ISOENZYME VARIATION IN *Piper* spp.

By

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THESIS

Submitted in partial fulfilment of the
requirement for the degree of

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Faculty of Agriculture
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Kerala, India

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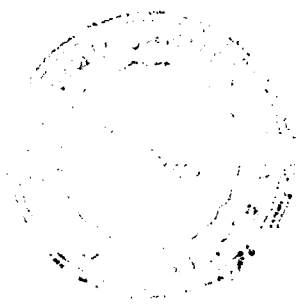
DECLARATION

I hereby declare that the thesis entitled "**Isoenzyme variation in *Piper* spp.**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.



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We, the undersigned members of the Advisory Committee of **Mr.Abraham Sebastian**, a candidate for the degree of **Master of Science in Horticulture** with major in **Plantation Crops and Spices**, agree that this thesis entitled "**Isoenzyme variation in *Piper* spp**", may be submitted by Mr.Abraham Sebastian in partial fulfilment of the requirements for the degree.

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ABRAHAM SEBASTIAN

dedicated to

My Loving Grandparents

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Introduction

INTRODUCTION

The genus *Piper* is enormously large accomodating more than 3000 (Purseglove, 1977) species which are distributed throughout the tropics and subtropics. It contains several economically and medicinally important species such as *Piper nigrum* (black pepper), *P. betle* (betel vine), *P. longum* and *P. cubeba*. Black pepper is one of the most important spice crop of the country and an important foreign exchange earner. However, the crop is being faced with many serious threats from pest and diseases especially foot rot. As resistance to foot rot is not reported within the species interogression of the genes from wild relatives is the probable solution to solve the problem. Studies on phylogeny and evolution are important as essential pre-requisites for scientific crop improvement programme.

Piper has been described as one of the most difficult genera to classify (Hooker, 1886) due to widely variable vegetative characters, closely packed spikete inflorescences, and extremely small floral parts. But most of the taxonomical works in the genus were based on the morphological parameters. Cytogenetical studies were also attempted, but mostly confined to observations on chromosome number.

Biochemical constituents such as flavonoides were also used to a limited extent for chemotaxonomical studies (Rahiman and Subbaiah, 1984).

Recently isoenzyme variations have been used as a powerful tool to complement and supplement conventional phylogenetic studies (Rick *et al.*, 1977; Rick and Tanksley, 1981; Gottlieb, 1971, 1977 and 1982; Crawford, 1983 etc.). The advantage of isoenzyme data over other phenotypic characters are manifold. These are basic biochemical constituents which are not affected by direct selection

These are basic biochemical constituents which are not affected by direct selection pressure during the course of domestication and evolution of a taxon providing a more accurate picture of the original variation present in population. Secondly, isoenzyme pattern is not affected by environment. Moreover, isoenzymes are usually direct product of single locus and relating the phenotypic variations with genotypic characters is relatively easier. Allozymes are usually inherited as codominants which help in distinguishing a homozygote from a heterozygote. Besides these, a relative case in electrophoretic analysis makes isoenzyme studies a powerful tool in phylogenetic studies. In the light of this, isoenzyme analysis was undertaken in the genus *Piper* with the major objectives:

to study the dynamics of variability at isoenzyme level in *Piper nigrum* Linn. and related taxa and to elucidate the species relationship in the genus *Piper*.

Review of Literature

REVIEW OF LITERATURE

2.1 Variation within *P. nigrum*

2.1.1 Morphology

In cultivated varieties of black pepper variations ranging from complete hermaphorodite to entirely male or female flowers were found (Blacklock, 1954). Sayed (1968) had reported that the main difference among various varieties of *Piper nigrum* was in the sexual composition of flowers in the spikes. Kanakamani (1985) divided the *Piper nigrum* varieties into five groups based on sexual composition. Each of the five groups were again subdivided based on internodal length of plagiotrops, petiole length and spike length which gave rise to a total of 40 groups out of the 45 types she studied. She also noticed variation in leaf shape, leaf margin, leaf base and colour of the leaf on the upper and lower side.

2.1.2 Cytology

Somatic chromosome number of *P. nigrum* has been reported variously by different workers (Table 1). Dasgupta and Datta (1976) had reported that chromosome length ranged between 0.77-1.9 μ in North Indian group with $2n = 48$ and 0.77-2.3 μ for South Indian group with $2n = 60$. North Indian group possessed four pairs of chromosome with secondary constriction and South Indian group with two pairs.

Varietal variation in length of chromosomes and number of nucleolar chromosomes were reported by Jose and Sharma (1983).

Table 1. Somatic chromosome numbers reported in *Piper* spp.

Species	Chromosome number	Reference
1	2	3
P. nigrum Linn.	36, 60	Dasgupta and Datta, 1976
	48	Sharma and Bhattacharyya, 1959;
	52	Mathew, 1958, 1972
	52	Martin and Gregory, 1962
	52	Samuel and Bavappa, 1981
	52	Jose and Sharma, 1983
	52	Samuel *et al.*, 1984
	52, 104	Samuel, 1986
	54	Sampathkumar and Navneethan, 1981
P. nigrum Wild	52, 104	Mathew, 1958, 1972
	52, 65	Samuel and Bavappa, 1981
	52, 48, 65	Samuel *et al.*, 1984
	104	Jose and Sharma, 1985
	104	Samuel, 1986
P. betle Linn.	26, 52	Samuel and Bavappa, 1981
	32	Johnson, 1910
	32	Janakiammal, 1945
	42, 58, 78	Jose and Sharma, 1983, 1985
	52	Samuel *et al.*, 1984
	52	Rahiman and Nair, 1986
	52	Samuel, 1986
	52, 65	Samuel and Morawetz, 1989
	64	Sharma and Bhattacharyya, 1959
	64	Dasgupta and Datta, 1976
	78	Mathew, 1958
	78	Okada, 1986
P. betle Wild	52	Jose and Sharma, 1985
P. betle var. Chava Wild	195	Jose and Sharma, 1983

Contd.

Table 1. Continued

1	2	3
P. longum Linn.	24	Tijo, 1948
	26	Samuel and Bavappa, 1981
	26	Samuel *et al.*, 1984
	44	Sampathkumar and Navneethan, 1981
	48, 96	Sharma and Bhattacharyya, 1958
	48	Dasgupta and Datta, 1976
	52	Mathew, 1958
	52	Jose and Sharma, 1983, 1985
	52	Rahiman and Nair, 1986
	53	Samuel and Morawetz, 1989
	60	Bai and Subramanian, 1985
P. argyrophyllum Miq.	26, 39	Samuel and Bavappa, 1981
	26, 39	Samuel *et al.*, 1984
	52	Rahiman and Nair, 1986
P. argyrophyllum Wild	52	Samuel, 1986
P. attenuatum Buch. Ham	26, 39	Samuel and Bavappa, 1981
	52	Jose, 1981
	52	Jose and Sharma, 1983
P. chaba Hunt	24	Janakiammal, 1945
	104	Jose and Sharma, 1985
P. colubrinum	26	NRCS unpublished

2.2. Phylogenetic and systematic studies in *Piper*

Hooker (1886) described 45 species in genus *Piper* of which 29 were assigned to Indian Peninsula. Gamble (1925) in 'Flora of the Presidency of Madras' described 13 species viz. *P. galeatum, P. trichostachyon, P. longum, P. hapnium, P. brachystachyon, P. schmidtii, P. hookeri, P. hymenophyllum, P. barberi, P. nigrum, P. attenuatum, P. wightii* and *P. betle* from South India. *P. bababudani, P. silentvalleyensis* and *P. pseudonigrum* were not included in Hooker's floras. They

were later described by Rahiman (1981), Ravindran *et al.* (1987) and Velayudhan and Amalraj (1992) respectively.

Hooker (1886) included *P. argyrophyllum* and *P. attenuatum* under the section Eupiper along with *P. nigrum*. *P. galeatum* was included in section Muldera along with *P. trichostachyon*. *P. longum, P. chaba, P. betle* and *P. hapnium* were members of section Chavica. Velayudhan and Amalraj (unpublished) observed that among the 15 species they collected from the Western Ghats six species viz. *P. nigrum, P. pseudonigrum, P. bababudani, P. trichostachyon, P. wightii* and *P. galeatum* appeared to be very closely related morphologically.

2.2.1 Morphology

Plants belonging to the genus *Piper* exhibit considerable variation in their morphological characters. Rahiman (1981) observed the morphological variations among them as follows:

All the species were climbers except *P. longum*, which was a creeper. *P. nigrum* and *P. bababudani* showed twining habit to a greater extent. Stem girth showed marginal increase from 0.05 cm in *P. longum* followed by *P. hapnium, P. attenuatum, P. argyrophyllum, P. nigrum, P. bababudani* reaching upto 7 cm in *P. trichostachyon* and *P. galeatum*. Samuel *et al.* (1983) reported marked difference in foliar characteristics such as leaf shape, size and venation pattern among the members of the genus *Piper*. The leaf shape varied from broadly ovate in *P. longum* and *P. betle* to lanceolate or ovate lanceolate in *P. argyrophyllum*. As per some of the earlier works, majority of the species had 5-9 principle nerves which were multiple palmately or rarely pinnately nerved. Stipules none to two, connate or adnate to the

petiole (Hooker, 1886; Hains, 1924; Gamble, 1925; Saldanha and Nicholson, 1976).

Most of the *Piper* species were unisexual. Inflorescences were cylindrical and erect in *P. longum* and *P. hapnium* while filiform and drooping in *P. nigrum, P. bababudani, P. argyrophyllum, P. attenuatum* and *P. galeatum*. Among the species *P. argyrophyllum* and *P. attenuatum* showed many similarities in morphological characters which made them difficult to distinguish in the case of male plants. But they could be distinguished in female plants based on spike and berry characters (Rahiman, 1981).

P. bababudani was reported by Rahiman in 1981, which according to him was distinguishable from *P. nigrum* by white flowering spikes, irregularly arranged bract, almost sessile stamens and bolder berries which never turn deep red. In other morphological characters they were very similar. *P. hapnium* and *P. longum* resembled in several morphological characters but for the climbing habit, amplexicaul auricles and absence of multiple nerved lamina in *P. hapnium* (Rahiman, 1981). *P. pseudonigrum* is a newly reported species (Velayudhan and Amalraj, 1992) and is related to *P. nigrum* and *P. trichostachyon* in many respects (Velayudhan and Amalraj, unpublished).

2.2.2 Biometrics

Based on D^2 analysis carried out in both male and female plants of eight species, Rahiman and Bhagavan (1985) found that *P. attenuatum* and *P. argyrophyllum* formed one cluster *P. trichostachyon* was clustering with *P. galeatum, P. nigrum* and *P. longum* retained their identity and formed separate clusters indicating that these species were distinct from all other species.

Ravindran *et al.* (1992) carried out centroid cluster analysis in 11 species of *Piper* based on observation of 30 characters. They had observed that *P. argyrophyllum* and *P. attenuatum* were closely related and formed a single cluster. *P. galeatum* and *P. trichostachyon* were part of second cluster. *P. nigrum* clustered along with *P. wightii* while *P. longum* stood alone.

2.2.3 Anatomy

Stem

According to Pal (1981) there was variation in layers of tunica in shoot apices of different species and *P. betle, P. nigrum*. Anatomical variation in *Piper* species was reported in stem endodermis development also (Bond, 1931).

Van Teighen (1908) grouped the plants in the genus *Piper* into three categories, viz. the plants having one central canal accompanied by peripheral canals, others having only central canal whilst in the third group they were absent. Murthy (1959) noted the presence of mucilage canal in *P. betle* and their absence in *P. longum*. Pal (1981) reported presence of mucilage canals in *P. betle, P. nigrum* and *P. longum*.

Leaf

Datta and Dasgupta (1977a) reported varying number of layers of cells in hypodermis in *P. nigrum, P. betle* and *P. longum*. According to him adaxial hypodermis was unilayered in *P. longum* 1-2 layered in *P. nigrum* and clearly 2-3 layered in varieties of *P. betle*. Abaxial hypodermis was unilayered in *P. longum*, 1-2 layered in all the varieties of *P. betle* and 2-3 layered in *P. nigrum*.

Leaf epidermal studies by Samuel *et al.* (1984) showed that epidermal cells of *P. betle, P. longum* and *P. argyrophyllum* were much larger than that of *P. nigrum* and *P. attenuatum*. Albeit, morphological features of *P. nigrum* and *P. attenuatum* were quite distinct there were strong resemblences in their leaf epidermal characteristics.

Root

Datta and Dasgupta (1977b) based on root anatomical studies, proposed the sequencial advancement among *Piper* species as *P. longum*, *P. nigrum* and *P. betle*.

2.2.4 Cytogenetics

2.2.4.1 Chromosome number

The cytological investigations by various workers showed wide variations in chromosome numbers of the same species in the genus *Piper* (Table 1).

2.2.4.2 Karyomorphology

According to Sharma and Bhattacharyya (1959), out of the four species of the genus *Piper* they studied, *P. nigrum* possessed one pair of chromosome characteristic of the species. They had also reported that chromosome sizes ranged from 0.6-1.7 μ in *P. longum* with 2n = 48 and 96 and 0.6-2.2 μ for another type with n = 24.

In *P. betle*, chromosome size range of 0.7-2.5 μ was reported for 2n = 78 varieties (Mathew, 1958) and 0.72-1.2 μ for 2n = 64 varieties (Dasgupta and

Datta, 1976). The karyotypes of *Piper* species showed a gross uniformity in size with 0.56-2.41 μ in *P. betle*, 0.56-2.05 μ in *P. nigrum*, 0.56-1.48 μ in *P. chaba*, 0.74-1.9 μ in *P. attenuatum* and 0.74-1.85 μ in *P. longum*. *P. attenuatum* showed three karyotypes while *P. nigrum* and *P. longum* showed only two karyotypes. *P. betle* mostly showed two karyotypes but one variety Jhol Bangla showed the additional karyotype which was present in *P. attenuatum* out of 30 varieties studied (Jose, 1981; Jose and Sharma, 1985).

2.2.4.3 Species affinities and evolutionary pattern in the genus *Piper* based on cytogenetics

Sharma and Bhattacharyya (1959) had reported that polyploidy might have played a distinct role in the origin of *P. longum*, *P. nigrum* and *P. betle*. Chromosome numbers were multiples of 12 and 16 in *P. longum* (2n = 24 and 96) whereas in *P. nigrum* it was multiples of 16, i.e. 48 and 128 respectively. Jose and Sharma (1985) reported chromosome numbers 24 to 195 in the genus *Piper* and suggested that polyploidy had been an important factor in evolution of *Piper*. According to them n = 12 might represent the basic set from which n = 13 might have been derived and 13 became deep seated in the genus *Piper*, possibly due to selective advantage.

Rahiman and Nair (1986) classified species as North Indian types with n = 12 and South Indian types with n =13. The North Indian types included the species from transgangetic provinces and South Indian types were from South Indian and Ceylon centres of distribution described by Hooker (1886). They have suggested that the species from these two centres had different evolutionary pathway starting from the basic number of 6 and 7. They also supported the earlier hypothesis of

Mathew (1958) that the commonly observed haploid number of 13 among the South Indian types might have arisen from the hybridisation of the types with n = 6 and 7.

In the light of an earlier report by Samuel and Bavappa (1981) of the occurrence of different species with 2n = 26, Rahiman and Nair (1986) suggested that 26 chromosome types could be diploids and the commonly observed 2n = 52 types could be considered as tetraploids. They have also suggested that the evolution of the genus *Piper* is complicated by different primary basic numbers, amphidiploidy and vegetative propagation.

Samuel and Morawetz (1989) have suggested that long term cultivation is responsible for intraspecific numeric variation in *P. nigrum, P. longum, P. betle* etc. since polyploidy, anorthoploidy or disploidy had been reported in these species. Based on the studies from both Old World and New World species and Okada's (1986) result, Samuel and Morawetz (1989) disapproved correlation between geographical distribution and ploidy indicated by Mathew (1958) based on different basic numbers.

Mathew (1958) had reported that in spite of gross homogeneity in chromosome morphology between different species, each species or variety was characterised by distinct karyotype of its own. The presence of a pair of chromosomes with supernumerary constrictions which was present only in *P. nigrum* also proved considerable structural alteration in the evolution of the species.

According to Jose and Sharma (1985) the genus *Piper* as a whole represented a homogeneous assemblage having general uniformity in karyotype and with difference in number of nucleolar chromosomes from one to four pairs. They were

of the opinion that gene mutation, imperceptible chromosome changes if any, had affected the evolution both at interstrain and interspecific levels. Occassional polyploidy and structural alteration involving principal chromosomes with secondary constriction had taken place. Samuel (1986) had opined that in the genus *Piper*, chromosomes of tetraploid species were much smaller than those of diploid species.

Samuel *et al.* (1986) reported significant interspecific DNA variation between species. They had also noticed that several New World diploid species had higher nuclear DNA than several New or Old World species. DNA content per basic genome was on the whole, lower in cultivated species. In *P. nigrum*, DNA content of wild accession of *P. nigrum* was approximately double that of cultivated tetraploid variety of same species.

2.2.5 Chemotaxonomy

The flavonoid analysis carried out by Rahiman and Subbaiah (1984) had revealed that *P. argyrophyllum* and *P. attenuatum* were biochemically related as they possessed a percentage similarity of 82 per cent and paired affinity of 33.33 per cent. *P. trichostachyon* showed a close similarity biochemically to *P. galeatum* with paired similarity of 89 per cent and paired affinity of 42.2 per cent. *P. nigrum* and *P. longum* were very distinct among eight species studied.

Samuel *et al.* (1984) reported chemotaxonomic markers by phenolic separation in different species of *Piper*. Species specific spots were found in *P. nigrum* and *P. betle*.

Ravindran *et al.* (1992) noted that *P. nigrum* was the only species having the alkaloid piperine and the whole set of terpenoids that contribute typical black pepper flavour.

2.2.6 Isoenzyme analysis

Kocchar *et al.* (1989) noted characteristic taxonomic markers for six varieties in betel vine by peroxidase pattern study. They had also observed that different types of same cultivar possessed the same band pattern in most of the cases.

Materials and Methods

MATERIALS AND METHODS

The present study was conducted in the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara, Trichur from June 1993 to December 1994.

3.1 Material

Material included 11 species of *Piper* including *P. nigrum* Linn. *P. nigrum* is the most important species of the genus and the most important spice crop of the Kerala showing enormous variation in morphological characters. Therefore, the taxon was studied in detail for within species variation as well. Twenty six varieties were studied in *P. nigrum*. The varieties were selected to represent the wide genetic base available in the germplasm collection maintained in the College of Horticulture which includes varieties from Kerala and neighbouring states (Table 2). In other species also the maximum accessions available in the collections were studied (Table 3).

3.2 Methods

For the separation of multiple forms of enzyme, polyacrylamide gel electrophoresis was carried out using vertical slab gel electrophoresis unit of Bio-tech.

