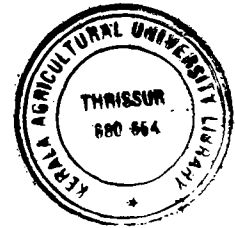# CHARACTERISATION OF FIELD ESTABLISHED TISSUE CULTURE DERIVED BLACK PEPPER (Piper nigrum L.) PLANTS USING MORPHOLOGICAL, CYTOLOGICAL AND MOLECULAR MARKERS

By

R. SUJATHA

THESIS

*Submitted in partial fulfilment of the
requirements for the degree of*

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*Faculty of Agriculture
Kerala Agricultural University*

DEPARTMENT OF PLANT BREEDING AND GENETICS

COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR - 680 656
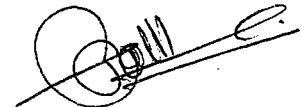KERALA, INDIA

2001

DECLARATION

I hereby declare that this thesis entitled **"Characterisation of field established tissue culture derived black pepper (*Piper nigrum* L.) plants using morphological, cytological and molecular markers"** 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 of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

Vellanikkara

15. 9. 2001

R. SUJATHA

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Vellanikkara
15-9-2001

CERTIFICATE

Certified that this thesis entitled "**Characterisation of field established tissue culture derived black pepper (*Piper nigrum* L.) plants using morphological, cytological and molecular markers**" is a record of research work done independently by **Mrs. R. Sujatha** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship, or associateship to her.
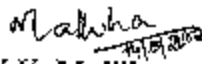
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(Chairman, Advisory Committee)

CERTIFICATE

We, the undersigned members of the Advisory Committee of Mrs. R. Sujatha, a candidate for the degree of Doctor of Philosophy with major in Plant Breeding and Genetics, agree that the thesis entitled "Characterisation of field established tissue culture derived black pepper (*Piper nigrum* L.) plants using morphological, cytological and molecular markers" may be submitted by Mrs. R. Sujatha, in partial fulfilment of the requirements for the degree.
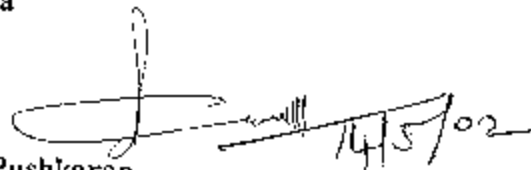
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It is in truth impossible to express fully in simple words what I owe the people who supported and helped me realise this dream. However, this work remains incomplete without my due acknowledgement of their efforts.
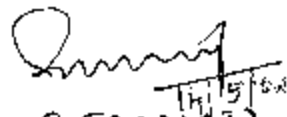
First and foremost it is Dr. V. K. Mallika, Professor, CCRP College of Horticulture, Vellanikkara and the Chairman of my Advisory Committee that counts. I owe her unfathomable gratitude for her unstinted interest, guidance, persuasion, incessant encouragement, expertise and above all for her patience and consideration that finally enabled me to carry out this task.

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R. Sujatha

❖ *For my family* ❖

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1. INTRODUCTION

Universally acclaimed as the 'King of Spices', black pepper (*Piper nigrum* L.) enjoys a unique position as a commercial crop of puissant history and is of great economic importance to several nations of the world (Pillai *et al.*, 1985). Until 18th century, black pepper cultivation and production had been the monopoly of India; the lion's share contributed by the state of Kerala. However, during the past two centuries, pepper cultivation has been taken up on a commercial scale by several other nations too. Presently, the total number of pepper producing countries in the world is ten.

Historical evidences show that black pepper cultivation and trade has been existing in India for the past 6000 years or more (Pillai, 1997). The plant *Piper nigrum* (*L.*) has been accepted as a native of India (Hooker, 1885 and Barber, 1906). The area under pepper cultivation in Kerala is approximately 2.38 lakh ha with a production of nearly 65,990 T (Madan, 2000). Despite these facts, the yield of the pepper vine in our country is alarmingly low: whereas the yield per vine is estimated in kilograms in other countries (eg. Thailand – 3.6kg per vine: Ghosh *et al.*, 1999), it is measured in grams in our country (Kerala – 283g per vine: Madan, 2000).

Our forefathers might have adopted this plant on realising the medicinal and culinary properties of this queer vine. Through a long and patient process of selection and rejection over several centuries, around 70 cultivars were adopted for cultivation in the different tracts of the state. These cultivars were highly variable in their morphological characters and yield potential (Mathai *et al.*, 1981;

Ibrahim *et al.*, 1984) and this variability has been preserved till date due to the vegetative means of propagation, which is the accepted practice in the crop.

In black pepper, efforts to fabricate varieties with high yield and quality through selection as well as hybridisation were initiated as early as in 1953 (Nambiar *et al.*, 1978) at Pepper Research Station, Panniyur, which gave birth to the first ever high yielding hybrid variety of pepper *viz.* Panniyur 1. Ever since this classic achievement, attempts were and are being made all over the world to generate high yielding black pepper varieties. So far, in Kerala itself, ten improved varieties have been released. The non-availability of sufficient quantity of planting materials is, however a major constraint in the wide spread popularisation of these high yielders.

It was the insatiable demand for planting materials of high yielding pepper varieties that forced the scientists to find alternate methods of rapid multiplication. The combined efforts of several workers during the early 1990's resulted in standardising the protocol for mass multiplication of black pepper (Babu *et al.*, 1993 and Joseph *et al.*, 1996) by adopting tissue culture (TC) techniques.

A thorough evaluation and characterisation of these micropropagated plants in relation to their performance in the field is a pre-requisite to establish their heritage and usefulness. But unfortunately, very scanty information is available regarding the field performance of *in vitro* raised perennials (Pandey and Singh, 1989). Hence the present study was taken up to establish the identity and heritage of *in vitro* raised black pepper plants belonging to four high yielding varieties *viz.* Panniyur1 (P_1), Panniyur2 (P_2), Panniyur4 (P_4) and Subhakara (Su), which were established in the field. These TC derived plants are characterised and compared

with conventionally propagated vines of the same age using four different marker systems.

Traditionally, genetic diversity in plants has been assessed by examining morphological or physiological traits. In black pepper also, crop improvement was mainly based upon morphological traits till recently. Though the efficiency of this marker system is amply justified by the evolution of several high yielders in black pepper, long periods of time required for the effective and efficient use of this conventional marker system is a major limitation.

Advances in biotechnology have provided several molecular markers useful in crop improvement programmes. These are versatile tools for fingerprinting and monitoring variations in plants, and in some cases, the only means to obtain a sufficient number of unbiased markers. Historically, isozymes were the first biochemical method applied to these types of investigations and had the advantages of affordability and easy applicability.

Among the diverse DNA markers identified during the past decade, RAPDs with the potentially unlimitted number of markers allow a finer distinction, especially if too little isozyme diversity exists. Babu (2000) tested the fidelity of TC plantlets of black pepper, which were in the hardening stage, using RAPD assay. But no such study is reported from field established plants of black pepper.

Variation in chromosome number is a frequent phenomenon in tissue culture derived plants. Hence, the characterisation will be fool proof only if cytological studies are also included.

Consequently, a combination of all the four types of markers *viz.* morphology, cytology, isozyme and RAPD analysis were tried in this study to

characterise and establish the genetic identity of black pepper vines selected for the study with the following specific objectives.

1. To establish the genetic identity of the 40 tissue culture (TC) derived and 20 conventionally propagated vines of four varieties of black pepper planted in the field, using a combination of conventional and molecular markers such as morphology, cytology, isozyme and RAPD.

2. To standardise the various techniques of molecular marker analysis in black pepper.

3. Detection of intra group and inter clonal variability of the conventionally propagated and TC derived plants.

4. Comparison of the efficiency of the different marker systems, alone and in combination with each other.

An attempt was also made to integrate the aforesaid four markers and to assess their comparative merits and demerits. It is quite likely that more progress could be made in black pepper genetics if single gene markers were made available to document the results of crop improvement efforts and also to study inter varietal and inter specific relationships.

2. REVIEW OF LITERATURE

The real applicability of micropropagated plants would ultimately depend on the comparative field performance with those derived from cuttings (Zaman *et al.*, 1997). To assess this, comprehensive characterisation is an essential pre-requisite. A large number of polymorphic markers are required to measure genetic relationships and genetic diversity in a reliable manner. Several strategies have been used to assess the genetic integrity of *in vitro* raised plants. Phenotypic identification based on morphological traits and karyotypic analysis of metaphase chromosomes to determine rearrangements and/or numerical variation in chromosomes were the methods employed earlier (Bhojwani *et al.*, 1986) and are still in use (Zaman *et al.*, 1997). Molecular characterisation of tissue culture derived plants by isozyme analysis as well as the recently developed and more specific DNA marker systems has been reported by many workers (Isabel *et al.*, 1993; Rani *et al.*, 1995; Angel *et al.*, 1996 and Todorovska *et al.*, 1997).

2.1 MORPHOLOGICAL MARKERS

Observation of phenotypes has been the classical approach to differentiate plant cultivars, morphological traits being the main target of such observations. The description of the plant morphology is an invaluable source of information of the genetic variability and has been the first criterion used to classify plant varieties.

According to Bretting and Widrlechner (1995) the data obtained using morphological, karyological and molecular markers were found useful in managing germplasms *ex situ*. Among these, morphological traits were the oldest and widely used genetic markers because of their simplicity and rapid, inexpensive assays. Even though molecular markers are more specific, morphological markers assisted with proper statistical

analysis provide valuable information in breeding of various crops such as maize (Desai and Singh, 2001) and *Brassica juncea* (Lakshmikant and Gulati, 2001), serve as single-gene controlled marker in linkage studies (Gaur and Gour, 2001) and help to assess genetic diversity in germplasm collections (Raje and Rao, 2001).

2.1.1 Morphology based investigations in black pepper

Piper nigrum is a unique crop and attempts to compare its genetic behaviour with that of any other crop may not be quite proper. Even though it belongs to the class dicotyledons, the anatomical characters are intermediate between those of dicotyledons and monocotyledons (De Waard and Zeven, 1969). It belongs to the family *Piperaceae* under the order Piperales, which is one of the most primitive branches originated from Ranales. The plant, a weak stemmed climbing vine, is a native of the evergreen forests of the Western Ghats of Kerala and is said to have been domesticated several thousand years ago (Purseglove, 1969). Krishnamurthy (1969) has reported that, pepper vine in its wild state is mostly dioecious, but most of the cultivated types are monoecious, a condition which probably originated from the wild ones as a result of continuous selection and vegetative propagation by man through ages. Even though geitonogamy is the rule in pepper (De Waard and Zeven, 1969) the original variability present in the wild types has been conserved as such in the present day cutivars due to the vegetative propagation practiced in the crop. Another reason for the wide variability observed is its suspected polyploid nature (De Waard and Zeven, 1969).

Mathai *et al.* (1981) reported more than 70 cultivated varieties of pepper. These local varieties were broadly divided into Malabar and Travancore cultivars (Ibrahim *et al.*, 1984). Kanakamany (1982) formulated a key for identification of black pepper types based

on morphological characters. Based on this key, the 45 types selected were found to fall into 42 groups.

Panniyur1 (P_1), the first hybrid of black pepper, obtained by artificial crossing of Malabar variety Uthirenkotta as female parent and the Travancore variety Cheriakaniakkadan as the male parent, was released in 1966. By virtue of the vegetative propagation method, heterosis could be fixed in the crop. The high yield and other desirable characters of P_1 have been reported by Ramankutty (1977), Nambiar *et al.* (1978), Sikka *et al.* (1984), Kannan (1985) and Ibrahim *et al.* (1986a). Pillai *et al.* (1987) assessed the heterosis of P_1. During the following years, several high yielding varieties have been released in black pepper, the salient features of which, as reported in various articles are tabulated as Table 1.

