.43 and 0.36.

5. Malate dehydrogenase showed four zones of activity and each zone showed a single band with Rm values 0.29, 0.23, 0.18 and 0.14. MDH-IV(1) is a conspicuous band present in all the seven rootstocks.

6. Of the five enzyme system examined PPO, MDH, ACP and PER were useful in discriminating among the rootstocks.

7. In the five enzyme systems, 16 loci were studied out of which, 9 loci were monomorphic and 7 loci were polymorphic, ACP-III, EST-I, PER-I, PER-II, PER-III, PER-V and PPO-II.

8. Genetic variation among the various apple rootstocks were explained on the basis of various parameters. The percentage of polymorphic loci varied from 13.33 per cent to 35.71 per cent. Polymorphic index ranged from 0.028 to 0.066. The value of Wright's fixation index was -1 for all the rootstocks which indicates the excess of heterozygotes and the pairwise comparison of diversity index on the basis of isozymic pattern showed a range from 7 to 14.

9. Similarity index calculated for the apple rootstocks varied from 0.583 to 0.9. M.26 and MM.111 showed maximum similarity. The genetic distance between various rootstocks was calculated and M.7 had maximum genetic distance (0.257) and closely related rootstocks were M.9 and MM.106.

10. On the basis of principal component analysis the rootstocks can be divided into two groups. The first group contained rootstocks M.4 & M.7 and the second group contained M.3, M.9, M.26, MM.106 and MM.111.

The data obtained from the characterisation of the five enzyme systems revealed that enough genetic polymorphism exists within the rootstocks to permit the identification of most of the rootstocks. Out of the five enzyme systems studied, 4 systems PPO, ACP, MDH and PER were useful for differentiating among the rootstocks. Among the seven rootstocks, three may be distinguished on the basis of one enzyme system only (M.3 with MDH or with PER, M.7 with PPO or PER and MM.111 with MDH), making the analysis economical and applicable in small laboratories as well as in large scale operations. More enzyme systems are needed to be resolved to get more polymorphic loci for identification of rest of the rootstocks.

It is suggested that evaluation of more enzyme systems and use of more precise and reliable techniques like DNA fingerprinting will be helpful in distinguishing intracultivar variability more accurately.

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* Originals not seen

APPENDICES

APPENDIX-I

EXTRACTION BUFFER

(Arulsekar and Parfitt, 1986)

Tris citrate pH 8.0

Tris base	0.05M
Citric acid	0.007M
Cystein HCl	0.1%
Ascorbic acid	0.1%
Polyethylene glycol (MW, 6000)	1%

Firstly, Tris base and citric acid were dissolved in water and the pH was adjusted to 8.3 with citric acid. Then, cystein HCl, ascorbic acid and polyethylene glycol were dissolved and the final pH was adjusted to 8.0.

APPENDIX-II

BUFFER SYSTEM

(Scandalios, 1969)

TRAY BUFFER

Lithium borate	pH 8.3
Lithium hydroxide	12.0 gm
Boric acid	118.9 gm
Distilled water	10 lts.

Dissolved the above components in 9.5 litres of water and adjust the pH to 8.3, then make the final volume to 10 litres.

GEL BUFFER

Tris citrate	pH 8.3
Trizma base	6.2 gms
Citric acid	1.46 gm
Distilled water	1 litre

Dissolve all the above components in 950 ml of distilled water, adjust the pH to 8.3 and make the final volume to 1 litre.

The gel buffer consists of 90% tris citrate buffer and 10% tray buffer (Lithium borate).

APPENDIX-III

GEL PREPARATION

STOCKS

[A] **Acrylamide + Bisacrylamide**

Acrylamide	30 gm
Bisacrylamide	0.8 gm

Dissolve in small amount of distilled water and make the final volume to 100ml. Store at 4°C in amber colour bottles.

[B]	**Gel buffer**	[APPENDIX-II]
[C]	**Ammonium per sulphate**	70 mg/50 ml of distilled water
[D]	**TEMED**	1 to 2 drops

Gel preparation

1. Spacer (4%) - 2.75 ml [A] + 8.25 ml [B] + 10 ml [C] + 2 drops of TEMED
2. Resolving gel-I (7.7%) - 15 ml [A] + 15 ml [B] + 30 ml [C] + 2 drops of TEMED
3. Resolving gel-II (9.2%) - 18 ml [A] + 12ml [B] + 30 ml [C] + 2 drops of TEMED
4. Resolving gel-III (10.2%) - 20 ml [A] + 10 ml [B] + 30 ml [C] + 2 drops of TEMED

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ABSTRACT

Molecular characterization of clonal apple rootstocks using isozymes was carried out to identify isozyme polymorphism in seven clonal apple rootstocks and to identify the most characteristic and stable enzyme marker for individual rootstock.

Five enzyme systems were studied out of which polyphenol oxidase, malate dehydrogenase, acid phosphatase and peroxidase were useful in discriminating among the rootstocks. The peroxidase enzyme system showed the maximum variation and the esterase showed the least variation among the rootstocks. Among the seven rootstocks three may be distinguished on the basis of one enzyme system only (M.3 with MDH or PER, M.7 with PPO or PER and MM.111 with MDH).

Genetic variation among the rootstocks was explained on the basis of various parameters. The percentage of polymorphic loci varied from 13.33 per cent to 35.71 per cent. The value of Wright's fixation index was -1 for all rootstocks which indicate the excess of heterozygotes and the pairwise comparison on the basis of isozyme pattern showed a range from 7 to 14.

M.26 and M.111 showed maximum similarity. The genetic distance between various rootstocks was calculated. M.3 and M.7 had maximum genetic distance (0.257). On the basis of principal component analysis the rootstocks were divided into two groups. The first group contained rootstocks M.4 and M.7 and the second group contained M.3, M.9, M.26, MM.106 and MM.111.

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