Acrylamide monomers ($CH = CHCONH_2$) were polymerised with bisacrylamide [$CH_2(NH\ CONH = CH_2)_2$ bis] to obtain the gel. Freshly prepared

Table 2. *P. nigrum* varieties used in the study

Varieties	Place of collection	Remarks
1. Kalluvally Type 1	Thaliparamba	
2. Kottanadan	PRS, Panniyur	
3. Karimkotta	Vilakode	
4. Neelamundi Type 2	PRS, Panniyur	
5. Perumunda	Travancore	
6. Arakkulamunda	Kottayam	
7. Veluthanamban	Thodupuzha	
8. TMB-2	Thaliparamba	
9. Doddiga	Mysore	
10. Shimoga	Mysore	
11. Malligesera	Mysore	
12. Ceylon	Sri Lanka	
13. Sullia	Sullia, Mysore	
14. Nilgiri-4	Nilgiris	
15. Cheriyakaniyakkadan	Kottayam	
16. Panniyur-1	PRS, Panniyur	Hybrid of Uthiramkotta x Cheriyakaniyakkadan
17. Panniyur-3	,,	,,
18. Uthiramkotta	Thalipparamba	
19. Panniyur-2	PRS, Panniyur	Selection from Balankotta
20. Panniyur-4	,,	Selection from Kuthiravally Type 2
21. Panniyur-5	,,	Selection from Perumkodi
22. Narayakodi Type 1	Travancore	
23. Sreekara	NRCS, Calicut	Selection from Karimunda
24. Shubhakara	,,	,,
25. Panchami	,,	
26. TMB-5	Taliparamba	

Table 3. *Piper* spp included in the study

Name
1. *P. pseudonigrum* Velayudhan and Amalraj
2. *P. nigrum* Linn.
3. *P. bababudani* Rahiman
4. *P. galeatum* C.DC.
5. *P. longum* Linn.
a) Type Sheemathippali
b) Type Panniyur
c) Type Mulayam
d) Male type
6. *P. colubrinum* Link
7. *P. hapnium* Miq
8. *P. chaba* Hunter
9. *P. betle* Linn.
a) Type-1
b) Type-2
c) Type-3
10. *P. attenuatum* Buch-Ham
a) Type-1
b) Type-2
c) Type-3
11. *P. argyrophyllum* Miq.
a) Type-1
b) Type-2
c) Type-3
d) Type-4

ammonium persulphate was used as catalyst and N, N, N', N' tetra methylene diamine (TEMED) as chain initiator.

Polyacrylamide gel was preferred because of its chemical inertness, high resolution, ease in handling and easiness in preparation.

3.2.1 Enzymes assayed

Electrophoresis and isoenzyme variation determination were done for the following enzymes

1. Peroxidase
2. Esterase
3. Glutamate oxaloacetate transaminase

The above enzymes were selected as they are commonly occuring plant enzymes.

3.2.2 Preparation of the sample

Two noded cuttings were raised in polybags filled with potting mixture consisting of sand soil and farmyard manure in 1:1:1 ratio. To select the ideal part for assay plant parts viz. root stem and leaf were used. In the case leaf different maturity stages such as very tender, tender, mature and dark green were also included. First two leaves from the tip of a flushing shoot were treated as very tender. The two subsequent leaves i.e., third and fourth from the tip were taken as tender leaves. These generally attained full size but were tender. The mature leaf was that leaf which had already turned to green colour but not much brittle and had a light texture with lesser cuticular wax. Dark green leaves were thick and with considerable wax

coating and dark green colour. The samples for analysis of each enzyme were collected at the desired stage from one year old rooted cuttings. The plant sample collected was washed to remove dust, dirt and other extraneous matters. It was then rinsed with distilled water. The rinsed material was gently pressed between blotting paper to remove water.

The sample was then weighed and ground in a pre-cooled mortar, along with 0.040 g of insoluble PVP and extraction buffer (0.1 M Tris hydrochloride pH 7.6) taken in suitable proportion, below 4°C by keeping in an ice tray. Insoluble PVP was used to coagulate the polyphenols and thus prevent oxidation by polyphenol oxidase enzyme. Since insoluble PVP was used it was removed during subsequent centrifuging. From the different proportions tried it was found that a sample buffer ratio of 4:5 was ideal to get sufficient volume of extract in required concentration.

Neutral sand was added for easy grinding especially for samples of root and mature leaf. The homogenised material was centrifuged at 15000 r.p.m. for 20 minutes in a Remi centrifuge below 4°C. After centrifuging, the supernatent was removed into vials labelled and stored below subzero temperature (refregerator freezer chest). Fresh samples were collected each day as it was found that in the case of *Piper* spp. the stability of enzymes was very poor even under subzero temperature.

3.2.3 Preparation of the gel

Reagents

The analyses of the enzymes were carried out in anionic system.

The following stock solutions were prepared

Solution A

Tris 36.6 g

TEMED 0.23 ml

I N HCl 48 ml

Volume made upto 100 ml with distilled water

pH 8.9

Solution B

Acrylamide 28.0 g

N' N' methylene bis acrylamide 0.735 g

Volume made upto 100 ml with distilled water

Solution C

Ammonium persulphate - 0.14 g

Volume made upto 125 ml with distilled water

Solution D

Acrylamide 18.0 g

Bis-acrylamide 0.47 g

Made up the volume to 100 ml with distilled water

Working solution

	Acrylamide concentration	
	7.5%	8.5%
Solution A (ml)	2	2
Solution B (ml)	4.285	4.860
Solution C (ml)	9.715	9.150

Working solution was prepared by mixing the stock solutions A, B and C in the above quantities to get the required gel concentration. Solution 'C' was prepared fresh every time. Solution A and B were stored in amber coloured bottles.

Stalking gel solution

Stalking gel solution contained

Solution A (ml) 2

Solution D (ml) 4

Solution C (ml) 10

Electrode buffer

Stock solution

Tris - 6 g

Glycine - 28.8 g

Volume made upto one litre with distilled water keeping the pH at 8.3. The stock buffer was diluted 1:9 before use.

The slab gel unit of 'Biochem' was used in the study. The size of slab gel was 16 cm x 14 cm. After preparing the working solution it was gently poured in between the glass plates kept in polymerisation stand. Polymerisation was achieved within three fourth of an hour to one hour. For peroxidase and glutamate oxaloacetate transaminase stalking gel to a width of 1-1.5 cm was also used for better resolution of bands. For esterase use of stalking gel did not give any added advantage and hence was not used.

After polymerisation, the gels along with glass plates were removed to electrophoretic apparatus. The upper and lower trays of the unit were filled with electrode buffer. Upper trough was connected to cathode and the lower one to the anode.

Electrophoresis was carried out at 5°C. A constant current of 25 mA per slab was maintained throughout the run. Bromophenol blue (0.002%) in imidazole buffer (pH 7.0) was used as the tracer dye.

3.2.4 Enzyme assays

3.2.4.1 Peroxidase

Gel concentration of 7.5 per cent acrylamide was found best for the peroxidase enzyme separation in *Piper* spp.

Gel buffer - Tris hydrochloride pH 8.9.

Electrode buffer - Tris-Glycine pH 8.3

Staining solution (modified from Shaw and Koen, 1968)

100 ml solution contained

0.2 M Acetate buffer pH 5.6 - 100 ml

Benzidine - 0.1 g

Hydrogen peroxide 3% - 0.4 ml

Fresh stain was prepared each time. Acetate buffer and benzidine were mixed, heated to boil, cooled, filtered and then hydrogen peroxide was added to the

mixture. The gels were immersed in staining solution for about one hour and de-stained in 7 per cent acetic acid. As the bands faded on standing. for long time photographs were taken on the same day of staining.

3.2.4.2 Esterase

Gel concentration: same as that for peroxidase

Gel buffer and electrode buffer: the same buffers as that of peroxidase were used.

Staining solution (modified from Shaw and Koen, 1968)

100 ml of staining solution contained

Phos A - Na_2HPO_4 (0.2 M), pH 8.8	- 10 ml
Phos B - NaH_2PO_4 (0.2 M), pH 4.16	- 50 ml
Fast blue RR	- 100 mg
α-naphthyl acetate in 50% acetone	- 2 ml
Distilled water	- 40 ml

After the run was over, the gels were taken out and incubated in the staining solution at 37°C for 45-60 minutes till brown bands appeared. The gels were destained in 7 per cent acetic acid. The bands remained stable for 2-3 days.

3.2.4.3 Glutamate oxaloacetate transaminase

Gel concentration: 8.5% acrylamide

Gel and electrode buffer: same as that of peroxidase

Staining solution (Shaw and Koen, 1968)

100 ml of staining solution contained

L-aspartic acid	- 532 mg
α-keto glutaric acid	- 72 mg
Pyridoxal 5' phosphate	- 50 mg
Fast violet B-salt	- 400 mg
0.1 M Phosphate buffer pH 7.0	- 100 ml

Fast violet B-salt was added just before use. Gels were incubated in the staining solution for 15 to 20 minutes till reddish orange bands developed. After staining, the gel was fixed in glycerine. The bands faded on standing. So photographs were taken within few hours of staining.

3.3 Nomenclature of Isozymes adopted in the present study

The enzymes were designated by following abbreviations

1. Peroxidase	- PRX
2. Esterase	- EST
3. Glutamate oxaloacetate transaminase	- GOT

Numbering

For numbering of enzymes all the isoenzymes of an enzyme in the species studied were pooled. The fastest moving anodal band numbered 1 (eg. PRX-1). The slower ones were given subsequent numbers.

3.4 Measurement of similarity

The measurement of electrophoretic similarity among varieties of *P. nigrum* L. and among *Piper* spp. was calculated by making pairwise comparison of the genotypes using method of Sokel and Sneath (1963) using the formula

$$SI = \frac{\text{Number of homologous bands}}{\text{Number of homologous bands} + \text{Number of non homologous bands}}$$

Average of similarity indices for all enzymes were computed and also pooled data compared.

Results and Discussion

RESULTS AND DISCUSSION

The results of the present study are presented under three major heads was follows:

1. Isoenzymes in different plant parts and at different stages of development of leaves
2. Isoenzyme variation within *Piper nigrum* L.
3. Isoenzyme variation and species relationship in the genus *Piper*

4.1 Isoenzyme in different plant parts and at different stages of development of leaves

To study the banding pattern for the three enzymes viz. peroxidase, esterase and GOT in different plant parts leaf, stem and root were analysed. In the case of leaf, different maturity stages such as very tender, tender, mature and dark green were also included. For standardizing the plant part and stage of growth of leaf for each enzyme analysis, the variety Panniyur-1 was taken as a standard.

4.1.1 Peroxidase

In the analysis of isoenzyme variation in different plant parts at different stages of development ten isoenzymes could be observed in *P. nigrum*. These were numbered PRX-1 to 10 for convenience and later changed based on pooling and numbering the isoenzymes in different species.

The activity of peroxidase in stem was very weak. In leaf, the tender and very tender leaf showed only negligible activity of peroxidase. PRX-1, PRX-2 and

PRX-3 were visible in these cases (Fig.1 and Plate 1). There was an increase in the activity of this enzyme with advancement of maturity of leaf. However, the roots were found to be the most ideal part for peroxidase enzyme showing better clarity in separation probably due to less chlorophyll interference. Dark green leaf was found to be the second best for peroxidase and was used as the sample material whenever destructive sampling and collection of root was not possible due to scarcity of material.

Peroxidase enzyme in plants is considered as IAA oxidase and it is a general observation in plants that activity of this enzyme increases with increasing maturity.

4.1.2 Esterase

In the case of esterase enzyme, four isoenzymes were observed in *P. nigrum* and were named EST-1, EST-2, EST-3 and EST-4. As in the case of peroxidase enzyme, these numbering were changed when species and varieties were compared. The activity of esterase enzyme were found to be highly varying at different stages of maturity and in plant parts (Fig.2 and Plate 2). In the case of stem samples, activity of only EST-4 was observed. EST-1 was visible only in mature and dark green leaves. However, activity of EST-1 was not always predictable. It appeared either singly or in groups and hence was not considered in the present study. The activity of EST-2 was observed to be increasing with maturity of leaf. However, the band was clearly visible from tender stage onwards. Activity of EST-3 was very much unpredictable in more mature leaves and was very rarely observed in mature leaves.

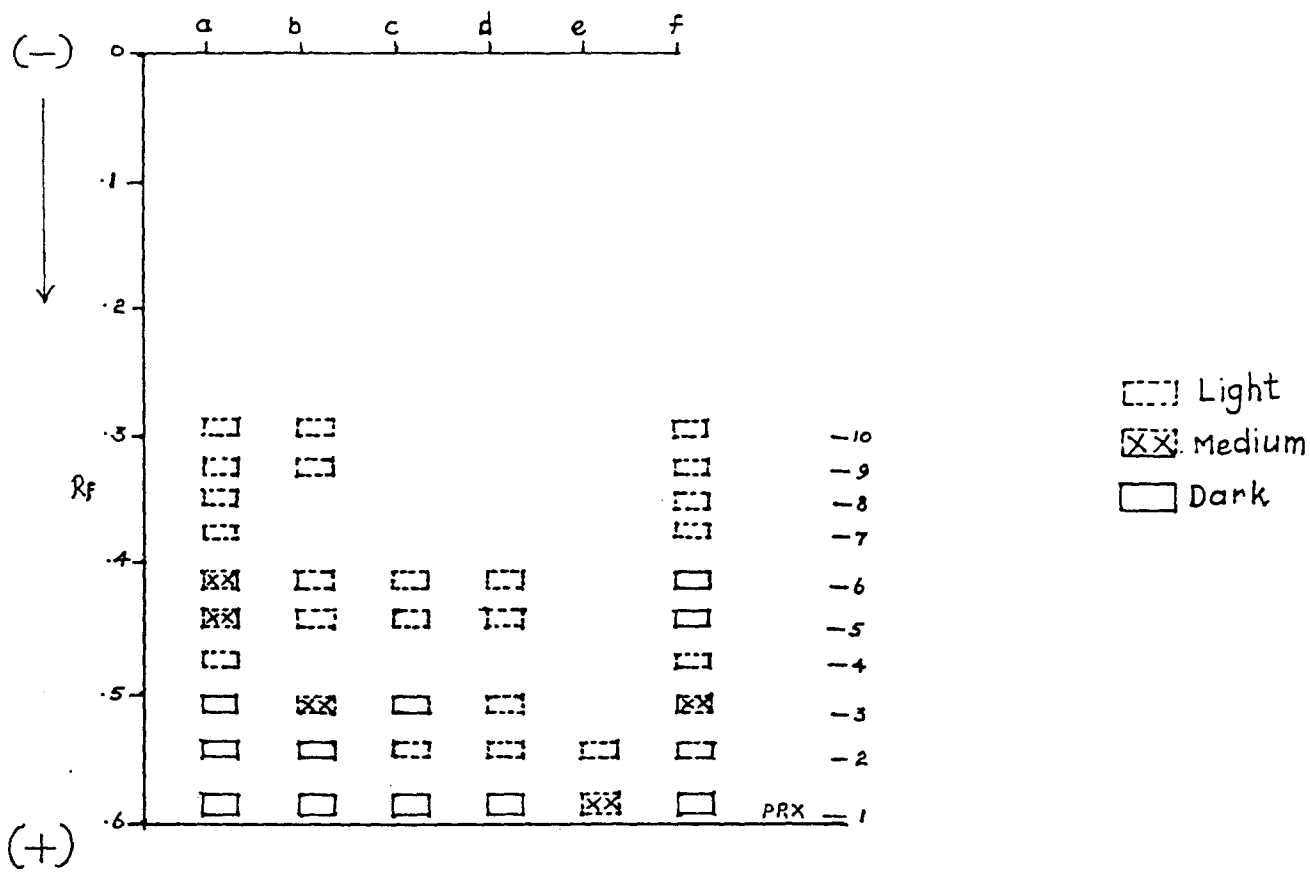


FIG.1. PEROXIDASE ZYMOGRAM IN ROOT, STEM AND AT DIFFERENT STAGES OF LEAF DEVELOPMENT IN *Piper nigrum* var Panniyur-1