Table 1. Salient features of the high yielding pepper varieties

High yielding variety	Parentage	Green yield (kg /vine)	Oleo-resin %	Drying %	Dry-yield potential (kg/ha)	Other specific features
P_1	UthirenkottaX Cheriyakaniakkadan	2.2	9.5	36.5	8800	Prefer open condition
P_2	O.P.* progeny of Balankotta	4.9	10.9	35.8	3313	Prefer open or partial shade, robust vine
P_3	Uthirenkotta X Cheriyakaniakkadan	4.9	12.7	27.8	3269	Prefer open conditions
P_4	O.P. progeny of Kuthiravaly	2.6	9.2	34.7	2443	Prefer open or partial shade
P_5	O.P. progeny of Perumkody	3.2	12.3	35.7	3242	Stable yielding, shade tolerant
KS_{14}	Selection of Karimunda	4.8	13.0	35.0	4200	-
KS_{27}	Selection of Karimunda	4.2	12.4	35.5	4487	-
Panchami	Selection of Aimpiriyan	5.2	12.5	34.0	6528	-

* Open pollinated.

2.1.2 Important characters for morphological scoring

Several vegetative and reproductive characters were given importance for characterisation of vines and for assessing the extent of variability in clonally propagated black pepper varieties. Many of these were of quantitative nature enabling statistical analysis for interpretation whereas several others were of qualitative nature, effective enough only to discriminate the varieties.

2.1.2.1 Vegetative characters

Presence or absence of anthocyanin pigmentation on the leaf sheath covering the young growing tip was reported to be controlled by a dominant-recessive intra-allelic interaction wherein the alleles governing pigmentation was observed to be dominant (Ibrahim *et al.*, 1986b).

Shape of leaf, leaf area, petiole characters, texture and colour of leaf etc. were reported to help in rapid identification of varieties in pepper (Purseglove, 1969; George and Mercy, 1978; Nambiar *et al.*, 1978; Kanakamany, 1982). Ibrahim *et al.* (1985a) have worked out a constant which when multiplied with the dimensions of the leaf would yield an estimate of leaf area.

Stem characters like internodal length, branching nature, direction of growth of branches etc. were reported to vary with cultivars (Kanakamany, 1982; Ibrahim *et al.*, 1986a; Sujatha and Namboodiri, 1995).

2.1.2.2. Reproductive characters

Inflorescence characters like number and length of spike, sex type of flower, stamen and stigmatic characters etc. were reported to be of economic importance as they influence yield (Nambiar *et al.*, 1978; Chandy *et al.*, 1979; Kanakamany, 1982). Similarly the

number of berries per spike, percentage of developed and underdeveloped berries per spike, 100 berry weight, 100 berry volume, drying percentage as well as chemical composition also manifest pronounced variation (Nambiar *et al.,* 1978; Kanakamany, 1982; Raja *et al.,* 1983; Sujatha and Namboodiri, 1995).

Jose and Sharma (1983) studied correlation between karyotype and chemical constituents (oleoresin, oil and piperine) of 14 genotypes of *Piper betle,* three of *P. nigrum* and one each of *P. longum, P.cubeba* and *P. attenuatum. P. betle* varieties were reported to have high oleoresin and essential oil content, but no piperine. *P. nigrum* had all the three chemicals. Piperine was also absent in *P. longum* but present in *P.attenuatum. P.cubeba* had the highest content of oleoresin and essential oil though piperine was in negligible amount.

They have also reported a correlation between the chemical constituents and chromosome number in the genus *Piper.* During the process of evolution and the diversification of the genus, increase in chromosome number has shown to be directly related with decrease in chemical content.

2.1.3 Morphology - conventional clones vs. micro clones

The protocol for mass multiplication has been already developed in black pepper (Babu *et al.,* 1993 and Joseph *et al.,* 1996). Babu (2000) tested the fidelity of tissue culture (TC) plantlets of black pepper, which were in the hardening stage, using RAPD assay. He reported no conclusive evidence of variation among the TC plantlets within each variety (P_1, P_2, P_3, P_4 and Karimunda). According to him a thorough evaluation and characterisation of tissue culture plants in the field is an absolute necessity to establish whether they are true-to type or not.

Increased vigour of micro-clones compared to conventionally propagated clones under field condition has been reported in blackberry (Swartz *et al.*, 1983), apple (Zimmerman, 1986) and mulberry (Zaman *et al.*, 1997).

Morphological markers are still relied upon for characterisation due to the simplicity and low cost. With proper statistical analysis they could give a more or less representative picture of the genetic constitution.

2.2 CYTOLOGICAL ANALYSIS

Chromosome number and cyto-morphological traits have served as genetic markers, especially in polyploid crop complexes where they have been important tools for elucidating the systematics and evolution of these crops (Simmonds, 1976).

2.2.1 Karyological studies in Piperaceae

The chromosome number in family *Piperaceae* is highly variable (review by Dasgupta and Datta, 1976 and Rahiman and Nair, 1983). Karyological analysis has been done in only 64 spp. out of around 2000 spp. and the results revealed the possibility of more than one basic number. Sharma and Bhattacharya (1959) suggested 12 as the basic number in *Piper*. Mathew (1958) and Smith (1966), on the contrary, recorded the basic number as 13. Dasgupta and Datta (1976) based on their review, found that 47.3 percent of species in *Piper* had x = 12 and only 19.04 percent had x = 13. So they concluded that, the other basic numbers have evolved from 12. They have also suggested that variation in the reports of chromosome number of the same species may be due to occurrence of chromosomal biotypes.

Jose (1981) stated that in *Piper*, there existed a large number of chromosome biotypes with intra-clonal variation. The small size of chromosomes and the difficulties in obtaining clear metaphase plates hinder the cytological studies in this genus.

StellaBai and Subramanian (1985), in a mitotic study of 21 species of *Piper* and *Peperomia*, also reported that chromosome numbers occur as multiples of 8, 11, 12, 13 and 14, of which, multiples of 11 and 12 were most frequent in *Peperomia* and *Piper* respectively.

Rahiman and Nair (1986) studied the cytology of eight *Piper* spp. from Western Ghats. They found that 6 spp. had 2n = 52 whereas the remaining two had 2n = 104. Based on the review of the chromosome number of *Piper* spp. so far recorded from India, they concluded that, mostly multiples of 12 chromosomes have been reported from North India and multiples of 13 from South India. Hence they assumed that the species from these two centres of distribution probably had different evolutionary pathway starting from basic number 6 and 7. Differences in karyotype at a varietal level have been reported in other crops such as *Pennisetum typhoides, P. americanum , Oryza sativa* etc. (Chandola and Jain, 1970; Virmani and Jill, 1972; Prabakaran *et al.*, 1991 and Versha and Ravi, 1995).

2. 2. 2 Karyology of *Piper nigrum*

Cytological investigations in cultivated varieties of *Piper nigrum* revealed varying somatic chromosome number such as 52 [Mathew (1958,1973), Martin and Gregory (1962), Samuel and Bavappa (1981), Jose and Sharma (1983)]; 48 (Sharma and Bhattacharya, 1959); 36, 60 (Dasgupta and Datta, 1976); 54 (Kumar and Navaneethan, 1981) and 52, 104 (Samuel, 1986). Rahiman and Nair (1983 and 1986) have reviewed the different reports about variation in chromosome number in *Piper* species and compiled the results in a tabular form for easy reference. This species is believed to be of ancient
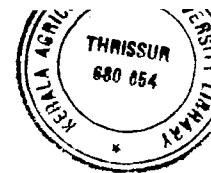
polyploid origin followed by diploidisation during the course of evolution. The basic chromosome number is reported to be 13 which might have arisen by inter specific hybridisation between types with n = 6 and n = 7 followed by doubling of the chromosome of the hybrid (Mathew, 1958 and 1973). He has also reported that the different varieties studied from South India had similar karyotypic features.

Dasgupta and Dutta (1976) classified the chromosomes in the genus *Piper* based on the positions of constrictions and lengths into six types (Type A, B, C, D, E, and F). They have described two types of *P. nigrum* with different chromosome numbers and different karyotypes *viz.*, *P. nigrum* Agartala (2n = 36, karyotype 2A, 4B, 26E and 4F) with chromosome length ranged between 0.77 to 1.9 μ and *P. nigrum* South India (2n = 60, karyotype 8B, 44E and 8F), with chromosome length varying from 0.77 to 2.3 μ.

Samuel and Bavappa (1981) using root tip squashes in aceto-orcein, confirmed the chromosome number of *P. nigrum* as 2n = 52. Since they could detect species with 2n = 26, 39, 65 and 78 also, the basic chromosome number is reported to be x = 13 and *P. nigrum* is considered as a tetraploid. They have also opined that the variation in chromosome numbers of the same species in different reports might be due to the presence of polyploid races or cytotypes at the intraspecific level.

Cytological studies in Panniyur1 (P$_1$) by Mathew and Mathew (1982) also showed presence of 52 chromosomes and the karyotype was very well comparable to other varieties. Jose and Sharma (1983) also reported the somatic chromosome number of *P. nigrum* as 52 from their studies involving three genotypes *viz.*, P$_1$, Cheriyakaniakkadan and Karimunda. They reported differences in the chromosome constitution among the species and even between the genotypes of the same species in chromosome number, basic set, number of nucleolar chromosome and even in size and morphology of chromosome.

2. 2. 3 Chromosome instability of *in vitro* raised plants

During *in vitro* culture, chromosomal rearrangements (deletion / translocation / inversion) aneuploidy and polyploidy are accepted phenomena, which leads to somaclonal variation (Larkin and Scowcroft, 1981; Karp *et al.*, 1989a, 1989b and Karp, 1995). This chromosome instability is more pronounced in callus and cell suspension cultures (review by Evans and Reed, 1981). Among the various methods developed to micropropagate plants, enhanced axillary branching culture has become the most important propagation method due to its simplicity, high rate of multiplication and low risk of genetic instability. Notwithstanding these considerations, there are numerous reports on the incidence of somaclonal variation among micropropagated plants as reviewed by Rani *et al.* (1995).

Numerical changes have been examined most often, as detection of structural changes requires sophisticated karyotype analysis. Also the comparatively few reports about the structural changes are noticed in species with a small number of large chromosomes.

Evans and Reed (1981) stated that endomitosis and endo-reduplication were the principal causes of polyploidy while aneuploidy may result from translocations, mitotic non-disjunction or deletions. They have listed more than 20 different crops, which were reported to have both euploid and aneuploid changes in chromosome number during *in vitro* culture. Also they found that, in general, the regenerated plants contain a normal chromosome complement when compared to the cultured cells. There are many other reports also related to the karyotypic analysis of metaphase chromosomes and/or numerical variation in the chromosomes of somaclones (Bhojwani *et al.*, 1986).

Variation in chromosome number in red clover somaclones was reported by Wang and Holl (1988). Cytological analysis of regenerated rye plants by Bebeli *et al.* (1990) detected chromosome losses and rearrangements. Son *et al.* (1993) reported the same

observations in poplar. Kawatta *et al.* (1992) analysed the ploidy level and pollen size in protoclones of *Oryza sativa* L. var. Norin-8, derived from a single homozygous seed. They observed predominance of tetraploids among the regenerated plants. Abnormal mitosis was observed in regenerated plants of *Trifolium alexandrianum* involving the occurrence of lagging chromosomes and aneuploid chromosome numbers (Cum, 1993).

2.2.4 Standardisation of protocol

2.2.4.1 Sample

Mathew (1958 and 1973), Dasgupta and Datta (1976), Jose (1981) and Nair *et al.* (1991) used the actively growing root tips from cuttings of several species of *Piper* and *Peperomia* as well as varieties of *P. nigrum* for somatic chromosome counts. For mitotic analysis of *in vitro* regenerated plants, Evans and Reed (1981) induced root formation *in vitro*. Root tips were collected after transition to the hardening stage and also from plantlets transferred to greenhouse. Roots were cut 2mm from the tip.

2.2.4.2 Cytogenetic techniques

Kao (1975) and Armstrong (1995) described in detail the various basic steps to be followed for karyological analysis. Evans and Reed (1981) suggested a protocol for root tip chromosome analysis for regenerated plantlets. The different steps involved are, pre-treatment, fixation, hydrolysis, and staining.

a) Pre-treatment

Pre-treatment is required to obtain separation of individual chromosomes by destroying spindle fibre, clarification of the chromosome segments by differential hydration and to get a large number of cells in metaphase stage (Sharma 1991). The concentration, period of treatment and the chemical used were reported to be specific for the organism

concerned. Also, cooling prior to fixation helped to spread out the chromosomes fairly well (Mathew, 1958).