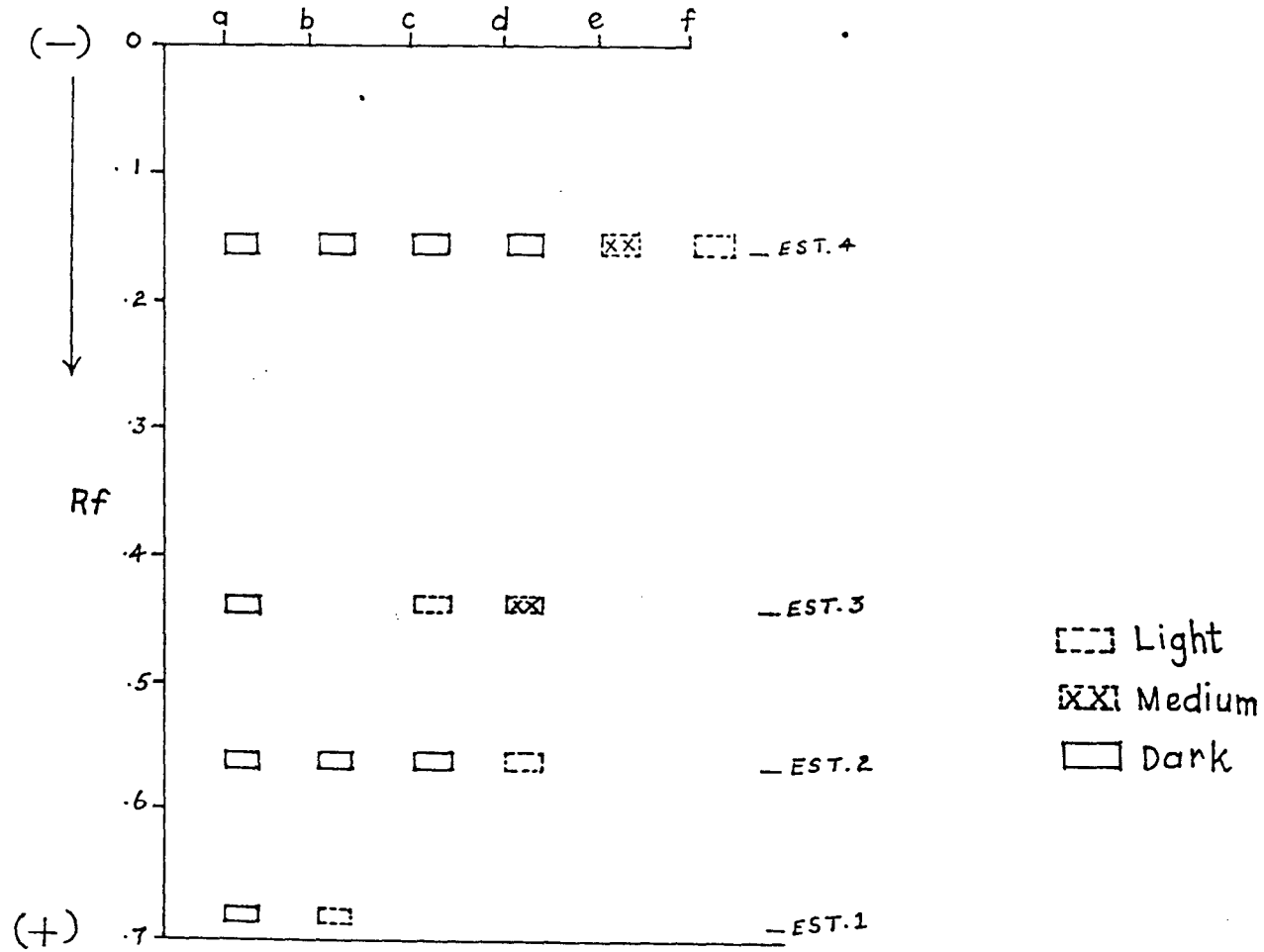
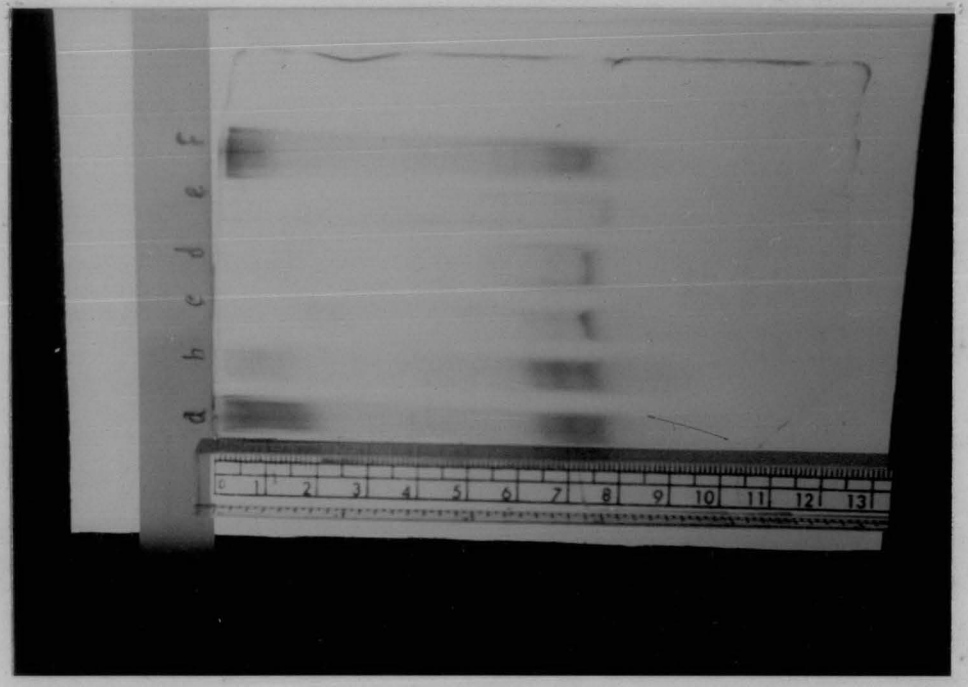
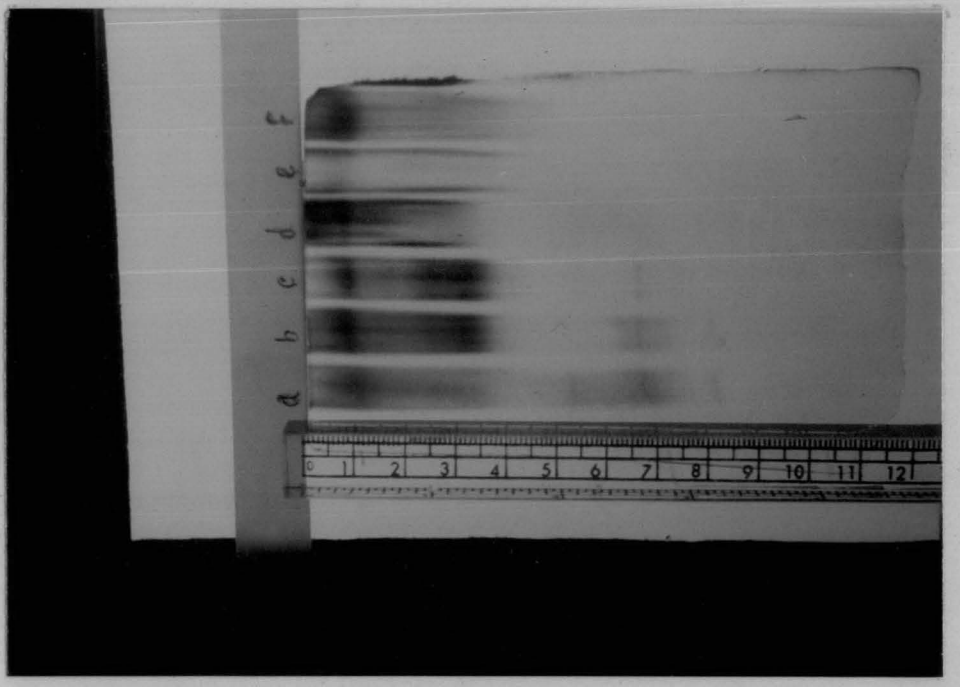


FIG.2. ESTERASE ZYMOGRAM IN ROOT, STEM AND AT DIFFRENT STAGES OF LEAF DEVELOPMENT IN *Piper nigrum* var Panniyur-1.

Plate 1. Peroxidase banding pattern in root, stem and different stages of development of leaf

Plate 2. Esterase banding pattern in root, stem and different stages of development of leaf


d b c d e f
0 1 2 3 4 5 6 7 8 9 10 11 12 13


d b c d e f
0 1 2 3 4 5 6 7 8 9 10 11 12

EST-4 was observed at all stages of maturity of leaf. Other plant parts such as stem and root showed very little activity of EST-4. So the option was to select the combination of EST-2 and 4 (tender leaf) or EST-3 and 4 (very tender leaf). In the case of very tender leaves the extract was highly viscous and difficult to handle. Therefore, tender leaves were selected for esterase analysis.

4.1.3 Glutamate oxaloacetate transaminase (GOT)

No variation was found in the GOT zymograms at different stage of growth of leaf and the activity was less in root and stem. Hence, tender leaves were selected for convenience of analysis. However in *P. attenuatum* and *P. betle* leaf showed very little activity and roots were used for analysis of GOT in these cases.

4.2 Isoenzyme banding pattern of *P. nigrum* L.

Twenty six varieties of *P. nigrum* representing a wide genetic base with regard to morphological characters from Kerala and neighbouring states were analysed for variation at three enzyme loci viz., esterase, peroxidase and GOT.

4.2.1 Peroxidase

Four variant isoenzymes were found in the varieties studied. Ten bands were found common in all the varieties, they were PRX-4, 6, 10, 13, 14, 17, 18, 19, 20, 21 and 22 (Fig.3; Plates 3, 4, and 5). PRX-22 was a common variant additionally observed which was found in eleven varieties (Table 4). PRX-23 was found in five varieties. PRX-25 was found in three varieties viz., Arakkulamunda, TMB-2 and Doddiga. Another variant PRX-24 was less frequent and found only in variety Shimoga among the 26 varieties studied.

The rarer isoenzymes observed had no geographical isolation. Varieties from northern or southern Kerala and Karnataka had the varient isoenzymes distributed in them.

4.2.2 Esterase

Tender leaf was taken for analysis of esterase isozyme. Five isoenzymes were observed in the 26 collections studied (Fig.4; Plates 6 and 7). EST-15 was common in all the twenty six types. Varieties could be grouped into two based on the presence of either EST-2 or EST-4 in them (Table 5).

These could be alleles of the same locus which only a genetical study can confirm. EST-3 was a rare variant observed in variety Arakkulamunda which appeared as a narrow band adjacent to EST-2. EST-16 was observed in Uthirankotta, Narayakodi Type-1 and Sreekara.

4.2.3 Glutamate oxaloacetate transaminase (GOT)

As it can be seen from Fig.5 and Plates 8, 9 and 10, the varieties showed considerable variation in the banding pattern of GOT enzyme. A total number of 15 isoenzymes were observed in the 26 varieties of *P. nigrum* analysed. Pooling them with general isoenzymes of *Piper* spp. studied, the isoenzymes present in *P. nigrum* were numbered GOT 2, 3, 4, 6, 7, 8, 10, 14, 16, 17, 18, 19, 21, 22 and 23. GOT-3 was observed in two varieties TMB-2 and TMB-5. GOT-6 was observed in Perumunda, Malligesera and Nilgiri-4. GOT-8 was visible in Sreekara and Shubhakara, which were selections from Karimunda. Cheriakaniakkadan, Panniyur-2 and TMB-5 possessed the GOT-10.

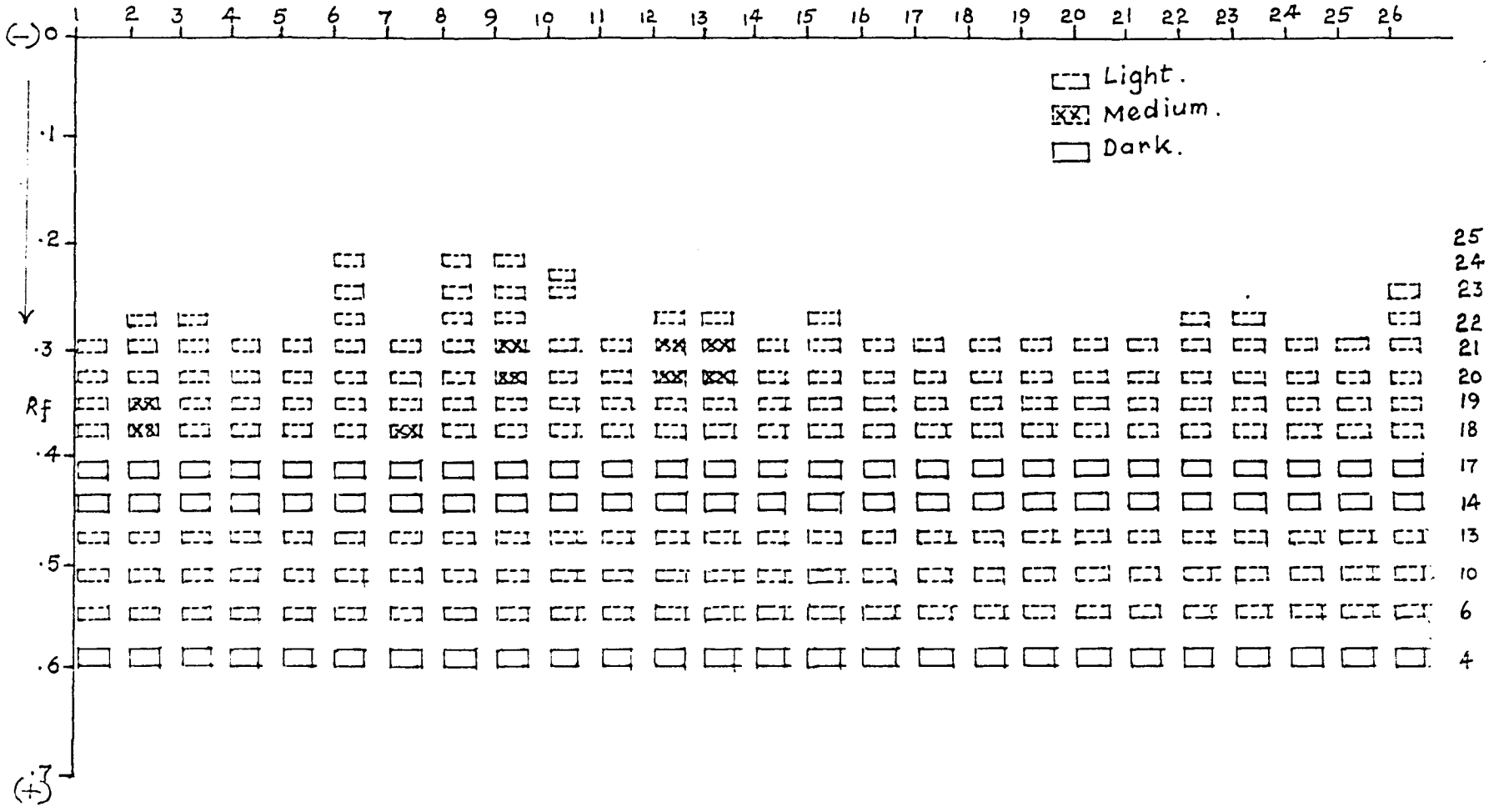


FIG.3. ZYMOGRAM OF PEROXIDASE IN *Piper nigrum* VARIETIES

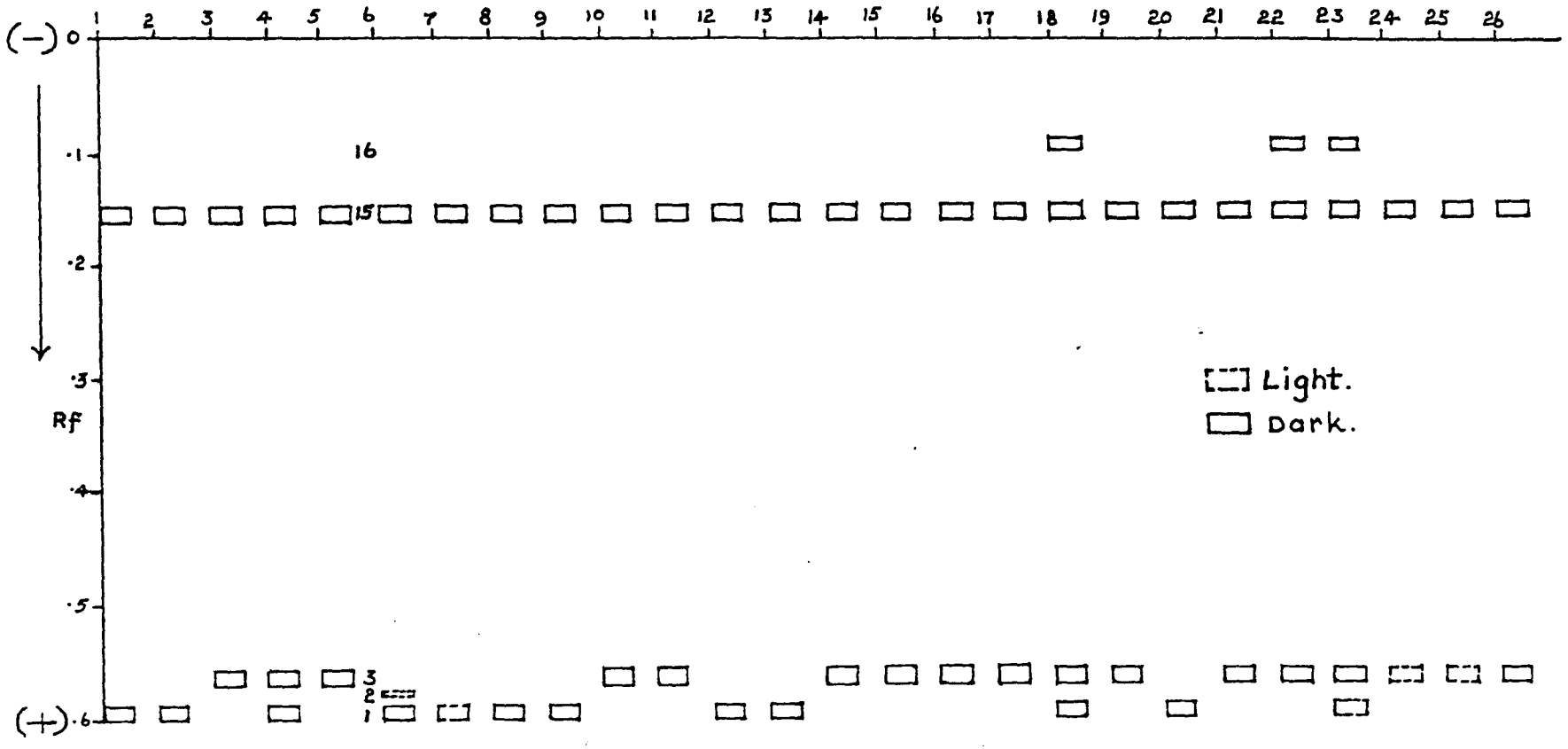
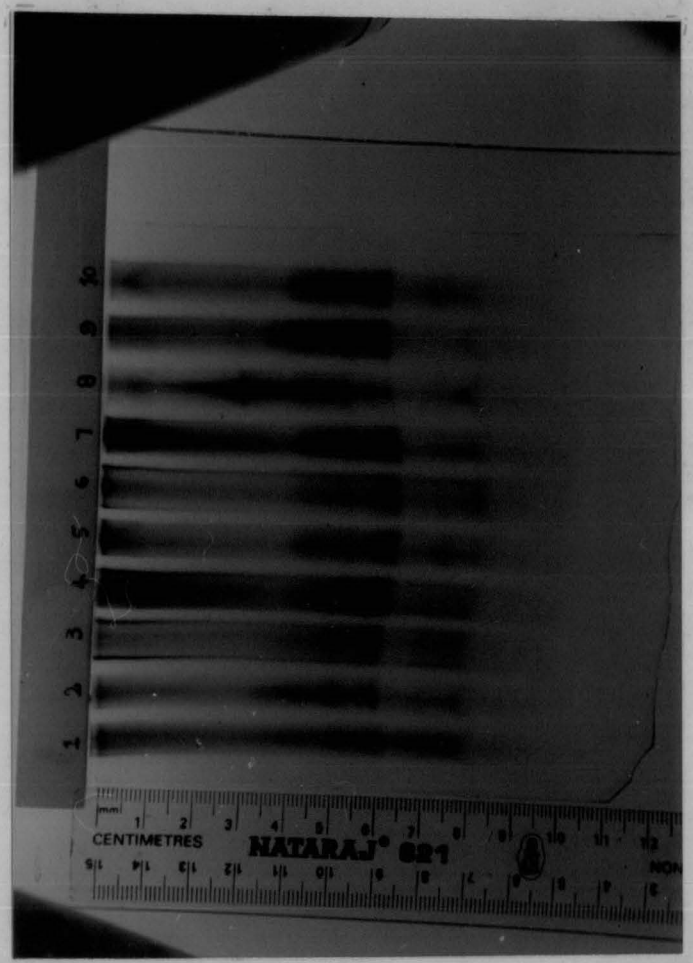


FIG. 4. ZYMOGRAM OF ESTERASE IN *Piper nigrum* VARIETIES

Plate 3 & 4. Peroxidase banding pattern in *P. nigrum* L. varieties


CENTIMETRES
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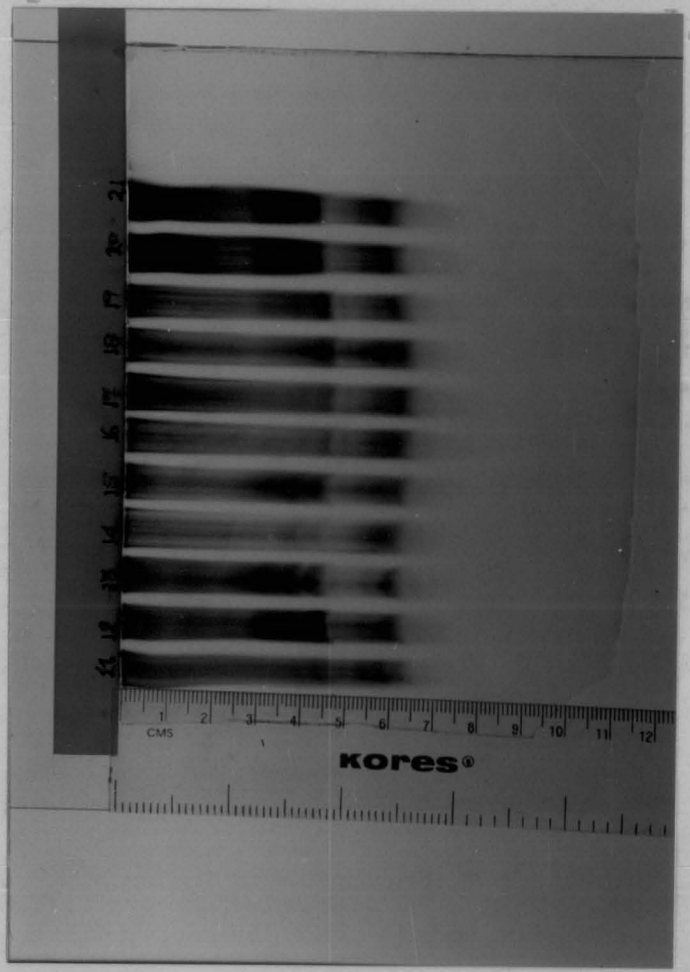
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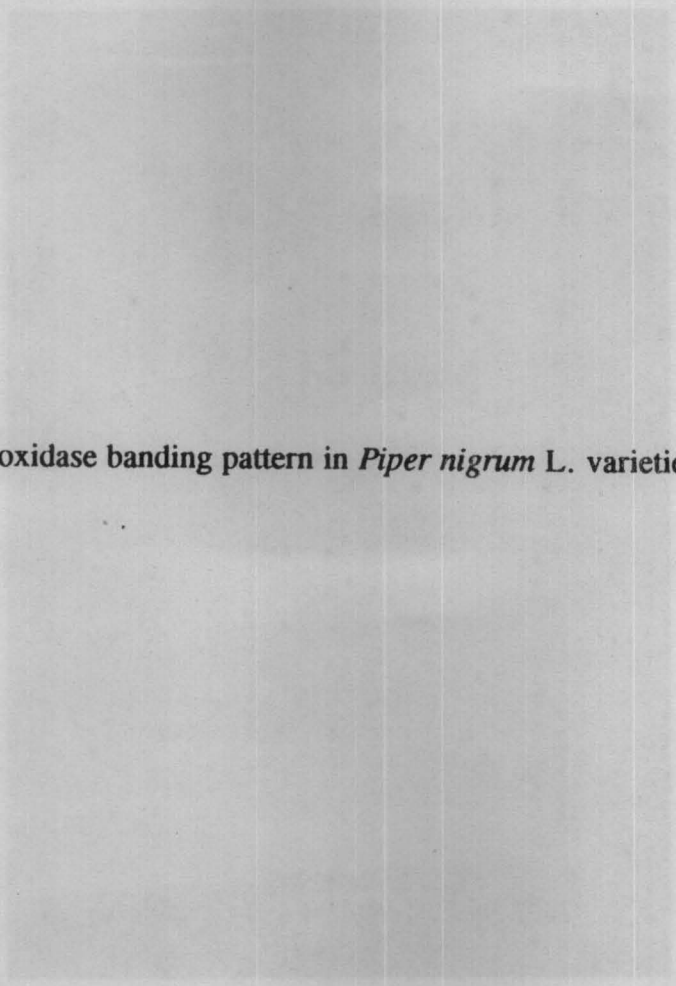
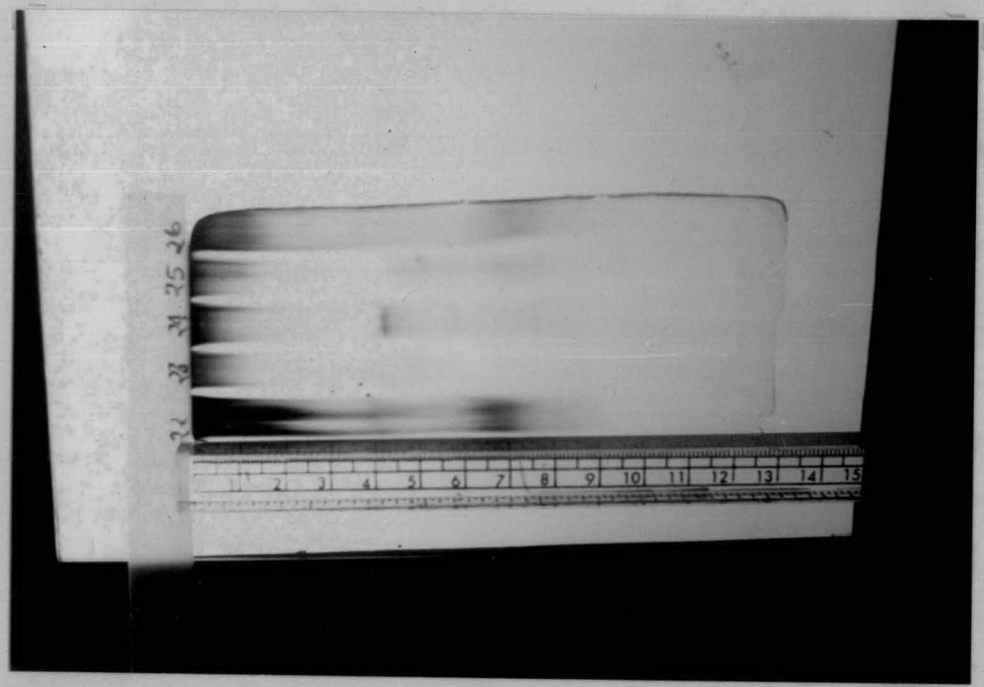


Plate 5. Peroxidase banding pattern in *Piper nigrum* L. varieties

Plate 6. Esterase banding pattern in *P. nigrum* L. varieties



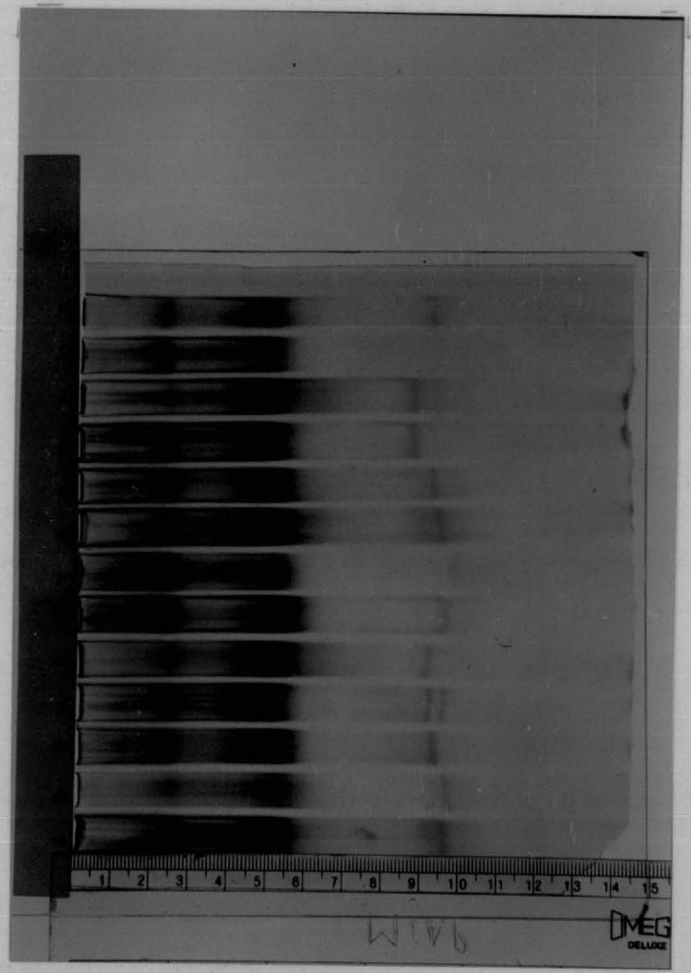


Plate 7. Esterase banding pattern in *Piper nigrum* L. varieties

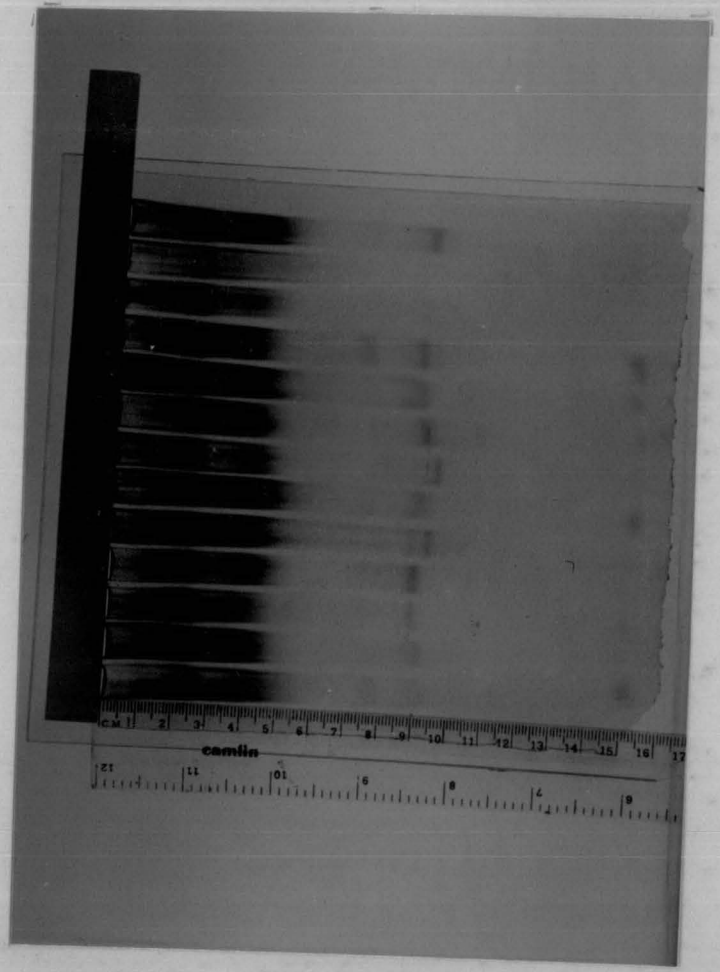

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Table 4. Grouping of *P. nigrum* L. varieties based on isoenzyme banding pattern of peroxidase

Varieties with only the ten isozymes	Varieties with PRX-22	Varieties with PRX-23	Varieties with PRX-25	Varieties with PRX-24
Kalluvally Type-1	Kottanadan			
Neelamundi Type-2	Karimkotta	Arakkula-munda	Arakkula-munda	
Perumunda	Arakkula-munda	TMB-2	TMB-2	Shimoga
Velutha-namban	TMB-2	Doddiga	Doddiga	
	Doddiga	Shimoga		
Malligesera	Ceylon	TMB-5		
	Sullia			
Nilgiri-4	Cheriyakaniya-kkadan			
Panniyur-1	Narayakodi Type-1			
Panniyur-3				
Uthirankotta	Sreekara			
Panniyur-2	TMB-5			
Panniyur-4				
Panniyur-5				
Shubhakara				
Panchami				

Table 5. Grouping of *P. nigrum* L. varieties based on presence of EST-2 or EST-4

Varieties with EST-2	Varieties with EST-4
Kalluvally Type-1	Karimunda
Kottanadan	Neelamundi Type-2
Neelamundi Type-2	Perumunda
Arakkulamunda	Shimoga
Veluthanamban	Malligesera
TMB-2	Nilgiri-4
Doddiga	Cheriyakaniyakkadan
Ceylon	Panniyur-1
Sullia	Panniyur-3
Uthirankotta	Uthirankotta
Panniyur-4	Panniyur-2
Sreekara	Panniyur-5
	Narayakodi Type-1
	Sreekara
	Shubhakara
	Panchami
	TMB-5

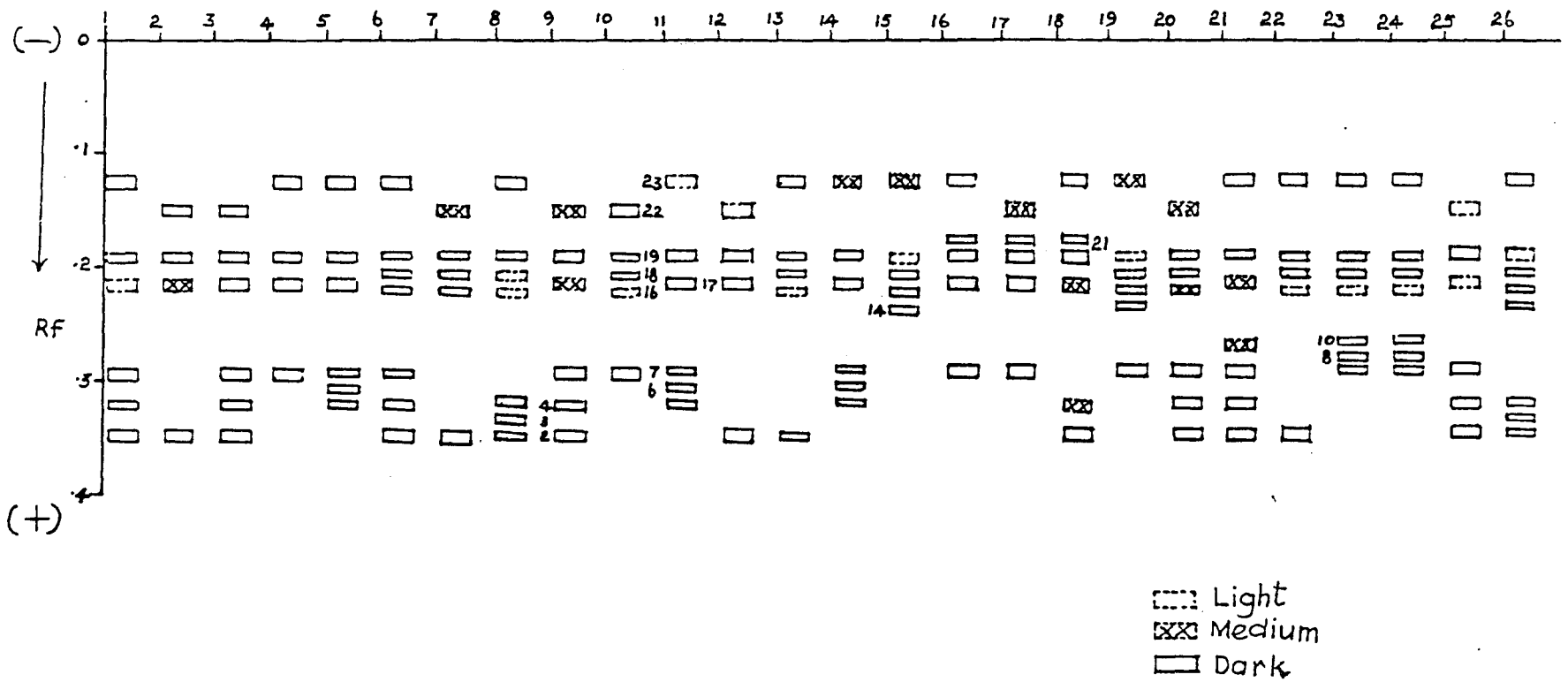

FIG. 5. ZYMOGRAM OF GOT IN *Piper nigrum* VARIETIES

Plate 8. GOT banding pattern in *P. nigrum* L. varieties

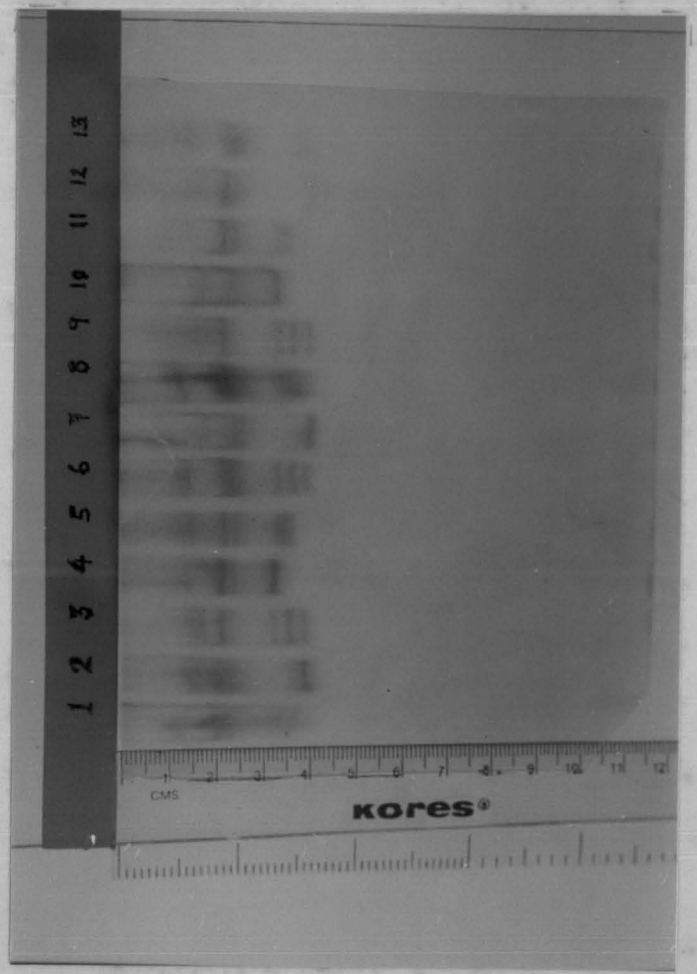
1 2 3 4 5 6 7 8 9 10 11 12 13
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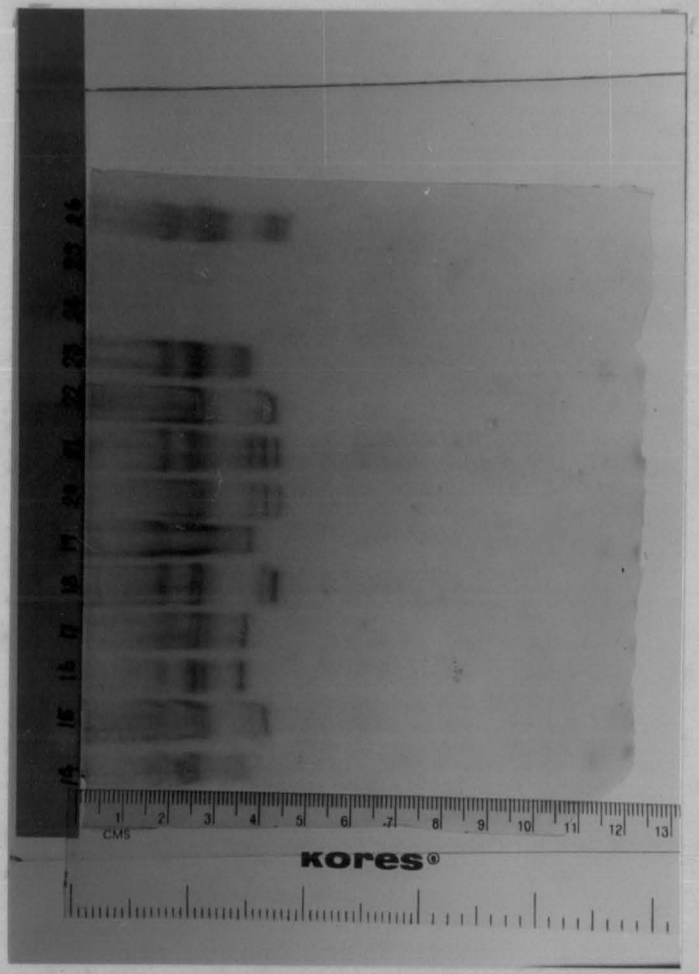
Plate 9 & 10. GOT banding pattern in *Piper nigrum* L. varieties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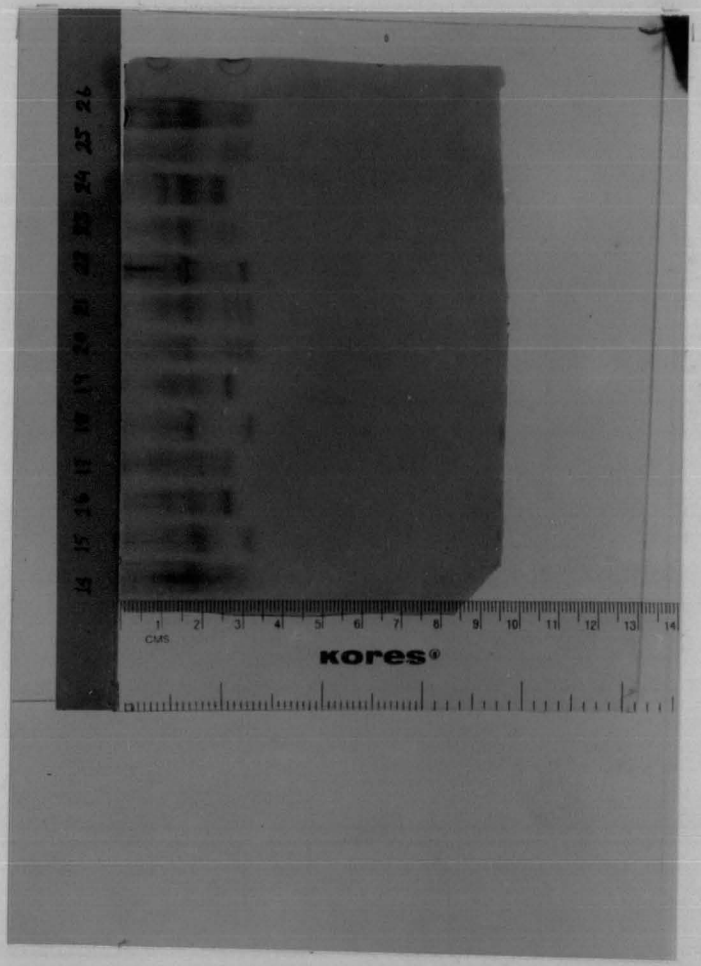
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Based on this complex pattern all the 26 varieties were grouped into eight groups (Table 6).