Colchicine, p-dichlorobenzene and oxyquinoline or hydroxyquinoline have comparatively wider field of application. There are several reports for the use of hydroxyquinoline as pre-treating agent as briefed in Table 2.

Table 2. Pre-treatment with hydroxyquinoline in *Piper* and *Peperomia*

Reference	Crop	Concentration	Time	Temperature
Mathew (1958)	*Piper* sp. *Peperomia* sp.	30mg/100ml	2 to3 h	0 ^{0}C
Mathew (1973)	*P. nigrum*	30mg/100ml	2 to3 h	0 ^{0}C
Mathew and Mathew, (1982)	*P. nigrum*	0.002 M	3 h	4 ^{0}C
Stellabai and Subramanian (1985)	*Piper* sp. *Peperomia* sp.	0.02 %	3 h	4 ^{0}C

Dasgupta and Datta (1976) pre-treated the root tips of *Piper* with aesculin for 1 to 3h. Jose (1981), in *Piper attenuatum*, used a mixture of saturated solution of p-dichlorobenzene and aesculin for 3 to 5h at 12 to 15^{0}C. Use of 1mg ml^{-1} of colchicine at room temperature for 2 to 6h has been suggested by Evans and Reed (1981) for pre-treatment of root tips of *in vitro* grown plantlets. Prabakaran *et al.* (1991) reported the use of 0.01 percent colchicine for 30 to 40 minutes for pre-treating the one-day-old roots obtained by germinating the seeds of *Pennisetum americanum* on moist filter paper. A solution of one percent α-bromonaphthalene at 4 to 5 ^{0}C was used for 4 h to treat the root tips of *Piper nigrum* (Nair *et al.*, 1991) and for 45 minutes in rice (Versha and Ravi, 1995).

b) Fixation

Fixatives are used to preserve the forms of cells and their contents as closely as possible to their living state and to render them resistant to further changes caused by subsequent processing. Different fixatives reported are,

1.Acetic alcohol: 1:1-*Piper* sp., *Peperomia* sp. (Dasgupta and Datta, 1976)

1:2- *Piper attenuatum* (Jose, 1981)

1:3- *Piper* sp., *Peperomia* sp. (Mathew, 1958;

StellaBai and Subramaniam, 1985)

1:3- *in vitro* regenerated plants (Evans and Reed 1981)

1:3- *Pennisetum americanum* (Prabakaran *et al.* 1991)

2.Ethanol: chlorofom: acetic acid: 3:1:1- *P. nigrum* (Nair *et al.*, 1991)

6:3:1Carnoy'sfixative-Rice (Versha and Ravi, 1995).

Evans and Reed (1981) stated that Carnoy's fixative might be preferred with preparations to be stained with acetocarmine.

c) Hydrolysis

For hydrolysis, the concentration, temperature and duration of treatment with hydrochloric acid (HCl) has to be standardised for each crop. In general, high temperature (60^0C) in combination with shorter time intervals and with lower concentrations of HCl has been suggested. Enzymatic hydrolysis using pectinase and cellulase was also done.

Sharma (1991) reported that heating or warming in dilute acids served the dual purposes of dissolving the middle lamella to separate the cells as well as cleaning the cytoplasm. But according to him, acid treatment might result in diminished stainability and interfere with the formation of bands, if chromosome banding had to be carried out later.

Various procedures for hydrolysis have been reported in different species of *Piper* and *Peperomia* (45% acetic acid for 15 min., by Dasgupta and Datta, 1976 and Jose 1981), *in vitro* regenerated plants (1N HCl, by Evans and Reed, 1981), *Penniseutum americanum* (1N HCl: 45% acetic acid - 9:1, by Prabakaran *et al.*, 1991), *Piper nigrum* (1N HCl, Nair *et al.*, 1991) and rice (1N HCl, Versha and Ravi, 1995).

d) Staining

The selection of dye or stain for a particular material usually depends on its chemical nature, the pH value of the fixative used and the chemical reactivity of stain on the material. Sharma (1991) provided a detailed description of all aspects regarding staining, including the types of stains, their mode of action, methods of staining etc.

Most commonly used chromosomal stains are aceto-carmine, aceto-orcein and basic fuchsin. Among these, basic fuchsin is the most specific but it also has certain disadvantages (Armstrong, 1995). The protocol for aceto-carmine and aceto-orcein are simple, quick and these are efficient stains (Armstrong, 1995). Among these, aceto-orcein was reported to give better results in *Piper* spp. (Anand, 1997).

Sharma (1991) described the mode of action of orcein as condensation of the dye at the polypeptide linkage of the chromosomal protein. Use of aceto-orcein for staining chromosomes have been reported in various species of *Piper* and *Peperomia* (Dasgupta and Datta, 1976; Jose, 1981; Samuel and Bavappa, 1981 and Stellabai and Subramanyan, 1985) and in *Pennisetum americanum* (Prabakaran *et al.*, 1991). Acetocarmine was used to stain the root tips of *Piper* spp. and *Peperomia* spp. (Mathew, 1958), *Piper nigrum* (Mathew, 1973 and Mathew and Mathew, 1982), *in vitro* regenerated plants (Evans and Reed, 1981) and rice (Versha and Ravi, 1995).

Hence it can be inferred from the above reports that, cytological analysis to determine the chromosome number and structural changes in plant regenerated from tissue cultured *in vitro* is a valuable tool to assess the genetic integrity. But the difficulty lies in its limited application in those species wherein, the chromosomes are small in size and/or the number is high due to polyploidy.

2.3   ISOENZYME ANALYSIS

Isozymes are different molecular forms of an enzyme produced by an organism to fulfil specialized metabolic requirements (Markert and Moeller, 1959). By definition, all isozymes of a particular enzyme system perform essentially the same catalytic function. Though it might seem wasteful for an organism to produce multiple molecular forms of any enzyme when one molecular form is sufficient to perform that particular function, this is actually an adaptation of living things. All living systems apparently require these multiple molecular forms in order to maximise biological capacity. They catalyse the same reaction, but under different metabolic conditions or in different places in the same cell, or in different cells, or in the same cell at the successive stages of differentiation. So it seems that the evolutionary pressures tailored these enzymes to fit the fastidious requirements of the cell's metabolic machinery (Markert, 1975 and Gottlieb, 1977).

Isozyme assay is now an important technique in many of the major fields of biology such as enzymology, biochemistry, cell biology, systematics, population biology, genetics and plant breeding. Since isozymes are co-linear with the gene and as they are direct gene products, the gap between gene (DNA) and the gene product (protein) is relatively small when they are used as the phenotypic markers rather than the secondary morphological characters or other biochemical markers such as pigments, oils etc which are further removed from the gene and are subject to more genetic- environment interplay in their final

expression. Hence, the equation between phenotype and genotype is simpler and better understood from electrophoretic evidence than the evidence obtained from morphological characters. Also, the alleles controlling these markers are co-dominant, with each genotype producing a distinct phenotype. They can be scored while very young, as they are available from vegetative tissue, especially leaves. The relative efficiency and cost effectiveness of the analysis is the reason for its wide use, particularly in studies of intra-specific variability.

2.3.1 Nomenclature

According to Markert (1975), although genetic and epigenetic isozymes have quite different origin, they are so closely related in biological significance that they should be described by a single general nomenclature. The term isoenzyme was officially recommended by the Standing Committee on Enzymes of the International Union of Biochemistry to describe the multiple enzyme forms occurring in a single species (Wilkinson, 1970). It was restricted to those forms, which were derived from the same organ and tissue of origin as well as being isodynamic (having the same catalytic action) to differentiate it from heteroenzymes, which were also isodynamic, but derived from different organs or even from different species. In a broad sense, the term 'isozymes' include electromorphs generated by different loci and different alleles of the same locus. But by genetic analysis, if bands were identified as the results of alleles of the same locus, they were designated as allozymes (Gottlieb, 1977; Weeden and Marx, 1984; Tanksley and Orton, 1983; Kephart, 1990).

The Committee has also approved the convention of numbering of isozymes in decreasing order of negative charge, i.e., in order of decreasing electrophoretic mobility. So the isozyme with greatest anodic mobility is assigned the number, one. Wieland and Pfleiderer (1957) introduced this system first. Conklin and Smith (1971), Boyle *et al.*

(1990) and Reyes *et al.* (1998) also adhered to this method whereas Torres and Tisserat (1980) followed another method. They named the locus that specified slow moving isozyme as 1, the faster one as 2 etc. Within the locus, they named the allele coding slow migrating isozyme as 's' and fast one as 'f'. Oliver and Martinez-Zapater (1985) designated isozymes by a hyphenated capital letter added to the symbol for each enzyme. Isozyme with most anodal migration was designated A, the next B and so forth. At each isozyme, the allele with the greatest relative mobility was called 'a' then, 'b', 'c' etc. Visedo *et al.* (1990) and Reyes *et al.* (1998) also followed this kind of nomenclature within the locus.

Isozymes could exist in different cell organelles and in different metabolic compartments within a single cell. The structure of enzyme, allelic state of coding gene and the number of structural gene copies (gene dosage) were reported to affect the number and/or intensity of enzymes (Gottlieb, 1977).

2.3.2 Advantages

Isozymes are produced in a wide variety of higher plants, are readily demonstrable by gel electrophoresis and are products of genes, which segregate in a mendelian manner. They are therefore useful as genetic markers at the molecular level for systematics, morphogenetics and physiology.

Rick and Fobes (1975) have listed out the various advantages and shortcomings of isozyme analysis compared to studies based on morphological data. Gottlieb (1977) also discussed the drawbacks in using morphological data such as small proportion of rare single-gene mutations that could be used as markers, polygenic nature of most of the phenotypic characters, which result in strong influence of environmental fluctuations etc, compared to isozyme markers. According to Torres *et al.* (1978a, 1978b) isozymes provide excellent, reliable and easily obtained single-gene markers for long-lived perennials. Also,

due to the co-dominant nature of the alleles genetic studies have to be carried out only up to F_1 generation.

According to Arulsekar *et al.* (1985), isozymes offer a powerful tool for providing co-dominant alleles at single marker loci that can be used for characterisation of cultivars, assessment of genetic purity in cultivar multiplication nurseries, estimations of out crossing rates and identification of cell protoplast fusion products.

2.3.3 Applications

2.3.3.1 As molecular markers for characterisation

As genetic markers, isozymes and allozymes have been successfully used for clonal and cultivar identification in many crops. Lyman and Ellstrand (1984) used isozyme phenotypes to elucidate clonal diversity in apomicts. Oliver and Martinez-Zapater (1985) have suggested the effectiveness and reliability of isozyme method for potato identification, particularly in varietal certification since it allows the quick test of the denomination of a given variety or the varietal purity of seeds and tissue culture stocks.

The concordance between morphology or cytology and isozyme / allozyme polymorphism was examined in a wide variety of wild and cultivated taxa (Bretting *et al.*, 1987). Weeden and Marx (1987) described the allozyme polymorphism of 11 enzyme systems and based on this, a linkage map of *Pisum sativum* was proposed. The study by Weeden *et al.* (1988) revealed that, isozyme loci could be used as convenient molecular markers to aid in the development of linkage map for *Vitis* and to tag genes controlling commercially important characters.

Studies of isozyme pattern in pineapple by DeWald *et al.* (1988) revealed that, the system could be used for cultivar identification. Also they obtained information concerning the origin and relationships between the commercially important cultivars.

Garvin *et al.* (1989) demonstrated indirect selection using isozyme markers in tomato, pea and bean. Wickneswari (1990) reported the use of isozyme markers in hybrid identification of *Acacia* and found that the enzyme was expressed in callus, juvenile and mature leaves and that, hybrids could be determined and verified at any stage of growth of the species.