Isoenzyme pattern for GOT in *Piper nigrum* gave a very complicated picture with 15 isoenzymes in different combinations in different varieties. GOT is reported to be a dimeric protein and such complicated patterns could be expected in a highly heterozygous population. A genetic analysis would have much simplified the existing situation.

4.2.4 Similarity among *P. nigrum* varieties

Similarity index among the varieties for isozyme banding pattern was calculated according to Sokel and Sneath (1968). The similarity index values for the three enzymes separately as well as the pooled data are presented hereunder.

4.2.4.1 Peroxidase

Range of similarity among the varieties for peroxidase zymogram was 0.7692 to 1 (Table 7). Three groups of varieties were obtained with similarity index 'one' among the members (Table 8).

Varieties Shimoga and TMB-5 did not fall in any of these groups due to a unique isoenzyme PRX-24 in Shimoga and absence of PRX-25 in TMB-5. The least similarity of 0.7692 was observed between varieties of group I and group III. Similarity of variety Shimoga with varieties of group II was also the same.

Table 6. Grouping of *P. nigrum* varieties based on isoenzyme banding pattern of GOT

Varieties with only GOT-2	Varieties with only GOT-7	Variety with only GOT-2, 3 and 4	Variety with GOT-2 and 4	Variety with GOT-3, 4 and 7	Variety with GOT-4, 6 and 7	Variety with GOT-7, 8 and 10	Variety with GOT-2, 4, 7 and 10
Kottanadan	Neelamundi Type 2	TMB -2	Uthirankotta	Kalluvally Type 1	Perumunda	Sreekara	Panniyur-5
Veluthanamban	Shimoga	TMB-5		Karimkotta	Malligesera	Shubhakara	
Ceylon	Panniyur-1			Arakkulamunda	Nilgiri-4		
Cheriakaniyakkadan	Panniyur-3			Doddiga			
Narayakodi Type 1	Panniyur-2			Panniyur-4			
				Panchami			

Table 7. Similarity index for peroxidase in *P. nigrum* L. varieties

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	1																									
2	0.9090	1																								
3	0.9090	1	1																							
4	1	0.9090	0.9090	1																						
5	1	0.9090	0.9090	1	1																					
6	0.7692	0.8461	0.8461	0.7692	0.7692	1																				
7	1	0.9090	0.9090	1	1	0.7692	1																			
8	0.7692	0.8461	0.8461	0.7692	0.7692	1	0.7692	1																		
9	0.7692	0.8461	0.8461	0.7692	0.7692	1	0.7692	1	1																	
10	0.8333	0.7692	0.7692	0.8333	0.8333	0.7857	0.8333	0.7857	0.7857	1																
11	1	0.9090	0.9090	1	1	0.7692	1	0.7692	0.7692	0.8333	1															
12	0.9090	1	1	0.9090	0.9090	0.8461	0.9090	0.8461	0.8461	0.7692	0.9090	1														
13	0.9090	1	1	0.9090	0.9090	0.8461	0.9090	0.8461	0.8461	0.7692	0.9090	1	1													
14	1	0.9090	0.9090	1	1	0.7692	1	0.7692	0.7692	0.8333	1	0.9090	0.9090	1												
15	0.9090	1	1	0.9090	0.9090	0.8461	0.9090	0.8461	0.8461	0.7692	0.9090	1	1	0.9090	1											
16	1	0.9090	0.9090	1	1	0.7692	1	0.7692	0.7692	0.8333	1	0.9090	0.9090	1	1	1										
17	1	0.9090	0.9090	1	1	0.7692	1	0.7692	0.7692	0.8333	1	0.9090	0.9090	1	1	1	1									
18	1	0.9090	0.9090	1	1	0.7692	1	0.7692	0.7692	0.8333	1	0.9090	0.9090	1	1	1	1	1								
19	1	0.9090	0.9090	1	1	0.7692	1	0.7692	0.7692	0.8333	1	0.9090	0.9090	1	1	1	1	1	1							
20	1	0.9090	0.9090	1	1	0.7692	1	0.7692	0.7692	0.8333	1	0.9090	0.9090	1	1	1	1	1	1	1						
21	1	0.9090	0.9090	1	1	0.7692	1	0.7692	0.7692	0.8333	1	0.9090	0.9090	1	1	1	1	1	1	1	1					
22	0.9090	1	1	0.9090	0.9090	0.8461	0.9090	0.8461	0.8461	0.7692	0.9090	1	1	0.9090	0.9090	0.9090	0.9090	0.9090	0.9090	0.9090	0.9090	1				
23	0.9090	1	1	0.9090	0.9090	0.8461	0.9090	0.8461	0.8461	0.7692	0.9090	1	1	0.9090	0.9090	0.9090	0.9090	0.9090	0.9090	0.9090	0.9090	1	1			
24	1	0.9090	0.9090	1	1	0.7692	1	0.7692	0.7692	0.8333	1	0.9090	0.9090	1	1	1	1	1	1	1	1	0.9090	0.9090			
25	1	0.9090	0.9090	1	1	0.7692	1	0.7692	0.7692	0.8333	1	0.9090	0.9090	1	1	1	1	1	1	1	1	0.9090	0.9090	1	1	
26	0.8333	0.9166	0.9166	0.8333	0.8333	0.9230	0.8333	0.9230	0.9230	0.8461	0.8333	0.9166	0.9166	0.8333	0.8333	0.8333	0.8333	0.8333	0.8333	0.8333	0.8333	0.9166	0.9166	0.8333	0.8333	1

Table 8. Groups of *P. nigrum* L. varieties having similarity index 'one' among the members for peroxidase zymogram

Groups of varieties		
Group I	Group II	Group III
Kalluvally Type-1	Kottanadan	Arakkulamunda
Neelamunda Type-2	Karimkotta	TMB-2
Perumunda	Ceylon	Doddiga
Veluthanamban	Sullia	
Malligesera	Cheriyakaniyakkadan	
Nilgiri-4	Narayakodi Type-1	
Panniyur-1	Sreekara	
Panniyur-3		
Uthirankotta		
Panniyur-2		
Panniyur-4		
Panniyur-5		
Subhakara		
Panchami		

4.2.4.2 Esterase

Range of similarity among varieties for esterase enzyme was 0.2000 to 1 (Table 9). Based on the maximum similarity (S.I. = 1) observed among members, *P. nigrum* varieties could be grouped into three as furnished in Table 10.

Least similarity was (S.I. = 0.2) was observed between Arakkulamunda and Narayakodi Type-1.

4.2.4.3 GOT

In the case of GOT, a similarity range of 0.0835 to 1 was observed (Table 11). Five groups of varieties were observed with similarity index 'one' among the members within the group (Table 12). The least similarity was observed between Panniyur-3 and TMB-5.

4.2.5 Similarity index among *P. nigrum* L. varieties for isoenzyme banding pattern

Similarity index for three isoenzymes put together ranged from 0.3978 to 1 (Table 13). Maximum similarity among 26 varieties studied were shown between Kottanadan and Ceylon and also among Perumunda, Malligesera and Nilgiri-4. The least similarity is between TMB-2 and Panniyur-3 (S.I. = 0.3978).

The wide variation among cultivated *P. nigrum* is in confirmation with Kanakamani's (1985) earlier observation. On the basis of morphological studies she got 40 groups, out of the 45 types studied and showing considerable variation in different morphological characters among the varieties of *P. nigrum*.

Table 9. Similarity index for esterase in *P. nigrum* L. varieties

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	1																									
2	1	1																								
3	0.3333	0.3333	1																							
4	0.6666	0.6666	0.6666	1																						
5	0.3333	0.3333	1	0.6666	1																					
6	0.6666	0.6666	0.2500	0.5000	0.2500	1																				
7	1	1	0.3333	0.6666	0.3333	0.6666	1																			
8	1	1	0.3333	0.6666	0.3333	0.6666	1	1																		
9	1	1	0.3333	0.6666	0.3333	0.6666	1	1	1																	
10	0.3333	0.3333	1	0.6666	1	0.6666	0.3333	0.3333	0.3333	1																
11	0.3333	0.3333	1	0.6666	1	0.2500	0.3333	0.3333	0.3333	1	1															
12	1	1	0.3333	0.6666	0.3333	0.2500	1	1	1	0.3333	0.3333	1														
13	1	1	0.3333	0.6666	0.3333	0.6666	1	1	1	0.3333	0.3333	1	1													
14	0.3333	0.3333	1	0.6666	1	0.2500	0.3333	0.3333	0.3333	1	1	0.3333	0.3333	1												
15	0.3333	0.3333	1	0.6666	1	0.2500	0.3333	0.3333	0.3333	1	1	0.3333	0.3333	1	1											
16	0.3333	0.3333	1	0.6666	1	0.2500	0.3333	0.3333	0.3333	1	1	0.3333	0.3333	1	1	1										
17	0.3333	0.3333	1	0.6666	1	0.2500	0.3333	0.3333	0.3333	1	1	0.3333	0.3333	1	1	1	1									
18	0.5000	0.5000	0.5000	0.7500	0.5000	0.4000	0.5000	0.5000	0.5000	0.5000	0.5000	0.5000	0.5000	0.5000	0.5000	0.5000	0.5000	1								
19	0.6666	0.6666	1	0.6666	1	0.2500	0.6666	0.6666	0.6666	1	1	0.6666	0.6666	1	1	1	1	0.5000	1							
20	1	1	0.3333	0.6666	0.3333	0.6666	1	1	1	0.3333	0.3333	1	1	0.3333	0.3333	0.3333	0.3333	0.5000	0.3333	1						
21	0.3333	0.3333	1	0.6666	1	0.2500	0.3333	0.3333	0.3333	1	1	0.3333	0.3333	1	1	1	1	0.5000	1	0.3333	1					
22	0.2500	0.2500	0.6666	0.5000	0.6666	0.2000	0.2500	0.2500	0.2500	0.6666	0.6666	0.2500	0.2500	0.6666	0.6666	0.6666	0.6666	0.7500	0.6666	0.2500	0.6666	1				
23	0.5000	0.5000	0.5000	0.7500	0.5000	0.4000	0.5000	0.5000	0.5000	0.5000	0.5000	0.5000	0.5000	0.5000	0.5000	0.5000	0.5000	1	0.5000	0.5000	0.5000	0.7500	1			
24	0.3333	0.3333	1	0.6666	1	0.2500	0.3333	0.3333	0.3333	1	1	0.3333	0.3333	1.0	1	1	1	0.5000	1	0.3333	1	0.6666	0.5000	1		
25	0.3333	0.3333	1	0.6666	1	0.2500	0.3333	0.3333	0.3333	1	1	0.3333	0.3333	1.0	1	1	1	0.5000	1	0.3333	1	0.6666	0.5000	1	1	
26	0.3333	0.3333	1	0.6666	1	0.2500	0.3333	0.3333	0.3333	1	1	0.3333	0.3333	1.0	1	1	1	0.5000	1	0.3333	1	0.6666	0.5000	1	1	1

Table 10. Group of *P. nigrum* in varieties having similarity index 'one' among members for exterase enzyme

Group I	Group II	Group III
Kalluvally Type-1	Karimkotta	Uthirankotta
Kottanadan	Perumunda	Sreekara
Veluthanamban	Shimoga	
Doddiga	Malligesera	
Sullia	Nilgiri-4	
TMB-2	Cheriyakaniyakkadan	
Ceylon	Panniyur-1	
Panniyur-4	Panniyur-3	
	Panniyur-2	
	Panniyur-5	
	Shubhakara	
	Panchami	
	TMB-5	

Table 11. Similarity index for GOT in *P. nigrum* L. varieties

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	1																									
2	0.4285	1																								
3	0.7142	0.6666	1.0																							
4	0.6666	0.3333	0.4285	1																						
5	0.7142	0.2500	0.5000	0.6666	1																					
6	0.6250	0.2222	0.4444	0.3750	0.4444	1																				
7	0.7222	0.5000	0.3750	0.1250	0.100	0.5000	1																			
8	0.4444	0.2222	0.3000	0.2222	0.3000	0.7500	0.5000	1																		
9	0.7142	0.6666	1	0.4285	0.5000	0.4444	0.3750	0.3000	1																	
10	0.2222	0.2857	0.3750	0.2857	0.2222	0.5000	0.6666	0.3333	0.3750	1																
11	0.7142	0.2500	0.5000	0.6666	1	0.4444	0.1000	0.3000	0.5000	0.2222	1															
12	0.4285	1	0.6666	0.3333	0.2500	0.2222	0.5000	0.2222	0.6666	0.2857	0.2500	1														
13	0.3750	0.2857	0.2222	0.2857	0.2222	0.7142	0.6666	0.7142	0.2222	0.4285	0.2222	0.2857	1													
14	0.7142	0.2500	0.5000	0.6666	1	0.4444	0.1000	0.3000	0.5000	0.2222	1.	0.2500	0.2222	1												
15	0.3333	0.2500	0.2000	0.2500	0.2000	0.6250	0.5714	0.6250	0.2000	0.3750	0.2500	0.2500	0.8333	0.2000	1											
16	0.5714	0.2857	0.3750	0.8000	0.5714	0.3333	0.1111	0.2000	0.3750	0.2500	0.5714	0.2857	0.2500	0.5714	0.2222	1										
17	0.3750	0.5000	0.5714	0.5000	0.3750	0.2000	0.2500	0.0909	0.5714	0.4285	0.3750	0.5000	0.1111	0.375	0.1000	0.666	1									
18	0.7142	0.4285	0.5000	0.4285	0.5000	0.4444	0.2222	0.4444	0.5000	0.1000	0.5000	0.4285	0.3754	0.5000	0.3333	0.571	0.375	1								
19	0.3333	0.1111	0.2000	0.4285	0.3333	0.6250	0.3750	0.4444	0.2000	0.5714	0.3333	0.1111	0.5714	0.3000	0.7142	0.375	0.2222	0.2000	1							
20	0.4444	0.3750	0.6250	0.2222	0.3000	0.7500	0.7142	0.5555	0.6250	0.7142	0.3000	0.3750	0.5000	0.3000	0.4444	0.500	0.3333	0.3000	0.4444	1						
21	0.8571	0.3750	0.6250	0.4285	0.6250	0.5555	0.2000	0.4000	0.6250	0.2000	0.6250	0.3750	0.3333	0.6250	0.3000	0.50	0.3333	0.6250	0.3000	0.4000	1					
22	0.3750	0.2857	0.2222	0.2857	0.2222	0.7142	0.6666	0.7142	0.2222	0.4285	0.2222	0.2857	1	0.2222	0.8333	0.25	0.1111	0.3750	0.5714	0.5000	0.3333	1				
23	0.3000	0.1000	0.1818	0.3750	0.3000	0.5555	0.3333	0.4000	0.1818	0.5000	0.3000	0.1000	0.5000	0.3000	0.4444	0.33	0.2000	0.1818	0.6250	0.4000	0.4000	0.5000	1			
24	0.3000	0.1000	0.1818	0.3750	0.3000	0.5555	0.3333	0.4000	0.1818	0.5000	0.3000	0.1000	0.5000	0.3000	0.4444	0.33	0.2000	0.1818	0.6250	0.4000	0.4000	0.5000	1	1		
25	0.7142	0.6666	1	0.4285	0.5000	0.4444	0.3750	0.3000	1	0.3750	0.5000	0.6666	0.2222	0.5000	0.2000	0.3750	0.5714	0.5000	0.2000	0.5555	0.6250	0.2222	0.1818	0.1818	1	
26	0.4000	0.2000	0.2727	0.2000	0.2727	0.6666	0.4444	0.8750	0.2727	0.3000	0.2727	0.2000	0.6250	0.2727	0.7500	0.1818	0.0833	0.4000	0.5555	0.5000	0.3636	0.625	0.3636	0.3636	0.2727	1

Table 12. Groups of *P. nigrum* L. varieties having similarity index 'one' among the members for GOT zymogram

Groups of varieties				
Group I	Group II	Group III	Group IV	Group V
Kottanadan	Karimkotta	Perumunda	Sullia	Sreekara
Ceylon	Doddiga	Malligesera	Narayakodi Type-1	Shubhakara
	Panchami	Nilgiri-4		

Table 13. Average similarity among *P. nigrum* L. varieties for peroxidase, esterase and GOT

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	1																									
2	0.7791	1																								
3	0.6521	0.6666	1																							
4	0.7777	0.6362	0.6666	1																						
5	0.6825	0.4974	0.6030	0.7777	1																					
6	0.6969	0.5783	0.5134	0.5490	0.4878	1																				
7	0.7407	0.5030	0.5391	0.5972	0.7777	0.6452	1																			
8	0.7378	0.6894	0.4931	0.5526	0.4675	0.8055	0.7564	1																		
9	0.6378	0.8375	0.7264	0.6214	0.5341	0.7036	0.7147	0.7666	1																	
10	0.4629	0.4627	0.7147	0.5952	0.6851	0.6501	0.6110	0.4041	0.4959	1																
11	0.6825	0.4974	0.8030	0.7777	1	0.4878	0.4777	0.4675	0.5341	0.6851	1															
12	0.7791	1	0.6666	0.6362	0.4974	0.4394	0.8030	0.6894	0.8375	0.4627	0.4974	1														
13	0.7613	0.7619	0.5185	0.6204	0.4581	0.7422	0.8585	0.8534	0.6894	0.5103	0.4581	0.7619	1													
14	0.6824	0.4974	0.8030	0.7777	1	0.4878	0.7777	0.4675	0.5341	0.6851	1	0.4974	0.4581	1												
15	0.5252	0.5277	0.7333	0.6085	0.7030	0.5737	0.6045	0.6014	0.4598	0.7147	0.7030	0.5277	0.7222	0.7030	1											
16	0.6349	0.5093	0.7613	0.8223	0.8571	0.4508	0.4814	0.4341	0.4925	0.6944	0.8571	0.5093	0.4974	0.8571	0.7407	1										
17	0.5694	0.5807	0.8268	0.7221	0.7916	0.4064	0.5277	0.3978	0.5579	0.7539	0.7916	0.5557	0.4511	0.7916	0.7000	0.8000	1									
18	0.7380	0.6124	0.6363	0.7261	0.6666	0.5370	0.5740	0.5712	0.5897	0.4777	0.6666	0.6125	0.5948	0.6666	0.6111	0.6904	0.6250	1								
19	0.6666	0.5622	0.7030	0.6983	0.7777	0.5480	0.6805	0.6267	0.5452	0.8015	0.7777	0.5622	0.7156	0.7666	0.9047	0.7916	0.7407	0.5666	1							
20	0.8148	0.7613	0.6224	0.6295	0.5444	0.7206	0.9047	0.7749	0.7980	0.6269	0.5444	0.7613	0.8030	0.5444	0.5925	0.5111	0.5555	0.6000	0.5925	1						
21	0.7301	0.5391	0.8446	0.6983	0.8750	0.5249	0.5110	0.5008	0.5758	0.6777	0.875	0.5291	0.5252	0.8750	0.7666	0.8333	0.7777	0.7083	0.7666	0.5777	1					
22	0.5213	0.5119	0.6295	0.5648	0.5992	0.5867	0.6095	0.6034	0.4396	0.6214	0.5992	0.5119	0.7500	0.5992	0.8029	0.6085	0.5622	0.6780	0.7156	0.5530	0.6363	1				
23	0.5696	0.5333	0.5505	0.6779	0.5696	0.6005	0.5807	0.5020	0.5093	0.5897	0.5696	0.5333	0.6666	0.5696	0.6178	0.5837	0.5363	0.6969	0.6780	0.6030	0.6030	0.7500	1			
24	0.5444	0.4474	0.6969	0.6805	0.7666	0.5249	0.5555	0.5008	0.4281	0.7777	0.7666	0.4474	0.5907	0.7666	0.8148	0.7777	0.7333	0.5606	0.8750	0.5777	0.8000	0.6918	0.8030	1		
25	0.6825	0.6363	0.9696	0.6983	0.8333	0.4878	0.5694	0.4674	0.7009	0.7361	0.8333	0.6363	0.4381	0.8333	0.7333	0.7916	0.8571	0.6666	0.7333	0.6295	0.8750	0.5992	0.5302	0.7272	1	
26	0.5222	0.4833	0.7297	0.5656	0.7020	0.6132	0.5370	0.7104	0.5096	0.7153	0.7030	0.4833	0.6249	0.7020	0.8611	0.6717	0.6388	0.5777	0.7962	0.5555	0.7323	0.7360	0.5934	0.7323	0.7026	1

Eventhough Gottlieb (1981) reported a high level of smilarity (S.I. = 0.95) among the conspecific taxa at electrophoretic loci, high amount of variation within the species has been observed in the present study. This could be due to vegetative propagation in the species where variability created get fixed. Such variation in isoenzyme banding pattern is observed in other vegetatively propagated species also like banana (Bhat *et al.*, 1992a and b), pineapple (Deward *et al.*, 1988), sugarcane (Feldmann, 1985) and grapes (Parfitt and Arulsekar, 1989; Weeden *et al.*, 1988).

Isoenzyme analysis is of considerable use in cultivar identification using isoenzyme finger printing. In the present study, PRX-24 was a cultivar specific isoenzyme in variety Shimoga which was not found in any other variety. However, there is no doubt that for proper isoenzyme finger printing the entire germplasm should be analysed for more enzymes. Presence of wide variability in enzyme banding patterns of *P. nigrum* studied shows that the task may be easy to solve in in *P. nigrum*.

4.3 Isoenzyme variation and species relationship in the genus *Piper*

Eleven species of the genus piper were compared for three enzyme systems namely peroxidase, esterase and GOT.

4.3.1 Peroxidase

In the peroxidase zymogram, a total number of 31 isoenzymes were found in the species studied and were numbered as PRX-1 to 31 (Fig.6; Plates 11 and 12). Among the species, PRX-1 to 3 were recorded only in *P. betle* types. PRX-4 was

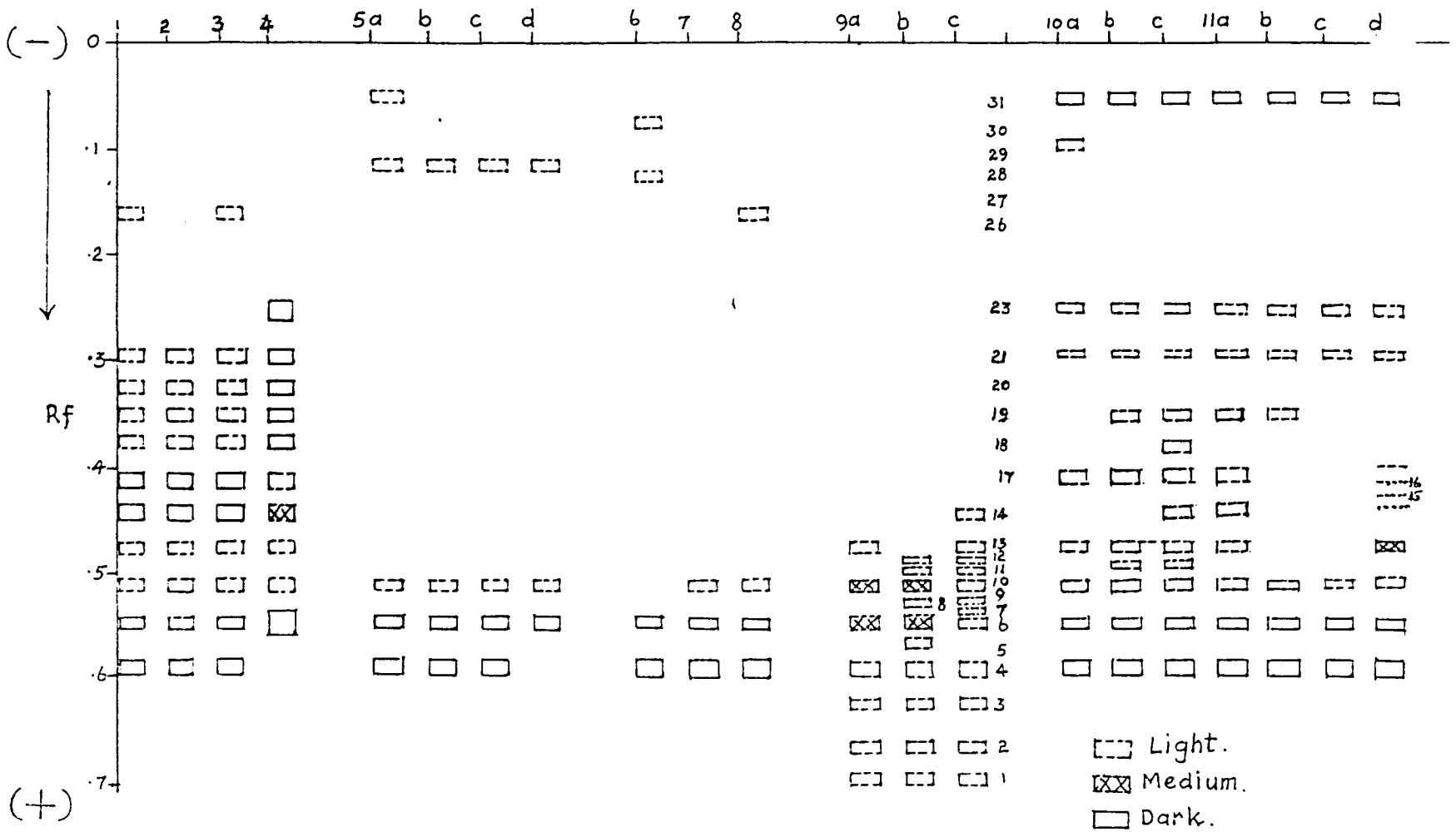
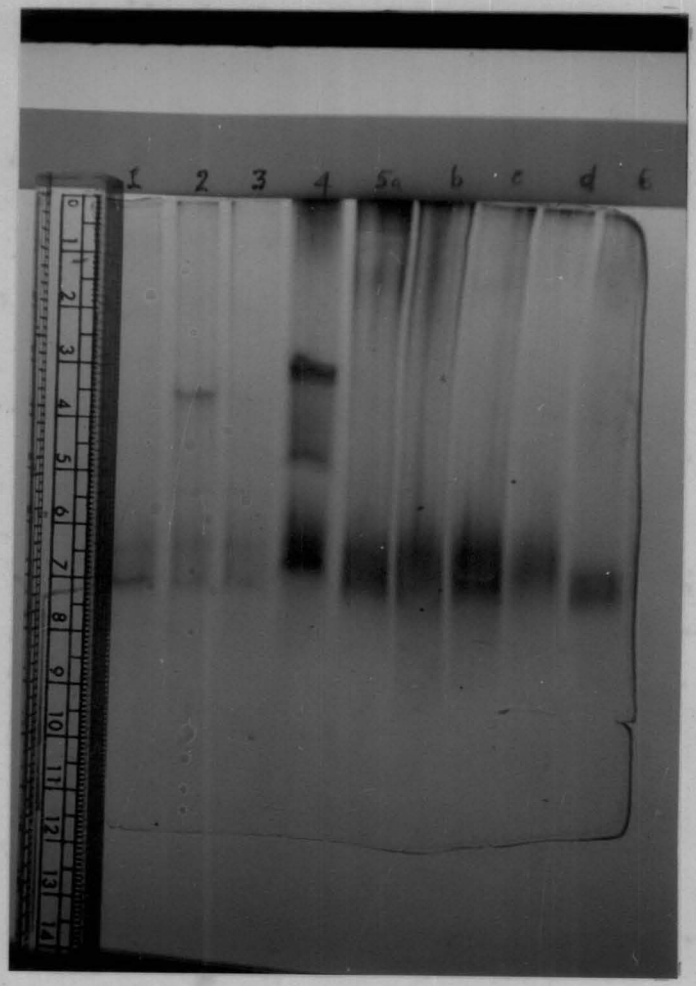
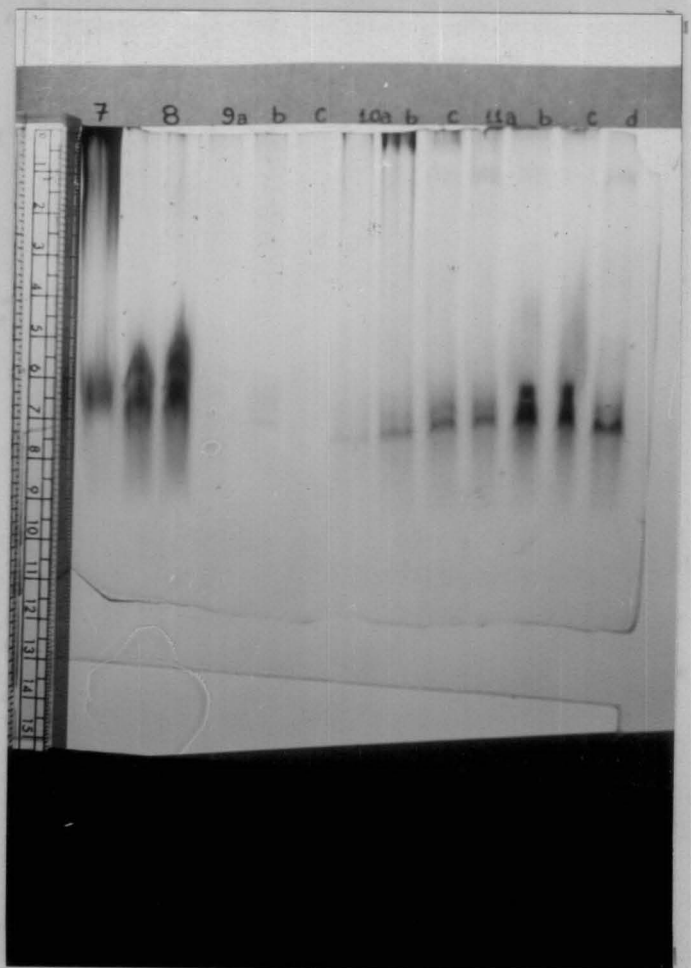


FIG. 6. ZYMOGRAM OF PEROXIDASE IN *Piper* Spp.

Plate 11 & 12. Peroxidase banding pattern in *Piper* spp.


1 2 3 4 5a b c d e


7 8 9a b c 10a b c 11a b c d

present in all taxa except *P. galeatum* and *P. longum* male type. PRX-6 was present in all the species where as PRX-10 was lacking in *P. colubrinum*, a South American species. PRX-7, 8 and 9 were present only in *P. betle* types.

P. bababudani and *P. pseudonigrum* possessed identical peroxidase pattern with a similarity index of 'one' (Table 14). *P. nigrum* is also very closely related to these species with similarity index of 0.9090. *P. galeatum* differed from *P. bababudani* and *P. pseudonigrum* in the absence of PRX-4 and PRX-26 and presence of PRX-23. Similarity of *P. galeatum* with *P. nigrum* is 0.8181 and that with other two species was 0.7500. The peroxidase profile of *P. argyrophyllum* and *P. attenuatum* were similar which support a high level of morphological similarity between the species. Within species variation was observed in *P. betle* types as in the case of *P. nigrum*. *P. Chaba* and *P. hapnium* are closely related with similarity index 0.7500. The difference was only in the presence of an additional band PRX-26 in *P. chaba*. The morphologically similar species *P. hapnium* and *P. longum* showed a similarity index ranging from 0.5000 to 0.7500 among the different types of *P. longum* in peroxidase pattern.

4.3.2 Esterase

Different species differed much widely in esterase pattern unlike peroxidase (Fig.7; Plates 3 and 4; Table 14). However, certain exceptions are also there. *P. argyrophyllum* and *P. attenuatum* showed a similarity index as high as 'one' when different types such as *P. attenuatum* type-3 and *P. argyrophyllum* type-4 were considered confirming their high morphological similarity. *P. bababudani* showed a similarity index of 'one' with *P. nigrum* and 0.6666 with *P. pseudonigrum*. *P.*

Table 14. Similarity indices for peroxidase, esterase and GOT in *Piper* spp.