Bhat *et al.* (1992a, 1982b) reported that unique isozyme banding patterns were ideal for clonal identification in banana. They identified 19 cultivars based on this. Genetic characterization of 15 clones of *Musa* sp. was done by Reyes *et al.* (1998) using isozyme markers to facilitate subsequent analysis of the genetic variability in the collection.

2.3.3.2 Developmental studies

Scandalios (1969) reported about the variation in staining intensity (activity of enzymes) and isozyme patterns in the course of development of an organism. Based on his studies, he concluded that, (1) electrophoretically identical isozymes may be present in different tissues, but varying in concentration, (2) an isozyme may be absent at a specific developmental stage or occur in such a low concentration that it cannot be detected and (3) electrophoretically distinct isozymes may occur in various tissues.

Conklin and Smith (1971) stated that, in developmental studies, the changing pattern of the electrophoretic bands of isozymes could be interpreted as evidence of different gene activations at the various ontogenetic stages sampled. Studies on peroxidase in tobacco by Thorpe *et al.* (1978) revealed that changes in isoperoxidases preceded the morphological appearance of the organs like vegetative bud, floral bud, root and callus formation during *in vitro* culture of tobacco and thus, could be considered useful markers of the differentiation process. Everette *et al.* (1985) stated that some of the isozymes were expressed only at particular stages of development and differentiation.

2.3.3.3 Systematics and evolution

Gottlieb (1977) discussed the applications of electrophoretic data in systematics and evolution of plants. Torres *et al.* (1978b) based upon their studies in *Citrus* concluded that molecular genetics of isozymes could be a very significant tool for examining problems in the taxonomy and breeding of the crop. Allozymes, as opined by Oliver and Martinez-Zapater (1985), have proven to be a very useful tool in deciphering the evolutionary relationships within different groups of plant as well as animal taxa.

2.3.3.4 Breeding

Studies of population genetics of commercially important species could be used to enhance their management and accelerate progress from tree improvement programmes. Use of isozymes in population genetic studies have made considerable progress in tree breeding (Boyle *et al.*, 1990).

In breeding for quantitative traits (QTs) improvement, isozymes as biochemical markers possess distinct advantages (Arunachalam *et al.*, 1996). They have identified 5 parameters to give 5 basic QTs from electrophoretic gel patterns *viz.*, number of bands, relative mobility, standard error (relative mobility), relative absorption and standard error (relative absorption). This correlation between isozyme data and the QTs could be used to predict the plant performance and select segregants based on isozyme data.

2.3.4 Peroxidase (PRX) (donor: H_2O_2 Oxido-reductase) (EC.1.11.1.7.)

2.3.4.1 Properties

Peroxidases utilise hydrogen peroxide to oxidise a wide range of hydrogen donors such as phenolic substances, aromatic primary, secondary and tertiary amines, leuco-dyes, certain heterocyclic compounds such as ascorbic acid and indole, certain inorganic ions like

iodine, cytochrome C, nitrite etc., but not H_2O_2 itself or alcohols (Scandalios, 1969; Liu, 1975). Recognition that PRX activity actually existed in multiple forms came from Jermyn and Thomas (1954) who observed five components of PRX isozymes.

Scandalios (1969) opined that PRX, like esterase group, should also be classified as a "family of enzymes" except in cases of allozymes or isozymes with high degree of chemical-physiological relationship. Liu (1975) also considered the PRX system as a family of homologous enzymes which had different substrate specificities and performed different physiological functions in the cell. In all higher plants investigated, it was reported that there existed a multitude of peroxidase isozymes, which were highly polymorphic and ubiquitous (Brewbaker and Hasegava, 1975; Liu, 1975).

Electrophoretic studies revealed a monomeric structure for the enzyme with a molecular weight of about 40,000 and involvement of more than one structural gene in the production of multiple forms of isoperoxidases (Scandalios, 1969; Rick et al., 1974; Smith and Conklin, 1975). It is characteristic of peroxidase that its isozyme pattern is complicated and difficult to interpret (Liu, 1975). This ambiguity in interpretation was reported to be due to the fact that the individual peroxidase isozymes had different reactivities with the various substrates used to measure their activity such as o-dianisidine and oxalocetate, benzidine and guaicol etc, which in turn might be due to differences in enzyme active sites that determine the specificity for particular hydrogen donors. He also reported that the electrophoretic mobility of peroxidase isozymes could be altered considerably with no loss of activity merely by storage at cold room temperature at pH 7 or higher.

The activity was reported to be affected by thiols, strong acids or alkalies, mechanical breakdown by heating, photoperiod etc. (Scandalios, 1964) and also by IAA, diseases or wounding and X ray irradiation (Wilkinson, 1970; Visedo et al., 1990). Studies in various crops revealed that, in general, peroxidases were highly thermostable and

long lived, remaining at high concentrations throughout the maturation of tissues (Brewbaker and Hasegawa, 1975).

Many researchers have studied the role of peroxidase in plant system. It was found to be involved in lignification and auxin metabolism (Thorpe et al., 1978), defensive response of plants and cell wall biosynthesis (Lagrimini et al., 1987), to act as IAA oxidases and to be associated with photosynthetic tissues (Brewbaker and Hasegawa, 1975).

2.3.4.2 Tissue specificity

Scandalios (1969) has shown that, in maize, the pattern of peroxidase isozyme production was usually tissue and organ specific. The number, migration rate and intensity were reported to be different in different parts like stem, leaves, silk, roots, endosperm etc. Peroxidase activity of pith, cortex and leaf of dwarf tomato plants were reported to be about three times greater than those of corresponding tissues of normal plants (Evans and Alldridge, 1965). In peas and beans, multiple forms of peroxidases have been found to vary with different organs as well as during different stages of development (Racusen and Foote, 1966; Siegel and Galston, 1967). In maize, Scandalios (1969) reported 8 to 12 bands in endosperm whereas only 3 to 6 bands were found in pollen. Mature leaves were reported to have more number of bands and increased intensity compared to younger leaves in many crops such as *Phaseolus* (Racusen and Foote, 1966), *Nicotiana* (Hart and Bhatia, 1967), *Hordeum* (Upadhyaya and Yee, 1968), *Triticum* (Bhatia and Nilson, 1969), Datura (Conklin and Smith, 1971), *Piper* spp. (Sebastian, 1995), Turmeric (Joseph, 1999).

Lavee and Galston (1968), Birecka et al. (1972) and Thorpe et al. (1978) reported an increase in activity of peroxidase with increase in age in vegetative and flowering tobacco plants and tobacco pith. They found that in a plant, the peroxidase activity as well

as band number decrease acropetally. There were eight to nine distinct anodic and five cathodic bands at bottom region whereas at the top, only low levels of three anodic bands were reported. Brewbaker and Hasegava (1975) studied peroxidase activity in 21 different tissues and organs of maize and found that there were 13 peroxidase isozymes, with each plant part showing one or two specific bands.

2.3.4.3. Isoperoxidases in plant system

Many scientists reported the activity of peroxidases in different crop plants. McCune (1967) separated six fractions of peroxidase by starch gel electrophoresis from corn leaf sheath. As many as eight cationic and five anionic peroxidase isozymes have been reported in various tissues of the wild rice (*Oryza perennis*) by Chu (1967).

Endo (1967) studied the effect of peroxidase banding pattern in maize seedlings. There were six anodal and six cathodal bands in the shoot and internode homogenates. In 10 herbaceous species of *Datura*, seedling at one-leaf stage showed only one fast moving anodal band whereas at four-leaf stage more number of bands were seen. Similarly, in mature plants, basal leaves gave more number of bands compared to apex leaves (Conklin and Smith, 1971). They also reported a decrease in the number of bands associated with senescence. Both greenhouse and field grown plants showed the same bands, as were found in plants grown under controlled conditions. The 4th leaf from top showed unique banding pattern for each species. Altogether they reported 19 bands, 12 anodal and 7 cathodal, but no single band was common to all. The distance from origin for anodal bands varied from 7 cm for fastest to 0.7cm for the 12th (slowest) band and for cathodal bands, it varied from 0.6 cm for 13th (slowest) band to 3.1cm for 19th band (fastest of cathodal).

Bonner *et al.* (1974) successfully classified 60 cultivars of banana based on peroxidase pattern and they found that this classification agreed with traditional taxonomic

grouping. They could also assign three classes of unknown origin to different genomic groups. Liu (1975) reported that in the horseradish peroxidase system, the most anodally migrating isozyme was distinct with greater density and had a specific activity 37.6 times greater than the less dense cathodically migrating peroxidase. Also, these isozymes were reported to have different active site architecture and different reactivity with substrates.

Smith and Conklin (1975) compared the intensity of a particular band in the trisomic vs. diploid *Datura*. Of the 13 significant differences, 11 were less intense and two were more intense. Similarly, the peroxidase zymogram of tetraploids and two of the trisomics, (the phenotypes of which markedly differed from that of diploid) did not differ significantly. So they concluded that not every morphological change was correlated with a change in peroxidase isozyme activity. With the assumption that zymogram bands and their intensity was a direct product of gene activity, they assigned the structural genes for peroxidase in *Datura* to specific chromosomes.

Brewbaker and Hasegava (1975) identified 13 major peroxidases in *Zea* sp., three cathodal and 10 anodal when electrophorased at pH 8.1. Genetic polymorphisms have been discerned for nine of the maize peroxidases. Thomas and Stoddart (1980) have reported that the enzyme activities could be directly related to the shelf life of leaves, the one with high activity of enzymes having shorter shelf life and the one with low activity having longer shelf life.

In betel vine, Kochhar *et al.* (1984) found that six cultivars showed distinct isozyme pattern, which could be considered as biochemical taxonomic markers. There were bands common in all cultivars that indicated the common origin from the same ancestor. But within each cultivar, the different types with different names and showing some differences were not found to differ in peroxidase banding pattern.

Weeden and Marx (1987) obtained several zones of peroxidase activity in pea. Genetic analysis was performed only on two most anodal peroxidases, PRX-1 and PRX-2. PRX-1 formed an intense, sharp band near the dye (Rf = 0.7 – 0.8) whereas PRX-2 formed a lightly staining broader zone with a mobility of approximately 0.6. Weeden et al. (1988) observed two zones of peroxidase activity in the cathodal region in grape leaves whereas in *Acacia*, Wickneswari (1990) reported four loci.

Reyes et al. (1998) reported that out of the 23 enzyme systems evaluated in *Musa*, only nine showed useful banding pattern. Out of the nine, two were reported to be monomorphic viz. peroxidase and Rubisco. For peroxidase, there were two zones of activity but only one zone PRX-1 had good resolution. The other zone was very attenuated with low resolution in the mid region of the gel, which they could not interpret.

2.3.5 Glutamate Oxaloacetate Transaminase (GOT) [EC 2. 6.1.1.]

2.3.5.1 Properties

The enzyme glutamate oxaloacetate transaminase (GOT- Sadasivam and Manickam, 1996) is also known as L-aspartate: 2-oxoglutarate aminotransferase or in brief, Aspartate Amino Transferase (AAT) (Wilkinson, 1970). This is a key enzyme, shunting metabolites into several different metabolic pathways. It catalyses the reversible conversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate.

Wilkinson (1970) reported the existence of two components of AAT, a fast migrating component on the anode side of origin that exist in cytoplasm (c-AAT) and a slow migrating component on the cathode side that exist in mitochondria (m-AAT). He has given a detailed description of the electrophoretic, chromatographic and other physico-chemical properties of these two fractions. Lyslova et al. (1975) also conducted a similar study in animal tissue and the results agree with that of Wilkinson (1970).

Regarding the pH optima of the two fractions, Wilkinson (1970) stated that, in crude tissue extracts, at pH 6, activity was almost exclusively due to m-AAT when the concentration of 2-oxoglutarate was high and aspartate concentration was low. At pH 7.4, with excess of both substrates, the sum of the activities of both isozymes could be determined. They also found that when pH was reduced below 7.4, activity of c-AAT fell rapidly and at pH 6, only about 40% of initial activity remained whereas no such fall occurred when m-AAT was similarly treated. Lyslova *et al.* (1975) defined the optimum pH range as 6.3 to 7.4 for m-AAT and 7.4 to 7.8 for c-AAT.