		1	2	3	4	5a	b	c	d	6	7	8	9a	b	c	10a	b	c	11a	b	c	d
1	PRX	1																				
	EST	1																				
	GOT	1																				
2	PRX	0.9090	1																			
	EST	0.6666	1																			
	GOT	0.4285	1																			
3	PRX	1	0.9090	1																		
	EST	0.6666	1	1																		
	GOT	0.6666	0.3333	1																		
4	PRX	0.7500	0.8181	0.7500	1																	
	EST	0.5000	0.3333	0.3333	1																	
	GOT	0.1111	0.	0.1428	1																	
5a	PRX	0.2307	0.2500	0.2307	0.1538	1																
	EST	0.1250	0.1428	0.1428	0.3333	1																
	GOT	0.3750	0.2857	0.1250	0.1250	1																
b	PRX	0.2500	0.2727	0.2500	0.1666	0.8000	1															
	EST	0.1000	0.1111	0.1111	0.4000	0.7500	1															
	GOT	0.2500	0.1428	0.1428	0.1428	0.8000	1															
c	PRX	0.2500	0.2727	0.2500	0.1666	0.8000	1	1														
	EST	0.1000	0.1111	0.1111	0.4000	0.7500	1	1														
	GOT	0.2500	0.1428	0.1428	0.1428	0.8000	1	1														
d	PRX	0.1666	0.1818	0.1666	0.1818	0.6000	0.7500	0.7500	1													
	EST	0.2500	0.2857	0.2857	0.4444	0.6250	0.5000	0.5000	1													
	GOT	0.4285	0.3333	0.1428	0.	0.2857	0.1428	0.1428	1													
6	PRX	0.1538	0.1666	0.1538	0.0769	0.2857	0.3333	0.3333	0.1666	1												
	EST	0.	0.	0.	0.1666	0.1666	0.125	0.1250	0.1428	1												
	GOT	0	0	0	0	0	0	0	0	1												
7	PRX	0.2727	0.3000	0.2727	0.1818	0.6000	0.7500	0.7500	0.5000	0.4000	1											
	EST	0	0	0	0.1428	0.1428	0.1111	0.1111	0.1250	0.5000	1											
	GOT	0.1250	0.1666	0	0	0.1428	0	0	0.1666	0	1											
8	PRX	0.3636	0.2727	0.3636	0.1666	0.5000	0.6000	0.6000	0.4000	0.3333	0.7500	1										
	EST	0	0	0	0.1666	0.1666	0.1250	0.1250	0.1428	1.	0.5000	1										
	GOT	0.1250	0	0.1666	0.1666	0	0	0	0	0.2000	0	1										
9a	PRX	0.2666	0.2857	0.2666	0.2000	0.3000	0.3333	0.3333	0.2222	0.2000	0.3750	0.3333	1									
	EST	0	0	0	0.1111	0.2500	0.2000	0.2000	0.2222	0.2500	0.5000	0.2500	1									
	GOT	0.1111	0	0	0.3333	0.2857	0.3333	0.3333	0.1428	0.1666	0	0	1									
b	PRX	0.1666	0.1764	0.1666	0.1111	0.2500	0.2727	0.2727	0.1818	0.1666	0.3000	0.2727	0.6363	1								
	EST	0	0	0	0.1111	0.2500	0.2000	0.2000	0.2222	0.2500	0.5000	0.2500	1	1								
	GOT	0.1111	0	0	0.3333	0.2857	0.3333	0.3333	0.1428	0.1666	0	0	1	1								
c	PRX	0.2777	0.2941	0.2777	0.2222	0.2142	0.2307	0.2307	0.1538	0.1428	0.2500	0.2307	0.5384	0.5714	1							
	EST	0	0	0	0.1250	0.2857	0.2222	0.2222	0.2500	0.3333	0.2500	0.3333	0.7500	0.7500	1							
	GOT	-	-	-	-	-	-	-	-	-	-	-	-	-	-							
10a	PRX	0.4285	0.4615	0.4285	0.4615	0.4000	0.3000	0.3000	0.2000	0.1818	0.3333	0.2000	0.3076	0.1875	0.2352	1						
	EST	0.1666	0	0	0.2500	0	0.0909	0.0909	0.1000	0	0	0	0	0	0	1						
	GOT	0.1000	0	0.1250	0	0	0	0	0.1250	0.3333	0	0	0.1250	0.1250	-	1						
b	PRX	0.6153	0.6666	0.6153	0.6666	0.3636	0.2727	0.2727	0.1818	0.1666	0.3000	0.2727	0.2857	0.1764	0.2941	0.7272	1					
	EST	0.1666	0.2000	0.2000	0.2500	0	0.0909	0.0909	0.2222	0	0	0	0	0	0	0.3333	1					
	GOT	0.1000	0	0.1250	0	0	0	0	0.1250	0.3333	0	0	0.1250	0.1250	-	1	1					
c	PRX	0.6428	0.6923	0.6428	0.6923	0.3076	0.2307	0.2307	0.1538	0.1428	0.2500	0.2307	0.2500	0.1578	0.2631	0.6153	0.8333	1				
	EST	0	0	0	0.1111	0	0.0909	0.0909	0.1000	0	0	0	0	0	0	0.6000	0.6000	1				
	GOT	0	0.1250	0	0	0	0	0	0.1250	0.3333	0	0	0.1250	0.1250	-	0.6666	0.6666	1				
11a	PRX	0.5714	0.6153	0.5714	0.6153	0.3333	0.2500	0.2500	0.1666	0.1538	0.2727	0.4000	0.2666	0.1666	0.2777	0.6666	0.9090	0.9166	1			
	EST	0	0	0	0.1111	0	0.1000	0.1000	0.1111	0.	0	0	0	0	0	0.7500	0.4000	0.7500	1			
	GOT	0.1111	0.1428	0.1428	0	0.1250	0.1428	0.1428	0	0.1666	0	0	0.1428	0.1428	-	0.5000	0.5000	0.5000	1			
b	PRX	0.3846	0.4166	0.3846	0.4166	0.5000	0.3750	0.3750	0.2500	0.2222	0.4285	0.3750	0.2500	0.2142	0.1875	0.6	0.7000	0.5833	0.6363	1		
	EST	0	0	0	0.1250	0	0.1000	0.1000	0.1111	0	0	0	0	0	0	0.75	0.4000	0.7500	1	1		
	GOT	0.1250	0.1666	0.1666	0.1666	0.3333	0.1428	0.1428	0	0.2000	0	0	0.1428	0.4	-	0.1428	0.1428	0.1428	0.4	1		
c	PRX	0.2857	0.3076	0.2857	0.3076	0.5000	0.3750	0.3750	0.2500	0.2222	0.4285	0.3750	0.2500	0.2142	0.1875	0.7777	0.5454	0.4615	0.5000	0.7500	1	
	EST	0.1428	0.1666	0.1666	0.2222	0	0.1333	0.1333	0.2000	0	0	0	0	0	0	0.5000	0.8000	0.8000	0.8000	0.6000	1	
	GOT	0	0.1333	0	0	0	0	0	0	0.3333	0	0	0.1250	0.1250	-	0.5666	0.6666	0.6666	0.6000	0.1428	1	
	PRX	.4375	0.4666	0.4375	0.4666	0.3076	0.2307	0.2307	0.1538	0.1428	0.2500	0.2307	0.25	0.1578	0.2631	0.7500	0.6923	0.6000	0.6428	0.4615	0.5384	1
	EST	0	0	0	0.1111	0	0.0909	0.0909	0.1000	0	0	0	0	0	0	0.6000	0.6000	1	1	0.7500	0.8000	1
	GOT	0	0	0	0	0	0	0	0	0.3333	0	0	0.1250	0.1250	-	0.6000	0.6666	1	1	0.1428	0	1

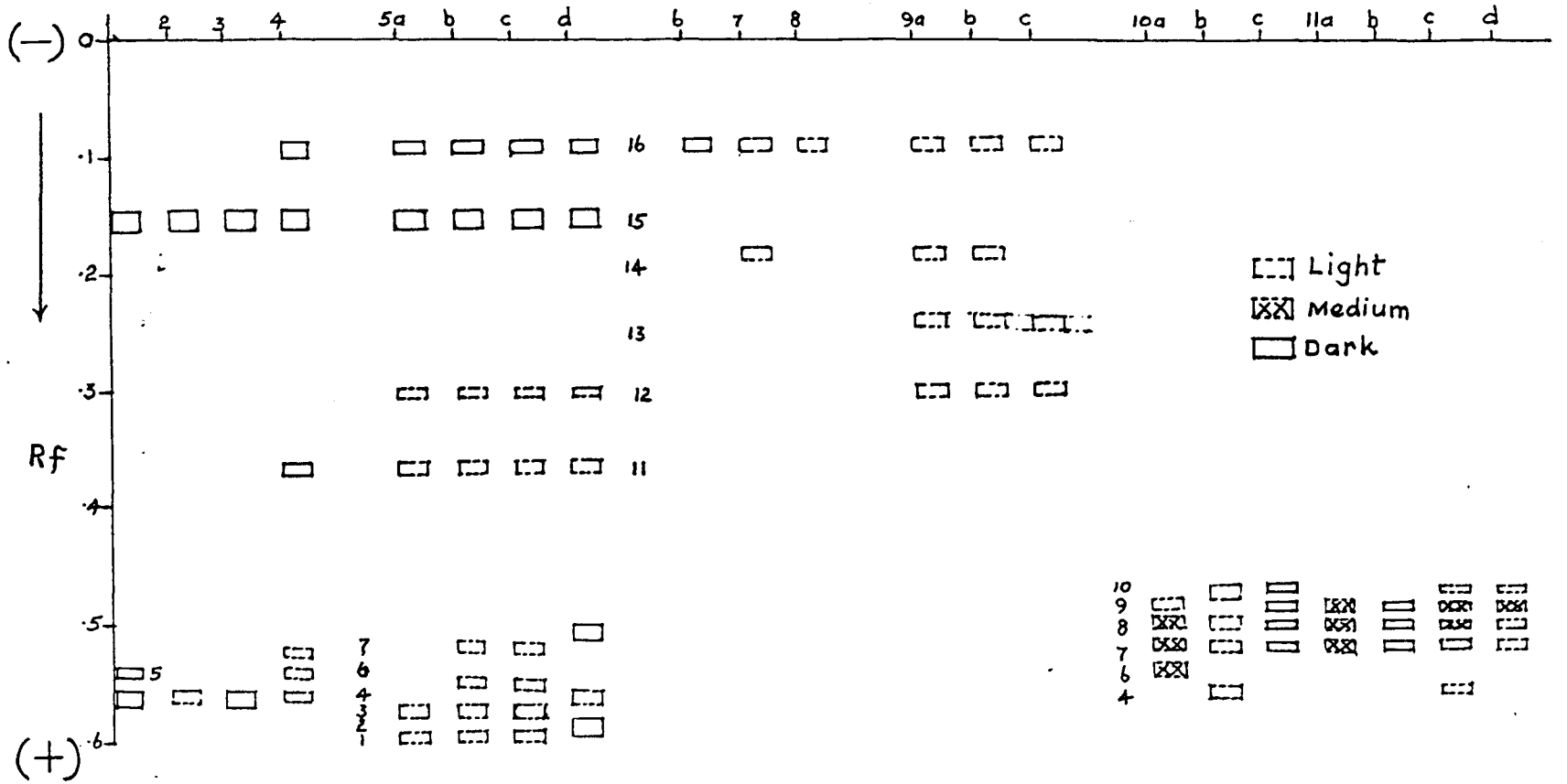
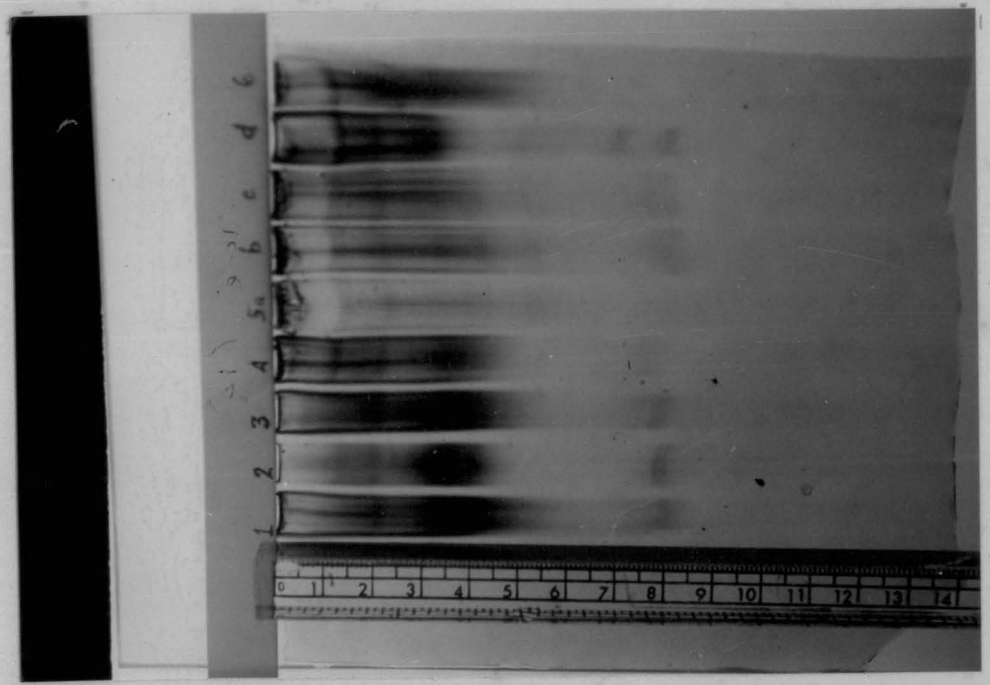
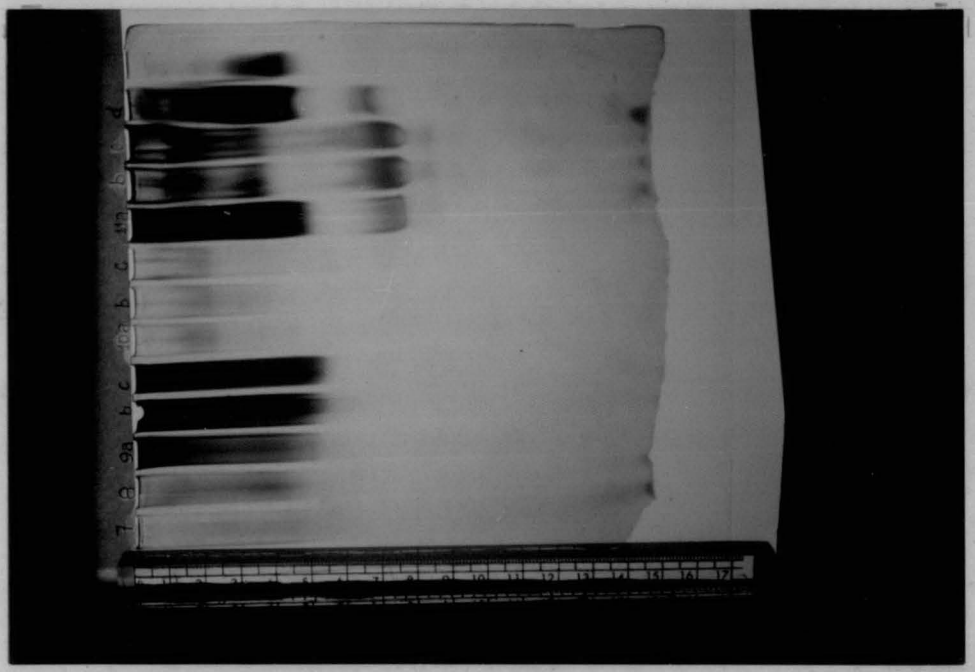


FIG. 7. ZYMOGRAM OF ESTERASE IN *Piper* Spp.

Plate 13 & 14. Esterase banding pattern in *Piper* spp.


1 2 3 4 5a b c d 6
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14


7 8 9a b c 10a b c 11 b d

pseudonigrum had a similarity index of 0.6666 with *P. nigrum* and 0.5000. *P. galeatum*. Within species, variation was very less in *P. betle* types (S.I. = 0.75-1) but much more in *P. attenuatum* (S.I. = 0.3333-0.6000), *P. longum* (S.I. = 0.5-1) and in *P. argyrophyllum* (S.I. = 0.6-1). EST-13 was present only in *P. betle* types. *P. colubrinum, P. hapnium* and *P.Chaba* differed much from the rest of the species in esterase pattern. *P. chaba* and *P. colubrinum* possessed only EST-16.

4.3.3 GOT

A total of 24 isoenzymes observed in GOT zymogram for *Piper* spp were numbered as GOT-1 to 24 (Fig.8; Plates 15 and 16). The similarity index ranged from 'zero' to 'one' (Table 14), the maximum being among *P. betle* types. Among the species, the highest similarity index of 0.6666 for GOT, was observed among *P. attenuatum* and *P. argyrophyllum* types and *P. pseudonigrum* and *P. bababudani*. *P. pseudonigrum* and *P. nigrum* had a similarity index of 0.4285 and that for *P. nigrum* and *P. bababudani* was 0.3333. *P. colubrinum,* an exotic species showed a comparatively higher similarity index (S.I. = 0.3333) with *P. attenuatum* and *P. argyrophyllum* types. *P. betle* also showed a similarity of 0.3333 with *P. galeatum* and *P. longum* types. *P. longum* male type which is popular in North India showed its difference from South Indian types with similarity index ranging from 0.1428 to 0.2857.

P. hapnium stands distinct from the rest of the species in having only three isenozymes i.e., GOT-5, 7 and 9. The similarity of *P. hapnium* with other species for GOT zymogram was mostly zero except with *P. nigrum, P. pseudonigrum* and *P. longum* types where a similarity of less than 0.2000 was observed. *P. chaba* showed its distinctness from rest of the species with a similarity index of '0' with

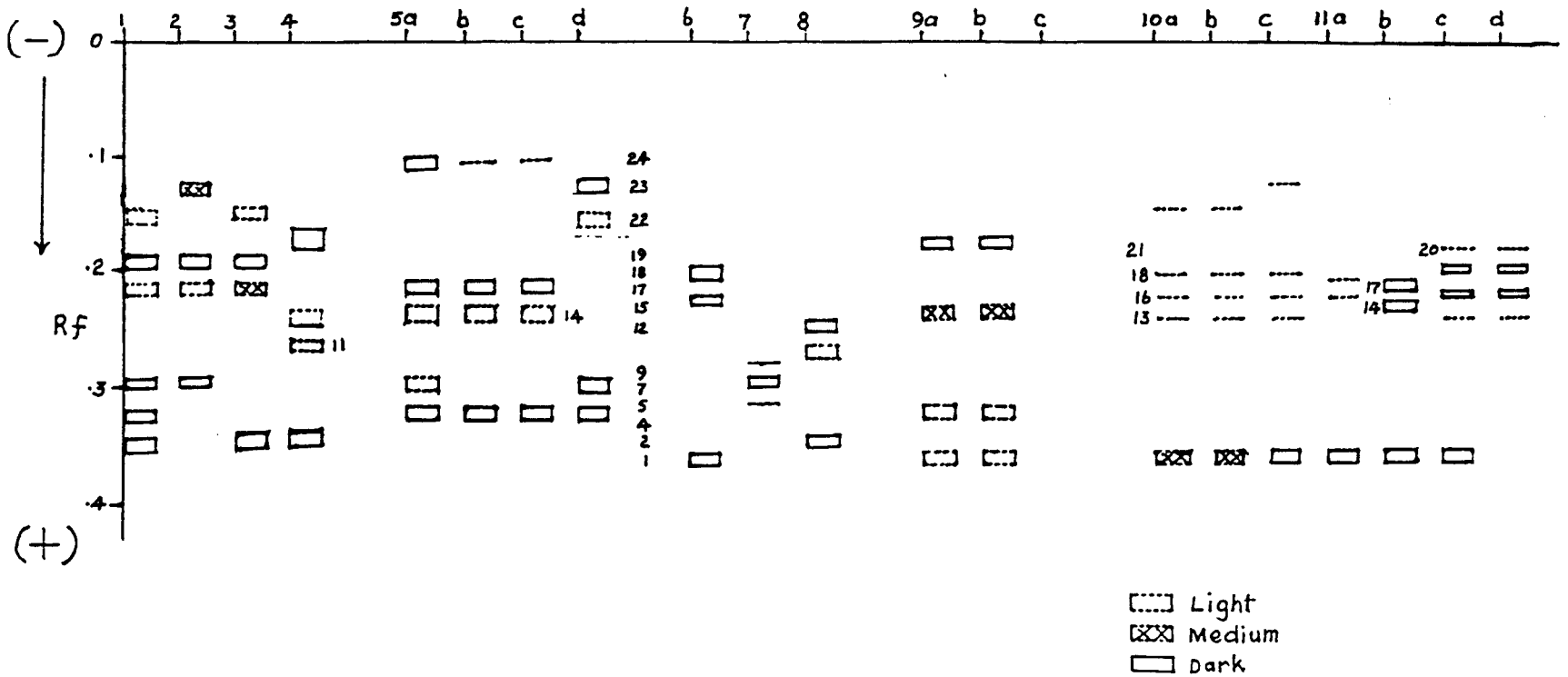
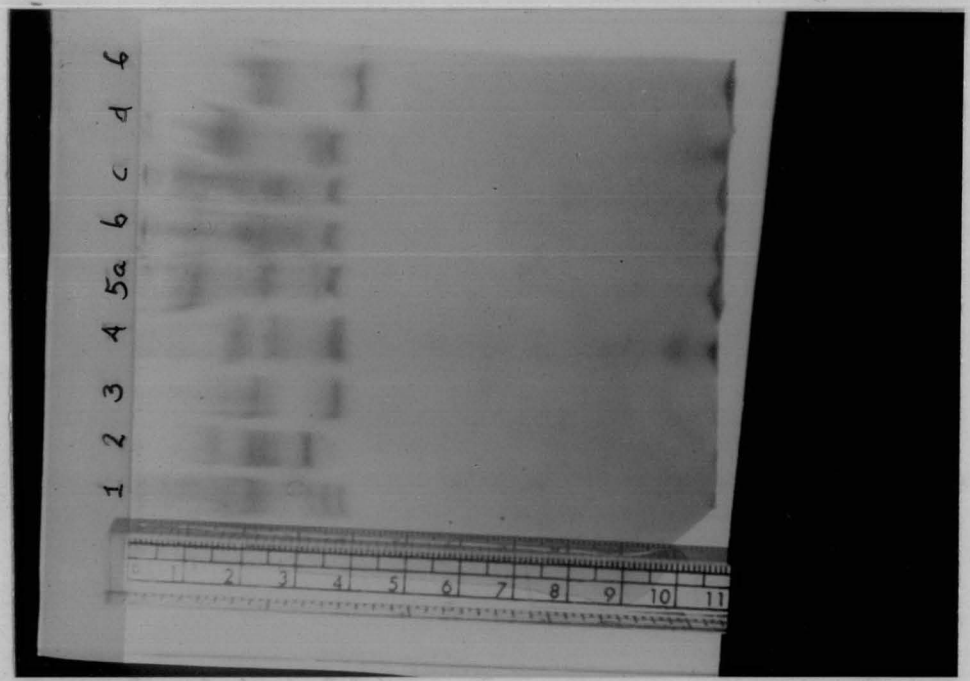
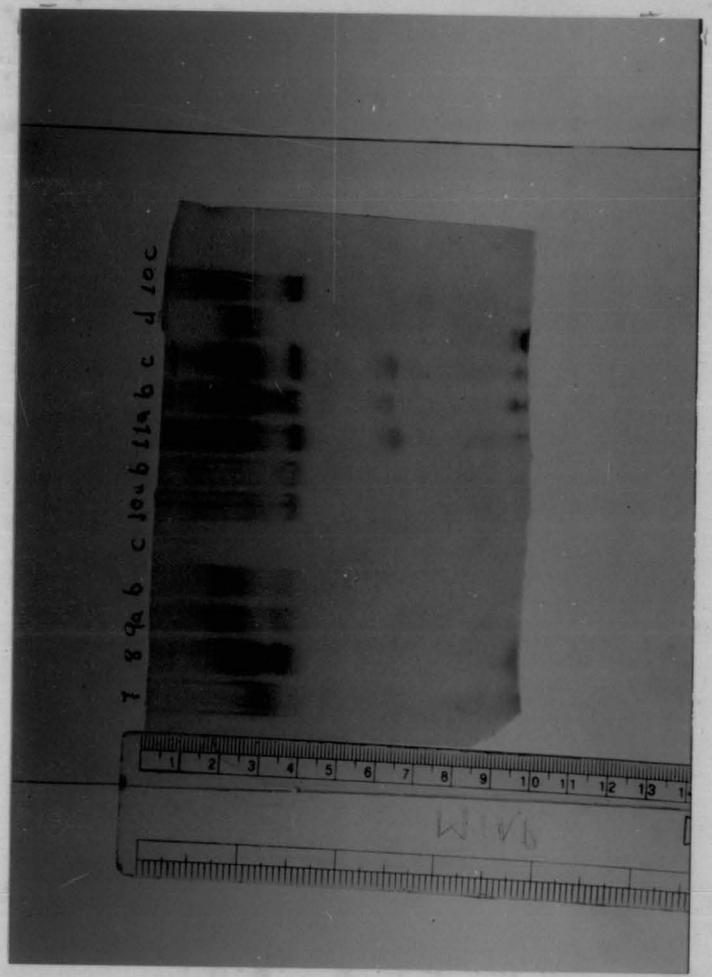