Electrophoretic studies in several crops revealed a dimeric structure for the enzyme (Torres and Tisserat, 1980; Weeden and Marx, 1984; Arulsekar *et al.*, 1985). Most of the reports indicated three to four distinct zones of activity representing the different forms such as mitochondrial, cytosolic, plastid specific etc. (Weeden and Marx, 1984; Arulsekar *et al.*, 1985; Weeden *et al.*, 1988). But Boyle *et al.* (1990) reported the presence of only two bands among which, one was very indistinct.

2.3.6 Technical details

2.3.6.1 Choice of Tissue

Electrophoresis can be performed successfully on extracts from a wide variety of plant tissues. The spectrum of isozymes expressed may vary in each case. The proper choice of the tissue, the mode of collection and proper storing prior to extraction determines partly, the success or failure of the method. Wendel and Weeden (1989) and Kephart (1990) have given a review of the methods followed with respect to these aspects.

The important factors affecting the choice of tissue were reported to be physiological and ontogenic condition of the sample and its availability. The sample should be at the same ontogenetic stage and from plants grown under the same environmental

conditions. Conklin and Smith (1971) and Wendel and Weeden (1989) also stressed on the point that different samples should be in the same physiological and ontogenic condition. Kephart (1990) reported that, environmental factors associated with both greenhouse and field collected material will affect observed levels of enzyme activity.

Depending upon the availability, any part of a plant such as leaf, shoot, root, seed, seedlings, callus, flower, etc. can be used. In general, fresh living tissue was reported to be the best material. For most of the enzyme systems, leaves were mostly selected, due to easy acquisition and storage, availability at all the stages of development and without the need for destructive sampling. The selection of the leaf should be based on the various aspects such as age, developmental stage, type of enzyme to be assayed etc. A concise list of references is self explanatory with regard to this aspect (Table 3).

Table 3. Type of leaf tissue used for PRX and GOT isozyme assay

Plant	Developmental stage	Enzyme	References
Datura	4th leaf down from shoot apex (mature plant under controlled conditions)	PRX	Conklin and Smith, 1971
Maize	Leaf tissue	PRX	Brewbaker&Hasegava, 1975
Citrus	Mature fully expanded, healthy leaves	GOT	Torres *et al.*, 1978b
Betlevine	Leaf from top, middle and basal portion	PRX	Kochhar *et al.*, 1984
Walnut	Leaves from juvenile and adult trees	GOT	Arulsekar *et al.*, 1985
Pea	Young leaves	GOT	Weeden and Marx, 1987
Pineapple	4th leaf at centre, counting from the first visible leaf	PRX	DeWald *et al.*, 1988
Grape	Young leaf within 5cm of the shoot tip	GOT	Weeden *et al.*, 1988
Acacia	Seed leaves excised from 1-2 weeks old seedlings germinated in sand beds	PRX	Wickneswari, 1990
Chenopodium foetidum	Leaves at different developmental stages, size and color	GOT, PRX	Visedo *et al.*, 1990
Banana	1st completely unfolded leaf from the top of the pseudostem of young plants	PRX	Bhat *et al.*, 1992a
	Tissue fragments from centre of leaves of *in vitro* plantlets 2 months old	PRX	Reyes *et al.*, 1998

2.3.6.2 Mode of extraction

Each taxon and tissue poses its own set of problems regarding the difficulty of cell breakage and associated inconveniences caused by endogenous tannins, phenols, phenol oxidases and other unidentified cellular constituents (Wendel and Weeden, 1989). Plant tissue generally requires more complex buffers and greater care during the extraction. According to Kephart (1990), this is partly due to the presence of chemical systems that hinder herbivores. Homogenisation of the tissue by grinding disrupts cellular compartmentalisation providing opportunities for interaction between proteins and various secondary components.

The important steps to be taken care of while extraction include the method of homogenisation, the buffer with suitable additives, centrifugation and storage. Tissue homogenisation is done by mechanical grinding to release enzymes from cell and organellar membranes. Kephart (1990) stated that this step was crucial because enzyme activity must be retained even though cell contents were no longer compartmentalised. For soft tissues or succulent vegetative parts, grinding in an appropriate buffer in a chilled mortar and pestle is sufficient to effect adequate cell breakage (Kochhar et al., 1984; Oliver and Martinez-Zapater, 1985; Visedo et al., 1990; Arunachalam et al., 1996).

2.3.6.3 Extraction buffer

Several investigators noted that, extraction buffer choice was one of the most important factors affecting enzyme resolution. Wendel and Weeden (1989) reported that, if the tissue to be homogenised was relatively free from phenolics and other interfering substances, best results were obtained with comparatively simple extraction buffers such as phosphate or Tris-HCl buffer (50 mM) containing sucrose or glycerol, mercaptoethanol or dithiothreitol (DTT) and small quantities of detergent. Kephart (1990), in a survey

conducted among the researchers about the different techniques practiced, found that 70 percent of the individuals used a relatively complex buffer with five or more added ingredients including a variety of protectants. She also observed that such complex buffers could be stored in refrigerator or by freezing for up to 1 to 4 months.

Many plant tissues were reported to contain compounds like phenolic acids, tannins, terpenes, pectins, resins, flavanoids, coumarins and even carotenoid pigments which interfered with enzyme activity (Van Sumere et al., 1975; Kephart, 1990). Most of the additives in extraction buffers were designed to reduce the interaction between phenols and proteins or their reaction products.

Several authors have discussed about the mechanisms by which phenolics interfere with protein extraction and methods to overcome or retard this interference (Van Sumere et al., 1975; Kelly and Adams, 1977; Kephart, 1990). They suggested the use of several buffer additives such as phenol-complexing agents [borate, germanate, polyvinylpyrrolidone (PVP), or polyvinylpoly-pyrrolidone (PVPP), bovine serum albumin (BSA)], phenol oxidase inhibitors [PVP, PVPP, thiols, cysteine, diethyldithiocarbamates (DIECA)] and various antioxidants and reducing agents [mercaptoethanol, DTT, ascorbate, bisulphite] for overcoming the phenolic interference.

Other common buffer additives include sucrose or glycerol, which act as enzyme-stabilising osmotica as well as detergents (Triton X-100, Tween-80), which help to enhance release of enzymes from membrane bound organelles (Wendel and Weeden, 1989).

A tabulated form of reference for the various types of extraction buffers and buffer additives used in various taxa is provided as Table 4.

Table 4. Types of extraction buffers with different buffer additives for isozyme assay

Buffer	Concentration	Additives	References and Taxon
Tris-citric acid	0.01M (pH 7.5)	BSA, mercaptoethanol, PVP 40, EDTA	Torres and Tisserat, 1980
	0.05M Tris- 0.008M citric acid (pH 8.3)	Cysteine-HCl, Ascorbic acid, PEG (mw3350), PVPP	Arulsekar *et al.*, 1985
Saline solutions	0.2M CaCl$_2$	Ascorbate / PVP / urea	Brewbaker and Hasegava, 1975
Tris-HCl	0.1M (pH 7.4)	-	Nisselbaum and Kopelovich, 1975
	0.1M (pH 8)	Triton X-100, mercapto ethanol	Weeden *et al.*, 1988
	0.1M (pH8)	DTT	Garvin *et al.*, 1989
	0.1M (pH 7.6)	-	Visedo *et al.*, 1990
	0.05M (pH 7.4)	Ascorbic acid	Arunachalam *et al.*, 1996
	0.1M (PH 7.5)	PVPP , Sucrose, DTT, Triton X-100	Reyes *et al.*, 1998
Sodium Phosphate	0.1M	-	Lyslova *et al.*, 1975
	0.2M (pH 6.1)	-	Thorpe *et al.*, 1978
	0.06M (pH 7)	-	Kochhar *et al.*, 1984
	0.1M (pH 6.8)	Sucrose, PVP40, PVP 360 ascorbic acid, EDTA, sodium disulphite, borax, egg albumin, DTT, DIECA, NADP, NAD, pyridoxal-5-phosphate	Wickneswari, 1990

As pointed out by Wendel and Weeden (1989) and Kephart (1990), the ratio of tissue to buffer volume should be appropriate. Plant samples ground in minimal volumes might be insufficiently buffered or protected from phenolics leading to low band intensity

and / or artifactual shadow or ghost bands. On the other hand, large volumes might dilute the amount of enzymes in each sample. The ratio of leaf tissue to buffer varies in different reports as 1:1 (Wickneswari, 1990; Visedo *et al.*, 1990), 1:2 (Oliver and Martinez-Zapater, 1985) 1:3 (Reyes *et al.*, 1998) and even upto 1:8 (Arulsekar *et al.*, 1985) and 1:20 (Thorpe *et al.*, 1978). But Kephart (1990) based on her survey reported that the usual range was between 1:1.5 and 1:2.5.

The next important step is to get a clear extract without any cell debris. Wendel and Weeden (1989) and Kephart (1990) observed that cellular debris should be removed by centrifugation as the small tissues present in homogenates could alter the resistance across the gel, resulting in streaking of the enzyme bands. The centrifugation speed and time also varied in different reports with most of them applying high speed for five to 20 minutes. (Kochhar *et al.*, 1984; Everett *et al.*, 1985 and Arunachalam *et al.*, 1996).

Bergmeyer *et al.*, (1974) discussed in detail the factors affecting stability of enzymes during storage such as extreme pH values, heat, freezing, organic solvents, detergents, specific enzyme inhibitors, action of micro organisms etc. He compiled the reports about stability of different enzymes in serum by several authors.

Wilkinson (1970) stated that addition of an enzymatically inert protein like albumin to the buffer solution effectively helps to overcome the relative instability of GOT in storage. Stuber and Goodman (1983), Wendel and Weeden (1989) and Kephart (1990) reported that superior results were obtained from freezing extracts rather than tissue samples especially in ultra cold temperature (-70 ^{0}c or below). According to them, the homogenates of many plant taxa could be stored thus for two to three years without considerable loss of activity.

2.3.6.4 Electrophoretic media

Most commonly used support media for gel electrophoresis were starch, agarose and polyacrylamide (Wieme, 1974; Wendel and Weeden 1989; Kephart 1990). In agarose gel, pores were reported to be very large (>120mμ) and hence were used to separate macromolecules such as nucleic acids, large proteins, protein complexes etc. In starch gel and polyacrylamide gel, the pores were much smaller, depending on the concentration of the gel forming mass. Ornstein (1964) and Davis (1964) introduced polyacrylamide gel as a medium for electrophoresis. Wilkinson (1970) has described the advantages and drawbacks of the different gel media in use. Polyacrylamide gel was reported to have high resolving power without any electro-osmotic flow as well as the ability to separate the enzyme molecules according to their charge as well as molecular size.

Blackshear (1984) pointed out that preference should be given to polyacrylamide gel electrophoresis for analysis requiring maximum resolving power due to stringency of molecular sieving, uniformity, transparency, inertness and the ability to concentrate proteins to very thin starting zones in stacking gel, prior to entering resolving gel.

2.3.6.5 Buffer systems

A buffer system consists of a gel buffer used in preparing the gel and an electrode buffer which is an ionised solution that conducts current through the gel during a run. The pH (which determines the net electric charge of molecule) and the molarity of the buffer system were reported to be the most crucial factors which determine the migration and separation of the enzymes as well as the resolution (Wilkinson, 1970; Andrews, 1981; Pasteur et al., 1988; Wendel and Weeden, 1989). The size and shape of the molecule, chemical properties of the buffer and the strength of electric field were the other important factors.

The various buffer systems employed in polyacrylamide gel electrophoresis (PAGE) by researchers for plant isozyme analysis are compiled in Table 5.

Table 5. Types of gel and electrode buffers used in PAGE

Gel conc. (%)	Gel buffer	Electrode buffer	Reference
8	Not specified	Tris 0.04M Glycine 0.388M pH 8.57	Hart (1975) (GOT- Wheat)
7	Not specified	Lithium borate pH 8.1	Brewbaker & Hasegawa (1975)
10	Lithium Hydroxide – Boric acid 0.025M pH 9.1	Not Specified	Kochhar *et al,* (1984) PRX (betel vine)
8	Tris HCl – 0.375 M pH 8.8	Tris 0.005 M Glycine 0.0384 M pH 8.3	Jarret & Litz (1986) (EST, PRX)
7.7	Tris –HCl pH 8.9	Tris 0.02M Glycine 0.19M, pH 8.3	Bhat *et al.,* (1992a) (EST, Catalase in Banana)
-	Tris - 0.09 M Boric acid – 0.09 M EDTA-0.025M pH 8.38	Same as gel Buffer	Kertadikara and Pratt (1995) (Teak)
7.7	Tris HCl – 0.375 M pH 8.9	Tris 0.02M Glycine 0.19M, pH 8.8	Bhat and Lakhanpaul (1995)

2.3.6.6 Sample loading

Wilkinson (1970) reported that, excessive dilution of sample might lead to denaturation and so greater amount of enzyme sample should be loaded to reduce the loss of activity. Kephart (1990) also made similar remarks and made a suggestion to increase the amount of sample applied to the gel or stained (by using thicker gel) or to minimise the amount of extraction buffer.

2.3.6.7 Run condition

Kephart (1990) pointed out that most of the enzymes usually assayed in electrophoretic studies migrate towards the anode at a rate that depends upon factors such as size of gel, amount of voltage applied, temperature and ionic strength of the buffer. The resistance to current flow generated by the gel was reported to be the reason for variable run times. Run time in different experiments varied from 3-10 h with most gels removed after 4-6 h or until dye front had moved 8-10 cm from the origin.

Gels could be run under constant voltage, power or amperage with minimum and maximum values ranging from 50 to 400V, 7 to 17 W and 15 to 75 mA (Kephart 1990). Amperage in excess of 75mA resulted in considerable heat generation, which denatured the enzymes whereas a low power output reduced resolution (Andrews, 1981).

Wieme (1974) stressed the importance of separation of heat sensitive enzymes only at low temperature. This could be done by keeping in a refrigerated chamber. Production of excess heat could be controlled by using a dilute buffer, thinner gel or reduced field strength. The optimum gel thickness for practical purposes was reported as 1.5mm. Wendel and Weeden (1989) suggested that the gel should be kept cool while casting (as gel polymerisation is an exothermic reaction) as well as before loading the sample to approximately 4^0C. They found that the bands were sharpest when electrophoresis was done at 4^0C and the gel was kept cool.

2.3.6.8 Enzyme visualisation

The commonly used procedure for detection of enzyme activity is the application of histochemicals to the electrophoretic support material after simple electrophoretic separation. Plant-derived enzymes present in the gel act on substrates provided in solution and their products interact with diazonium salts and other compounds that precipitate as

insoluble dyes. While staining protocols exist for over 200 different enzymes, only about 40 of these have proven useful in plants (Wendel and Weeden, 1989).

Several techniques of staining were described by Wieme (1974) but he stated that the procedure of incubating the gel in liquid substrate was the most widely used, as danger of elution of enzyme molecule was reduced in this. Kephart (1990), in her survey, pointed out that, most stains were incubated at 37^0C to hasten development. The precise temperature of incubation was not critical, but some stains (such as that of PRX, EST, PGI) get over-stained easily. In such cases, to slow down the development, she suggested to keep trays at room temperature or in refrigerators. For light sensitive stains, the tray should be maintained in the dark.

Wieme (1974), Liu (1975) and Brewbaker and Hasegava (1975) reported that peroxidase produced a distinctive colour reaction in the presence of H_2O_2 and certain phenolic compounds like benzidine, catechol, o-toluidene, ethanolic solution of o-dianisidine (3,3' dimethoxy benzidine) etc. These compounds were redox dyes and acted as hydrogen donors, which got oxidised into a strongly coloured compound. Liu (1975) opined that in the investigation of peroxidase isozymes in plant system, the use of any one substrate resulted in a failure to recognize and identify isozymes that were not reactive with that hydrogen donor. He tried three different substrates as stain in horseradish and pea $viz.$, eugenol-H_2O_2, tyrosine -H_2O_2, and the standerd redox dye, benzidine-H_2O_2. The result revealed that some PRX isozymes existed in the cell that were undetectable by conventional redox dye staining procedure.

Different staining procedures for peroxidase were suggested by Endo (1967), Shaw and Koen (1968), Conklin and Smith (1971), Weeden and Marx (1984, 1987), Wickneswari (1990), Visedo et al. (1990) and Reyes et al. (1998).

Visualisation of the zones of GOT activity is based upon the fact that in transaminase reaction, aspartate is converted into oxaloacetate and the later combines with fast violet B salt, forming a chromogenic (pinkish-red to red-orange) compound. Here, aspartate acted as the amino donor and ketoglutarate acted as the universal acceptor (Wilkinson, 1970). Wieme (1974) suggested soaking the gel in a wash liquid for 30 minutes to remove electrode buffer at least partially so that it will not interfere with enzymatic reaction. Suitable staining procedures for GOT were suggested by Shaw and Koen (1968), Torres *et al.* (1978b), Arulsekar *et al.* (1985), Visedo *et al.* (1990) and Sadasivam and Manickam (1996).

Oliver and Martinez-Zapater (1985) considered only the presence or absence of each allozyme irrespective of their staining intensity. According to them this would eliminate possible errors by visually assessing the intensity. Also, it helped to simplify the method of scoring, to computerize the data so as to sort and arrange different phenotype and to compare a new variety with all those previously studied.

To have a more quantitative specification of electrophoretic data and to distinguish allozymes from isozymes, several workers stressed the need for genetic analysis (Gottlieb, 1977; Torres *et al.*, 1978a, 1978b; Kephart, 1990). Based on the predictability of enzyme structure, localization and expected number of loci, hypothesis could be made about the observed phenotypes. Predictions not supported by full genetic analysis were reported as putative loci and genotypes.

2.3.6.9 Band intensity and resolution

Saunders *et al.* (1964) pointed out the causes of loss of activity or decreased activity as mechanical breakdown of the enzyme by heating, severe mechanical treatment or addition of strong acids or alkalies. An inadequate extraction buffer, an incompatible buffer system, a

volume to tissue ratio which was too high or too low, deteriorating plant tissue, type of plant tissue, absence of proper temperature control, inactivation by phenolic, improper pH, quality and concentration of gel, low substrate concentration, improper stain as well as staining conditions etc. were reported to be the most common reasons for reduced band intensity and resolution (Kephart, 1990). She has also suggested measures to overcome them.

2.3.7 Drawbacks of isozyme analysis

Although the presence of isozyme activity might be sufficient evidence for gene activity, Scandalios (1968) warned that the particular structural gene might not be active at the specific developmental stage at which the enzyme was detectable. Shaw (1970) pointed out that only about 30 per cent of the substitutions of nucleotides were expected to result in substitution of amino acids that caused changes in electrophoretic mobility. This was called, 'the redundancy of the genetic code'.

Smith and Conklin (1975) from their work on peroxidase banding pattern in *Datura* concluded that not every morphological change was correlated with a change in peroxidase isozyme activity.

Jarret and Litz (1986) and Bhat *et al.* (1992a) stated the possibility of failure of the enzyme studies to identify cultivars differing in only a few genes, especially in vegetatively propagated crops or somaclones. This underlines the need for more sensitive molecular techniques like DNA fingerprinting, which can distinguish unambiguously two closely related individuals.

Although DNA technologies are expanding, proteins and enzymes remain important as post transcriptional and translational products of an organism's DNA and as structural and enzymatic components of cells.

2.4 RAPD ANALYSIS

The maintenance, characterisation and evaluation of any germplasm are important but costly and time-consuming processes. Traditionally the characterisation of any genetic resource was carried out by using a combination of morphological and agronomic traits. The effectiveness of using these to estimate genetic diversity has been questioned by several workers (Gottlieb, 1977; Brown and Clegg, 1983). Due to the long generation time of most perennial crops also indicates that many of the morphological descriptors can only be assessed at maturity. Molecular markers that are not subjected to environmental influences provide an opportunity to examine more precisely the genetic relationship between accessions.

As a consequence of the development of the technology to amplify fragments of DNA enzymatically *via* the polymerase chain reaction (PCR) (Mullis and Faloona, 1987), several procedures of molecular fingerprinting have been described such as arbitrary primed PCR briefly described as AP-PCR (Welsh and McClelland, 1990), RAPD (Williams *et al.*, 1990), DNA amplification fingerprinting (DAF) (Caetano-Anolles *et al.*, 1991) etc. Halward *et al.*, (1992) opined that the most meaningful phylogenetic analysis should involve observations from as many types of data as were available including morphological, cytological, geographical, isozyme variations, restriction fragment length polymorphisms (RFLPs) and polymerase chain reaction (PCR) based methods.

In PCR based methods, polymorphisms between individuals can arise through: (1) nucleotide changes that prevent amplification by introducing a mismatch at one priming site; (2) deletion of a priming site; (3) insertion that render priming sites too distant to support amplification; and (4) insertions or deletions that change the size of the amplified product (Williams *et al.*, 1990).

Gallego and Martinez (1996) opined that, in the plant market there is an increasing demand for molecular methods to identify and define genotypes as a way to control the quality of the products and to prevent fraudulent commerce.

2.4.1 Advantages

A DNA polymorphism assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence was first described by Williams *et al.* (1990), which were called RAPD markers, after Random Amplified Polymorphic DNA. They have discussed the advantages of this method over molecular markers such as RFLP. Since then, the technique was used to reproducibly amplify segments of genomic DNA from a wide variety of species (Caetano-Anolles *et al.*, 1991; Martin *et al.*, 1991, Halward *et al.*, 1992). Their results show that single primers could be used to amplify genomic DNA and polymorphisms can be detected between the amplification products of different individuals.

Vierling and Nguyen (1992) pointed out that the polymorphism detected between amplification products of different individuals using the short, random, single primers made RAPD markers well suited for studies of genetic diversity, genetic relationships, genetic mapping, plant breeding, DNA fingerprinting and population genetics.

Mori *et al.*, (1993) reported that, compared to the analysis based on phenotypic data and isozyme data, DNA polymorphisms were independent from environmental conditions and had several advantages including their ubiquitous presence and unlimited numbers. Among the DNA markers, RFLP assay was reported to be laborious and time consuming whereas RAPD was simple, rapid and require minute quantity of genomic DNA.

Several reports are there comparing the advantages and drawbacks of the various techniques that can be used for assessing the genetic integrity, such as morphological traits,

karyological analysis, isozyme markers, RFLP, RAPD, VNTRs etc. (Newbury and Ford-Lloyd, 1993; Novy and Vorsa, 1995; Gallego and Martinez, 1996, Das *et al.*, 1999).

Todorovska *et al.* (1997) detected somaclonal variation in callus regenerated lines in barley and some of these tissue culture derived (TCD) lines showed valuable agronomic changes. In order to assess this genetic variability in 64 TCD lines of four barley varieties, they have used protein, RFLP and RAPD methods. The results indicated heritable polymorphism. They concluded that tissue culture might induce rearrangements in any part of the entire plant genome and hence, RAPD markers were better to detect somaclonal variation than the limited number of RFLP probes applied.

The use of RAPD markers during the last decade became widespread for the identification of somaclonal variants, evaluation of genetic integrity, assessment of micropropagation or *in vitro* regeneration protocols (Isabel *et al*, 1993; Heinze and Schmidt, 1995; Rival *et al.*, 1998) and identification of clonal plant material (Castiglione *et al.*, 1993; Rani *et al.*, 1995). RAPD markers, in particular, have been successfully employed for determination of intra- species genetic diversity in several plants, whereas fewer reports are available on determination of inter-species diversity (Goswami and Ranade, 1999).

A DNA based diagnostic assay like RAPD is able to identify genotypes directly and can therefore help mitigate complications arising from earlier cytological and morphological studies (Lim *et al.*, 1999).