FIG. 8. ZYMOGRAM OF GOT IN *Piper* Spp.

Plate 15 & 16. GOT banding pattern in *Piper* spp.


1 2 3 4 5a 6 c d 6


7 8 9a b c 10a b 11a b c d 12c

most of the species and a low similarity index of 0.2000 with *P. colubrinum*, 0.1250 with *P. pseudonigrum* and 0.1666 with *P. bababudani* and *P. galeatum*. *P. galeatum* showed a GOT pattern which was much different from *P. pseudonigrum*, *P. nigrum* and *P. bababudani* unlike in the case of peroxidase and esterase systems. GOT 11 and 14 found in *P. galeatum* were absent in three species while GOT 17 and 19 which were present in the latter groups were absent in *P. galeatum*.

4.3.4 Similarity index among *Piper* spp for isoenzyme banding pattern

Average similarity indices for the three enzymes were computed among the eleven *Piper* spp. (Table 15) to study the relative closeness and distinctness of different species. The groups of the species observed to be closely related are

Group I — *P. nigrum*, *P. pseudonigrum*, *P. bababudani* and *P. galeatum* (S.I. = 0.3838-0.7777)

Group II — *P. argyrophyllum* and *P. attenuatum* (S.I. upto 0.7222)

Group III — *P. chaba*, *P. hapnium* and *P. colubrinum* (S.I. = 0.3000-0.5111)

P. bababudani (Rahiman, 1981) and *P. pseudonigrum* (Velayudhan and Amalraj, 1992) are recently described species in the genus *Piper*. These species were very similar to *P. nigrum* except in some minor morphological characters. The highest similarity index of 0.7777 was observed between *P. pseudonigrum* and *P. bababudani* which showed their closensss confirming the morphological similarity. *P. nigrum* showed a similarity index of 0.7474 with *P. bababudani* and 0.6680 with *P. pseudonigrum*. *P. galeatum* is closer to *P. pseudonigrum* (S.I. = 0.4537) and *P.*

Table 15. Average similarity among *Piper* spp. for peroxidase, esterase and GOT

	1	2	3	4	5a	b	c	d	6	7	8	9a	b	c	10a	b	c	11a	b	c	d
1	1																				
2	0.6680	1																			
3	0.7777	0.7474	1																		
4	0.4537	0.3838	0.4087	1																	
5a	0.2435	0.2261	0.1661	0.2040	1																
b	0.2000	0.1755	0.1679	0.2364	0.7893	1															
c	0.2000	0.1755	0.1679	0.2364	0.7833	1	1														
d	0.2817	0.2669	0.1983	0.2087	0.5025	0.4642	0.4643	1													
6	0.0512	0.0555	0.0512	0.0811	0.1507	0.1527	0.1527	0.1031	1												
7	0.1325	0.1555	0.0909	0.1082	0.2952	0.2870	0.2870	0.2638	.3000	1											
8	0.1629	0.0909	0.1767	0.1666	0.2222	0.2416	0.2416	0.1009	0.5111	0.4166	1										
9a	0.1259	0.0952	0.0888	0.2148	0.2785	0.2888	0.2888	0.1957	0.2055	0.2916	0.1944	1									
b	0.0925	0.0588	0.0555	0.1851	0.2619	0.2686	0.2686	0.1822	0.1944	0.2666	0.1742	0.8787	1								
c	0.1388	0.1470	0.1388	0.1736	0.2499	0.2264	0.2264	0.2019	0.2300	0.2500	0.2820	0.6442	0.6607	1							
10a	0.2317	0.1538	0.1845	0.2371	0.1333	0.1303	0.1303	0.1416	0.1717	0.1111	0.1000	0.1442	0.1041	0.1176	1						
b	0.2939	0.2809	0.3134	0.3055	0.1212	0.1212	0.1212	0.1763	0.1649	0.1000	0.0909	0.1369	0.1004	0.1470	0.6868	1					
c	0.2142	0.2724	0.2142	0.2678	0.1025	0.1072	0.1072	0.1263	0.1587	0.0833	0.0769	0.1250	0.0942	0.1315	0.6273	0.6999	1				
11a	0.2275	0.2527	0.2380	0.2467	0.1527	0.1642	0.1642	0.0925	0.1068	0.0909	0.0833	0.1364	0.1031	0.1388	0.6388	0.5030	0.7222	1			
b	0.1698	0.1501	0.1837	0.2060	0.2778	0.2916	0.2916	0.1203	0.1497	0.1428	0.1250	0.2166	0.2047	0.0937	0.4976	0.4142	0.4920	0.6787	1		
c	0.1428	0.1501	0.1507	0.1766	0.1666	0.1527	0.1527	0.1500	0.1851	0.1428	0.1250	0.1250	0.1130	0.0937	0.6481	0.6706	0.6427	0.5333	0.4975	1	
d	0.1458	0.1555	0.1458	0.1925	0.1025	0.1072	0.1072	0.0846	0.1031	0.0833	0.0769	0.0833	0.0526	0.1315	0.6166	0.5924	0.7000	0.5753	0.4038	0.7271	1

bababudani (S.I. = 0.4087) than *P. nigrum* which showed similarity index of only 0.3838. In earlier studies by Rahiman and Subbaiah (1984), Rahiman and Bhagavan (1985) and Ravindran *et al.* (1992) it was reported that *P. galeatum* was closely related to *P. trichostachyon*. Similarly *P. nigrum* was reported to be related to *P. wightii* (Ravindran *et al.*, 1992). Unfortunately, these two species could not be included in the present study.

P. attenuatum and *P. argyrophyllum* are other two species which are observed to be closely related in the present study. This also confirms the earlier reports by various workers. Even Hooker (1886) and Gamble (1925) had observed their similarity on morphological grounds. Rahiman and Subbaiah (1984) based on flavonoid analysis reported paired similarity of 82 per cent and paired affinity of 33.33 per cent between the two species. Paired similarity of 75 per cent and paired affinity of 30 per cent were considered as indices of closeness. Rahiman and Bhagavan (1985) based on D^2 analysis showed that these two species are biometrically related. Results of cluster analysis based on 30 characters by Ravindran *et al.* (1992) also showed close relationship of these two species and they formed a single cluster.

P. colubrinum, P. hapnium and *P. chaba* formed the third group with maximum similarity between *P. colubrinum* and *P. chaba* (S.I. = 0.5111). The similarity index of *P. hapnium* with *P. chaba* is 0.4166 and that with *P. colubrinum* is 0.3000. This isoenzyme similarity is in accordance with their morphological similarity. All the three species have erect cylindrical spikes. The maximum similarity observed in this group is between *P. chaba* and *P. colubrinum* which exhibit maximum morphological similarity also. In addition to the common spike characters, these two species have similar leaf characters as the leaves in the fruiting branches of *P. chaba*

is very similar in size, shape and texture to the leaves of *P. colubrinum*.

The present study also revealed the distinctness of *P. longum* and *P. betle* from the rest of the species as they stood individually having only low similarity index with others. The maximum similarity of *P. longum* and *P. betle* are with *P. hapnium* with similarity indices 0.2952 and 0.2916, respectively. The low similarity observed between *P. longum* and *P. hapnium* is contradictory to the high morphological similarity observed (Hooker, 1886; Rahiman, 1981). These two have similar spike characters, leaf characters and growth habit. Works carried out by various earlier researchers such as flavonoid analysis by Rahiman and Bhagavan (1985), D^2 analysis by Bhagavan and Subbaiah (1984) and cluster analysis by Ravindran *et al.* (1992) also showed the distinctness of *P. longum* compared to various other species. *P. longum* showed a similarity index of 0.1661 to 0.2817 with *P. nigrum* group and 0.1822 to 0.2888 with *P. betle*. *P. nigrum* group and *P. betle* are wider apart with similarity index around 0.1000. This is contradictory to the sequence of advancement proposed by Dasgupta and Datta (1977b) based on root anatomy where in the order was *P. longum, P. nigrum* and *P. betle*.

P. attenuatum possessed a slightly higher similarity with *P. nigrum, P. pseudonigrum* and *P. bababudani* than *P. argyrophyllum* had with these species with a similarity index more than 0.2000 in most cases. This supports the leaf epidermal similarity of *P. attenuatum* with *P. nigrum* reported by Samuel *et al.* (1984), eventhough the two species are morphologically distinct.

Among the species, least similarity was observed between *P. colubrinum* and *P. nigrum* group and more specifically *P. colubrinum* with *P. pseudonigrum* and *P. bababudani* (S.I. = 0.0512).

Summary

SUMMARY

The present study was undertaken with the objective of analysing isoenzyme variation in the genus *Piper* and supplementing the existing morphological and cytological observations. Twenty six varieties of *P. nigrum* from Kerala and near by states and eleven species of the genus *Piper* were included in the present study.

The selection of ideal part for analysis was done based on observations of isoenzyme banding pattern of stem, root and leaf. In the leaf, different maturity stages were also analysed to get the picture of isoenzyme variation at different stages of growth of leaf and to decide the stage of growth to be selected for analysis to get the best results. The experiment was done for the three enzymes studied viz. peroxidase, esterase and GOT. For peroxidase, root was selected as the best part and wherever destructive sampling was not possible dark green leaves were used as the best alternative. Esterase banding pattern was found to be considerably varying with maturity. For convenience and uniformity, tender leaf was selected as the ideal part. In the case of GOT, leaf was found to be the best sample material. GOT zymogram in *Piper* did not differ at different stages of maturity. Here also for convenience of analysis tender leaf was selected as sample. However, in *P. attenuatum* and *P. betle* activity of GOT was very less in leaf and root was used for analysis.

Isoenzyme banding pattern of peroxidase in *P. nigrum* varieties showed a set of 10 common bands and four variants PRX-22, 23, 24 and 25. PRX-22 was the

most common variant and PRX-24 was present only in variety Shimoga. The varieties were grouped into five based on the isoenzyme variation at peroxidase. In the case of esterase, varieties could be grouped into two based on the presence of EST-2 or 4 in them. For GOT, 15 isoenzymes were observed in *P. nigrum* varieties and they showed a highly complex pattern. Varieties were grouped into two with one group having GOT-3 and the other with GOT-7.

Similarity index among the varieties based on individual enzymes as well as pooled data for three enzymes were found. Similarity index for peroxidase in *P. nigrum* ranged between 0.7692 to 1. Based on peroxidase zymogram varieties were grouped in three with similarity index 'one' among the members within the group. Varieties Shimoga and TMB-5 stood individually. For esterase enzyme similarity index range was from 0.2000 to 1. Least similarity was between Arakkulamunda and Narayakodi Type-1. Three groups of varieties showed similarity index 'one' among the members. In the case of GOT banding pattern of *P. nigrum* varieties, similarity index ranged from 0.0835 to 1. Five groups of varieties were observed with similary index 'one' among the members within the group. Least similarity was between Panniyur-3 and TMB-5. Similarity indices of three enzymes put together ranged from 0.3978 to 1. Maximum similarity of 'one' was observed between Kottanadan and Ceylon and among Perumunda, Malligesera and Nilgiri-4. The least similarity was observed between Arakkulamunda and Panniyur-3. Wide variation has been observed in the isoenzyme pattern among 26 varieties studied confirming Kanakamani's (1985) earlier observation on morphological scoring.

Peroxidase pattern in *Piper* spp. showed that PRX 1, 2 and 3 were present only in *P. betle*. *P. bababudani* and *P. pseudonigrum* possessed identical peroxidase pattern (S.I. = 2). *P. nigrum* is closely related to these species with similarity index of 0.9090 and *Pl galeatum* showed a similarity index of 0.8181 with *P. nigrum*. The peroxidase profile of *P. attenuatum* and *P. orgyorphyllum* showed a high degree of similarity (S.I. upto 1) which support a high level of morphological similatiry. *P. chaba* and *P. hapnium* are closely related with a similarity index of 0.7500. In esterase pattern also, *P. attenuatum* and *P. argyrophyllum* showed a high degree of similarity with similarity index as high as 'one' when different types were compared. *P. bababudani* showed a similarity index of '1' with *P. nigrum* and 0.6666 with *P. pseudonigrum*, *P. pseudonigrum* had a similarity index of 0.6666 with *P. nigrum* and 0.5000 with *P. galeatum*. Among the species the highest similarity index of 0.6666 for GOT was observed among *P. attenuatum* and *P. argynophyllum* types and *P. pseudonigrum* and *P. bababudani*. *P. hapnium* and *P. chada* showed their distinctness from the rest of the species with similarity index 'zero' with most of the species.

Pooled analysis of similarity indices have shown that out of the 11 species studied 9 could be grouped into three groups. *P. nigrum*, *P. pseudonigrum*, *P. bababudani* and *P. galeatum* formed the first group with similarity index ranging from 0.3838 to 0.7777. *P. argyrophyllum* and *attenuatum* formed the second group with similarity index reaching upto 0.7222. The third group include *P. chaba*, *P. hapinum* and *P. colubrinum* with similarity index ranging from 0.3000 to 0.5111. *P. longum* and *P. betle* stood individually showing their distinctness from rest of the

species. The least similarity was observed between *P. colubrinum* on one side and *P. pseudonigrum* and *P. bababudani* on the other (S.I. = 0.0512).

Thus present study supports the wide morphological variation in the varieties of *P. nigrum* at isoenzyme as well. One grouping, based on isoenzyme similarity among the 11 species studied, *P. nigrum, P. pseudonigrum, P. bababudani* and *P. galeatum* formed the first group, *P. Argyrophyllum* and *P. attenuatum*, the second group and *P. chaba, P. hapnium* and *P. colubrinum* the third group. *P. longum* and *P. betle* showed their distinctness from the rest of the species. A further detailed study including more species and types within the species for more number of enzymes could be greatly helpful in understanding the sequential advance in the genus *Piper* which is highly confusing at present.

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* Originals not seen

ISOENZYME VARIATION IN *Piper* spp.

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ABSTRACT OF A THESIS

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ABSTRACT

The study on "Isoenzyme variation in *Piper* spp. was conducted in the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara, Trissur from June 1993 to December 1994. Material included 11 species of the genus *Piper* including *Piper nigrum* Linn. Maximum accessions available in each species were analysed for three enzymes viz., peroxidase, esterase and GOT using polyacrylamide gel electrophoresis.

The selection of ideal part for electrophoresis for each enzyme was done based on observations of banding pattern of stem, root and different maturity stages of leaf. Root was selected for peroxidase and tender leaf was selected for esterase and GOT in all species except in *P. attenuatum* Buch-Ham and *P. betle* Linn. where for GOT root was selected.

Isoenzyme pattern of 26 varieties of *P. nigrum* were compared for peroxidase esterase and GOT. When all the three enzymes are taken into account maximum similarity of 'one' was observed between Kottanadan and Ceylon and also among Perumunda, Malligesera and Nilgiri-4.

On grouping the 11 species of *Piper* based on isoenzyme similarity *P. nigrum* Linn., *P. pseudonigrum* Velayudhan and Amalraj, *P. bababudani* Rahiman and *P. galeatum* DC formed one group, *P. argyrophyllum* Miq. and *P. attenuatum*

Buch-Ham second group and *P. chaba* Hunter, *P. hapnium* Miq. and *P. colubrinum* Link. third group. *P. betle* Linn. and *P. longrum* Linn. showed their distrinctiness from the rest of the species. Least similarity was observed between *P. colubrinum* on one side and *P. pseudonigrum* and *P. bababudani* on the other side (Similarity Index 0.0512)