2.4.2 Applications in molecular breeding

Kaemmer *et al.*, (1992) reported that RAPD fingerprinting was effective to identify cultivars, species as well as duplications in germplasm collections, to study evolutionary relationship, to monitor genetic stability of tissue culture materials, as markers in cross and

mutation breeding programmes etc. They compared the two DNA based marker techniques *viz.*, RAPD and olegonucleotide fingerprinting which recognises simple repetitive DNA sequences and found that RAPD technique was superior as it did not require isolation of pure, high molecular weight DNA and also no hybridisation step was needed.

2.4.2.1 Fingerprinting and testing genetic stability

The most advanced area of research with respect to the use of RAPD technology is that of creation of DNA fingerprints for the study of individual identity and taxonomic relationship (Welsh and Mc Clelland, 1990; Hu and Quiros, 1991; Wilde *et al.*, 1992; Newbury and Ford-Lloyd, 1993; Cipriani *et al.* 1996; Khandka *et al.* 1996; Parani *et al.*, 1997).

a) In clones

In potato, the increasing number of genotypes, the limited number of scorable allozymes as well as available enzymes systems and the need for different assay procedures limits the use of isozymes. Compared with this, Mori *et al.* (1993) stated that, virtually unlimited number of RAPDs could be identified, by simply changing primers without changing other experimental conditions.

Kresovich *et al.*, (1994) reported that in clonally propagated vetiver grass, two seemingly unique accessions were found to be represented by the same genotype based on RAPDs. Analogous findings were reported in cranberry in which 17 RAPD profiles represented 22 cultivars; as for some cultivars the profiles were identical (Novy and Vorsa, 1995). On permutational analysis of the data, they found that this was due to cultivar misclassification, resulting in the representation of a genotype by several cultivar names.

Neto *et al.,* (1995) reported using only three primers for efficient finger printing of four cashew clones.

b) In tissue culture derived plantlets

The usefulness of RAPD in assessing the genetic stability of populations of black spruce derived by somatic embryogenesis was evaluated by Isabel *et al.* (1993) using only three arbitrary 11-mer primers. They could identify 10 markers (four monomorphic and six polymorphic) to evaluate the somatic embryos. No variation was detected within clones.

In micropropagated plants of *Populus deltoids* (poplar), Rani *et al.,* (1995) could detect a somatic mutation that occurred in very early stages. Out of the 23 plants 17 were uniform and six were found different from these. But among the six plants, all showed complete homology within the group confirming the fact that a single mutation separated the 23 plants into two groups. But these 6 plants were morphologically indistinguishable from the remaining 17, underlining the fact that RAPD technique is more efficient to determine the genetic fidelity in micropropagated plants.

In *Picea abies* (norway spruce), genetic fidelity of somatic embryogenesis was tested by RAPD analysis (Heinze and Schmidt, 1995). They scored fairly high numbers of bands but could not detect any crude aberrant. Campion *et al.* (1995) found no incidence of genetic instability during micropropagation of *Allium* gynogenic line, using RAPD.

In micropropagated Cavendish banana, production of dwarf off-types due to somaclonal variation was a serious drawback, as the fruit bunches produced by these dwarfs were of inferior commercial value, causing serious economic losses to the growers. The frequency of production of such off-types is reported to range from 3 to 25 per cent in banana (Hwang and Ko, 1987). Damasco *et al.* (1996) identified a 1.5kb band by RAPD analysis using the primer OPJ-04, which was consistently present in all normal but absent

in all dwarf plants of both cultivars. They have stated that this marker was the only available means of *in vitro* detection of dwarfs.

Angel *et al.* (1996) used RFLP and RAPD assay to test whether there was any DNA rearrangement in cassava germplasm maintained at CIAT, Colombia, for 10 years under slow growth *in vitro* conditions. They could not detect any variation, which confirmed the genetic stability of germplasm. Teparkum and Veilleux (1998) used RAPD analysis to genetically characterise anther derived monoploids. They have also suggested that total number of bands present in a monoploid could be used as an indirect method to confirm ploidy level determined by flow cytometry.

2.4.2.2 Genetic diversity analysis

The PCR has proved to be a powerful tool for the identification of polymorphism in cereals. Using wheat, barley, rye and wheat-barley addition lines, Weining and Langridge (1991) detected polymorphism using conserved, semi-random and random primers. With different combinations of primers, they were able to detect both inter-specific and intra-specific diversity.

Hu and Quiros (1991) used phylogenetic information from RAPD markers. They showed that the amplification products from only four random primers were sufficient to discriminate between 14 different broccoli and 12 different cauliflower cultivars.

Vierling and Nguyen (1992) reported the use of RAPD analysis for determining the genetic relationships among diploid wheat genotypes. It has been used also for the assessment of intra-specific genetic diversity and cultivar identification in crops like *Brassica* sp. (Hu and Quiros, 1991; Demeke *et al.*, 1992) Cocoa (Wilde *et al.*, 1992), poplar (Bradshaw *et al.*, 1994), prosopis (Goswami and Ranade, 1999) etc.

Klaas (1998) discussed in detail the use of RAPD in phylogenetic analysis in *Allium* along with a detailed review of the work. He stated that RAPD markers gave sufficient resolution between closely related species possibly up to the level of sections. Kumar *et al.* (1998) were able to distinguish 16 cultivars of *Heliconia* using a single decamer primer OPA-18. The phylogenetic tree derived from RAPD data showed that all the 16 cultivars were closely related. Moreover, two triploid cultivars showed identical RAPD profiles with 10 different primers in agarose and polyacrylamide gels suggesting that they were of the same genotype.

Lim *et al.* (1999) was able to distinctly separate two different *Vanda* spp. among which, one has recently been recognised as a separate genus, *Papilionanthe*. In Orchidaceae, RAPD analysis was found to be a fast and efficient tool, which help the breeders to determine the genetic background of their material.

2.4.2.3 Mapping and tagging genes

One of the practical uses of RAPD markers was in the creation of high-density genetic linkage maps, as these markers segregate in a mendelian fashion (Newbury and Ford-Lloyd, 1993). Within these maps close linkages might be detected between a molecular marker and a trait of interest in crop improvement. This has been used in tomato to tag the *Pseudomonas* resistance genes (Martin *et al.*, 1991). By using recombinant inbred lines for RAPD assay, Reiter *et al.*, (1992) were able to place over 250 new genetic markers in *Arabidopsis thaliana* by only 4 months.

Smith and Williams (1994) have provided a detailed review about the broad use of this technology in plant breeding. Takemori *et al.*, (1994) successfully employed RAPD assay to confirm the hybridity of protoplast fusion derived regenerants of potato using

single decamer primers. In their study, only four single and one mixed primers were needed for simple confirmation of somatic hybrids of 32 fusion combinations.

Using RAPD techniques in combination with bulked segregant analysis, Bennet *et al.*, (1995) were able to identify and map three RAPD markers linked to a dominant gene conferring resistance to black leaf spot in Chinese elm. They found that, from a total of 1210 fragments, only three (0.2%) were linked to the *Bls* gene. Selection of hybrid individuals possessing the two flanking markers will aid in the selection of high level of resistance. Such marker assisted selection (MAS) has been proposed to facilitate early selection in woody plants.

Lefebvre *et al.* (1997) reported the construction of two updated linkage maps of *Capsicum annuum* generated with two doubled haploid populations obtained from intra-specific F_1 hybrids using RFLPs, RAPDs, isozyme and phenotypic markers.

2.4.3. Standardisation of protocol

2.4.3.1 Source material for DNA isolation

Mori *et al.* (1993) reported that in potato, identical banding patterns were observed for any given cultivar using DNAs isolated from sprouting buds and expanded leaves and from plants grown in different locations. Isabel *et al.* (1993) isolated DNA from both *in vivo* and *in vitro* material from black spruce.

Takemori *et al.* (1994) reported that 10mg of fresh leaves of potato yielded 0.9 to 1.2 µg DNA. Novy and Vorsa (1995) reported storing the leaves at $^-73^0$ C after freezing in liquid nitrogen up to 2 months. Cipriani *et al.* (1996) collected leaves from one year old budwood of kiwifruit kept in jars of water at room temperature.

Most of the reports mentioned leaves as the ideal plant part for DNA isolation (Table 6).

Table 6. Source material for DNA isolation from plants

Reference	Crop	Part used
Vierling & Nguyen, 1992	Wheat	Fresh leaves
Mori et al., 1993	Potato	Sprouting buds, expanded leaves
Isabel et al., 1993	Black spruce	Needles, embryogenic cells, somatic embryos, mega gametophytes
Takemori et al., 1994	Potato	Fresh leaves
Bennet et al., 1995	Chinese-elm	Fresh leaves
Heinze and Schmidt, 1995	Norway spruce	Zygotic and somatic embryos, needles
Neto et al., 1995	Cashew	Leaves
Novy and Vorsa, 1995	Cranberry	Leaves
Rani et al., 1995	Populus	Fresh leaves
Cipriani et al., 1996	Kiwifruit	Young expanding leaves
Khandka et al., 1996	Asparagus	Young and fresh stems and leaves (stored at $^{-}80^\circ$ C)
Damasco et al., 1996	Musa spp.	Fresh leaves
Angel et al., 1996	Cassava	Fully expanded fresh young leaves
Gallego & Martinez (1996)	Rose	Fresh mature leaves
Walters et al., 1997	Navy beans	Young trifoliate leaves
Moodie et al., 1997	Wild mustard	Fresh/ frozen ($^{-}80^\circ$ C) leaves
Weir & Pierre 1997	Saskatoon	Frozen leaves ($^{-}70^\circ$ C)
Varghese et al., 1997	Rubber	Fresh mature leaves
Rival et al., 1998	Oil palm	Freeze dried leaves
Teparkum & Veilleux, 1998	Potato	In vitro plantlets
Goswamy & Ranade, 1999	Prosopis	Frozen ($^{-}70^\circ$ C) leaves

2.4.3.2 DNA isolation

Different researchers used DNA isolation procedure, which differs mainly in the quantity as well as type of chemicals used (Moodie et al., 1997; Varghese et al., 1997; Kumar et al., 1998; Lim et al., 1999; Das et al., 1999). The procedures suggested by Doyle and Doyle (1987, 1990) and Dellaporta et al. (1983) were the most commonly followed

with slight modifications for each taxa (Wilde *et al.*, 1992; Mori *et al.*, 1993; Cipriani *et al.*, 1996; Das *et al.*, 1999).

Gallego and Martinez (1996) stated that, among the various protocols tried to extract DNA from mature rose leaves, which were lignified and containing high amount of polyphenols, the above two methods yielded high quality DNA that gave consistent RAPD patterns in PCR. But they feel that these methods were too tedious and time-consuming to include in routine fingerprinting programmes. To overcome this, they reported that, addition of PVPP to the cetyl trimethyl ammonium bromide (CTAB) extraction buffer improved sample quality as well as reduced time of extraction.

Rogers and Bendich (1994) reported that the DNAs and RNAs produced by methods employing CTAB generally exhibited lower level of enzyme inhibition than did those by other methods. While the yields were lower with CTAB than from some of the other methods, the quantity of DNA was still adequate for most uses in molecular biology and the condition of the DNA was above average.

Moodie *et al.* (1997) reported that the isolated DNA samples could be stored at -80^{0} C for up to one year. Rival *et al.* (1998) reported a considerable improvement in yield of DNA (250 to 500µg g^{-1} dry weight) by a procedure described by them, compared to the previously reported yield in oil palm (20µg g^{-1} dry weight – Shah and Parveez, 1992)

Wettasinghf and Peffley (1998) investigated four DNA extraction protocols in onion to select the fastest and best method that would yield a high quantity of intact DNA with low RNA contamination, with the least expense. The protocol selected by them did not require phenol or chloroform; isolated high yield of DNA with no RNA contamination and extractions up to 50 samples for 8h day were possible.

Ellsworth *et al.* (1993) reported that contaminating RNA in the extracts might interfere with DNA amplification. Kumar *et al.* (1998) also stated that, the isolated genomic

DNA in *Heliconia* was cleaner and there was no visible background smear after RNase treatment and phenol-chloroform extraction. Also, the amplification was satisfactory and band profiles were reproducible after RNase treatment.

Lim *et al.* (1999) determined the quality of isolated DNA by observing the ratio of absorbance at A_{260} : A_{280} and by subjecting it to 0.8 per cent agarose gel electrophoresis. Goswami and Ranade (1999) used Hoechst 33258 as fluorochrome on a DyNa Quant 200 flourometer (Pharmacia – Hoefer) for measuring the concentration of DNA isolated and the quality was checked on one percent agarose gel in 0.5 X TBE according to Sambroke *et al.* (1989).

2.4.3.3 Reaction conditions

The banding patterns are repeatable for a plant genotype using a particular primer provided that all of the reaction characteristics are consistent. Variation in any one of the several parameters of the reaction can yield differing banding patterns and the investigator must decide upon a set of reaction conditions and rigidly adhere to them (Newbury and Ford-Lloyd, 1993; Karp *et al.*, 1997).

a)Thermal cycler

Penner *et al.* (1993) reported difficulties in obtaining identical patterns from the same set of primers and materials among different laboratories. In their study, the type of thermocycler used for RAPD analysis seemed to be a key determinant of the reproducibility of band patterns.

b) DNA polymerase

Schierwater and Ender (1993) have reported the dependence of the RAPD pattern on the source of DNA polymerase used for target amplification. Nanda and Jain (1994) also reported that the enzyme preparations of different companies amplified with different efficiency (i.e. yield of product per cycle) and fidelity (error-free incorporation of nucleotides) depending upon reaction conditions. They have also listed out the different types of DNA polymerase such as Taq DNA polymerase, Vent $_R^{TM}$ DNA polymerase as well as Pfu enzyme which were all isolated from different thermophilic marine bacteria and compared their efficiency. Taq DNA polymerase was reported to be the most common and had an extension rate of 2000 - 4000 bases per minute at 70 to 80°C.

Cipriani *et al.* (1996) compared the Ampli Taq Stoffel fragment (Perkin Elmer) with the Taq DNA polymerase (Boehringer Mannheim). They found that the former produced smaller fragments, gave an average number of bands per primer 19 percent higher and an average number of polymorphism per primer 40 percent higher than that obtained with later. Consequently, they selected the Stoffel fragment due its better performance.

Gallego and Martinez (1996), using rose DNA as template, compared four commercial enzymes viz. Amplitaq (Perkin Elmer), Dynazyme (Finnzymes Oy), Biotaq (Bioprobe) and Taq DNA polymerase (Hytest). Their results support the earlier reports. The quality of the RAPD pattern was found best for the Taq DNA polymerase of Hytest.

c) Reaction buffer

The Mg $^{2+}$ ion is known to affect the primer – template interactions (Welsh and Mc Clelland, 1990) polymerase activity and the melting temperature of double stranded DNA (Rolfs *et al.*, 1992).

Changes in the PCR reaction buffer conditions usually affect the quantum of amplification. Nanda and Jain (1994) reported that the concentration of $MgCl_2$ had a profound effect on the specificity and yield of amplification. Also, the presence of EDTA or other chelators disturb the Mg^{++} ion concentration, which should be in the range of 1.5 to 4mM. The annealing temperature should be 5-10°C below Tm (melting temperature) for primer. They have suggested a thumb rule for the calculation of Tm as 2^0c for every A or T and 4^0C for every G or C.

d) Template DNA

Takemori *et al.* (1994) tried various amounts of template DNA (0.01 to 50ng) for RAPD analysis in potato and found that amounts exceeding 0.1ng consistently gave stable banding pattern. The size of the template DNA was found to be optimum within a range of 0.5kb to 2.1kb. The DNA and $MgCl_2$ concentrations in the reaction mixture were reported to be important for successful DNA amplification in wheat (Devos and Gale, 1992).

Damasco *et al.* (1996) tried template DNA concentration of 5, 10, 25, 50, 75 and 100ng from normal Cavendish banana using different primers at 2.5mM $MgCl_2$ concentration as well as different concentrations of $MgCl_2$ (1.5, 2, 2.5, 3, 4 and 5mM) tested at 50ng DNA, with different primers. The results indicated that the DNA concentration between 25 to75 ng and $MgCl_2$ concentration between 2.5 to 4mM resulted in good amplification and products that were easy to score. But Gallego and Martinez (1996) reported no difference in the RAPD profiles of rose over a template concentration ranging from 5-100ng.

The optimum amount of DNA template for *Heliconia* was reported to be 100ng (Kumar *et al.*, 1998). No DNA bands were amplified in the absence of $MgCl_2$ and relative

intensity of bands was found to increase as $MgCl_2$ concentration increased from 1mM to an optimum of 3mM, beyond which it decreased.

e) Thermal cycle

With Ampli Taq Stoffel fragment (Perkin Elmer), Cipriani et al. (1996) have tested three annealing temperatures (35, 37 and 39^0C) using four different primers and found that 37^0C resulted in the best performance in terms of number of amplified fragments and reproducibility of results.

Gallego and Martinez (1996) have also tested several thermal cycle programmes that combined different durations of the amplification steps. Their results revealed that, the denaturing and annealing steps could be reduced from 1 min to 15 and 30 sec respectively without change in profiles. The best annealing temperature for the last 35 cycles was found to be 45^0C rather than 37^0C, which assured the consistency of the profiles.

Kumar et al., (1998) reported the optimal primer annealing temperature as 37^0C,at which, the number of bands varied from 4 to 20.

f) Electrophoresis

Novy and Vorsa (1995) separated the RAPD markers in polyacrylamide gels and visualised by silver staining. They stated that the increased resolution of polyacrylamide gels in conjugation with the 2-5 fold increased sensitivity of silver staining compared to ethidium bromide provided reproducible molecular markers and reduced the chance of mis-scoring of bands. But majority of the earlier reports suggested the use of agarose gel with ethidium bromide staining (Varghese et al., 1997; Teparkum and Veilleux, 1998; Goswami and Ranade, 1999; Lim et al., 1999).

As the interpretations of the RAPD data depend on the assumption that amplification products of equal size are homologous, mis-interpretation of the data-sets is to be expected in studies of distant species (Rieseberg, 1996). Klaas (1998) pointed out that a thorough analysis of homology or the genetic characterisation of single markers could reduce this, but then, it will make studies of little characterised species less feasible. This could be overcome by including at least two bands per taxon and several primers, preferably scoring many bands from only few PCR reactions.

2.4.4 Extent of polymorphism

Vierling and Nguyen (1992) in RAPD analysis of diploid wheat, *Triticum monococcum* and *T. urartu*, scored the data on the basis of presence or absence of amplification products. For each individual amplification, they designated the most cathodal product as 'a' and subsequent products as b, c, d and so on. If a particular product was present in a genotype, it was designated 1; if no shared products were present in other genotypes, they were designated 2. This scoring was done for each amplification product across all genotypes within a species. Using 60 random primers, they obtained 41 polymorphic and 62 monomorphic bands in *T. monococum* whereas 73 polymorphic and 53 monomorphic bands in *T. urartu*. The origin of *T. urartu* and the phenogram clustering based on RAPD data were found correlated. So they concluded that RAPD analysis is a powerful tool for determining the genetic diversity among diploid wheat genotypes.

In *Arachis*, Halward *et al.* (1992) reported that 1 to 3 major segments were amplified in any given sample, along with a number of bands of lesser intensity. Scoring was done based on presence (+) or absence (-) of bands. Tingey and del Tufo (1993) have described the advantages of RAPD assay in comparison with phenotypic assay and RFLP assay. They pointed out that, on an average, the frequency of finding RAPD polymorphisms

has been 0.3 per primer in *Arabidopsis thaliana,* 0.5 per primer in soybean, 1 per primer in corn and 2.5 per primer in *Neurospora crassa.*

In potato, Mori *et al.* (1993) selected 5 decamer primers which generated several monomorphic and polymorphic bands, out of which 15 were identified as useful RAPD which were polymorphic and shared among the cultivars. They did not select single, cultivar specific bands, as they were mostly minor bands and often un-reproducible. They also scored the bands based on presence (+) or absence (-).

A set of 7 arbitrary 11-mer primers were scored by Isabel *et al.* (1993) to select three primers for segregation studies in *Picea mariana* (black spruce). With the three selected primers they evaluated the genetic integrity in somatic embryogenesis derived populations. The presence or absence of bands in parents and embryos were used for the segregation analysis.

Takemori *et al.* (1994) identified somatic hybrids in potato based on the presence of specific bands. Out of the 20 different primers tried, 18 revealed polymorphism for tetraploid potatoes.

A total of 220 10-base primers were screened by Bennet *et al.* (1995) for Chinese elm RAPD assay. Out of this, 199 (90.5%) provided 1210 fragments ranging in size from 200-3000kb. From these 2 primers were selected which provided three fragments (markers) which were tightly linked to the resistant gene for black leaf spot, i.e. OPP 17 and OPC 20.

Rani *et al.* (1995) used 11 arbitrary decamer primers to generate 61 monomorphic and 13 polymorphic bands in micro propagated *Populus deltoides.* Of this, five primers produced polymorphic and monomorphic bands (OPC 7, 19; OPA 5,8,10). Out of the total 39 bands produced by these, 13 bands were found polymorphic in six plants out of the 23. In rest of the 17 plants, all bands were monomorphic. The marker profiles among these six

plants were identical with all five primers. They scored the bands based on presence or absence.

Neto et al. (1995) also scored the bands as present (+) or absent (-). Six decamer primers generated a total of 27 amplification products in seedling of four cashew clones. The number of distinguishable bands per primer varied from zero to four. Among the 27 bands, 21 were seedling specific. They have identified three primers as most efficient to fingerprint the four cashew clones.

Novy and Vorsa (1995) screened 23 cranberry clones using 12 decamer primers. They generated 116 scorable bands of which 52 (45%) were polymorphic. A range of 1-14 amplified bands per primer was observed.

Eighty decamer primers were used to assay four genotypes (2 male and 2 female) of each of the diploid species of Actinidia (kiwi fruit) (Cipriani et al., 1996). 30 out of the 80 were reported to produce species-species markers, i.e. bands monomorphic within the species where they were present and polymorphic between species. The scoring of the data was done based on presence or absence of bands and they could identify a total of 87 markers as potentially useful for taxonomic purposes.

Khandka et al. (1996) reported that out of the 31 decamer primers tried in asparagus, 26 primers produced bands while 5 gave either weak or no amplification. 12 out of 26 generated only monomorphic bands and were not used in statistical analysis. The remaining 14 primers generated a total of 119 bands among which, 33 were found polymorphic. The markers were scored by either presence (+ for faint and ++ for intense) or absence (0) of a given amplification product for each cultivar.

Damasco et al. (1996) screened 66 random decamer primers, which produced a total of 234 bands, to identify dwarf off-types of in vitro Cavendish banana plants. Of this, 19 (28.8 %) revealed polymorphism (34 bands) between normal and dwarf plants. The average

number of products was reported to vary between 1 and 10 per primer. The bands were scored based upon presence (+) or absence (-).

Angel et al. (1996) used 20 decamer primers to test the stability of 10-year-old in vitro stored cassava germplasm. Each primer generated between 2-10 bands and no polymorphism was detected within the accessions. In rose, 20 decamer primers (OPA kit) were used to amplify the genomic DNA of 25 cultivars (Gallego and Martinez, 1996). Three primers failed to give amplification, but the remaining 17 generated a total of 163 fragments out of which 73 were polymorphic. Out of the 20 primers, seven showed enough variability to allow complete differentiation of the 10 rose varieties. They have selected two out of this, OPA11 and OPA 17 to profile all the 25 cultivars. They could identify a set of 37 RAPD markers to distinguish unambiguously the 25 genotypes.

RAPD analysis was carried out in tissue culture derived barley lines to assess somaclonal variability (Todorovska et al. 1997), using 20 decamer primers of OPA kit. Out of these, two primers were selected which generated reproducible polymorphic bands. In navy bean RAPD assay, Walters et al (1997) screened 390 random decamer primers and selected 101 primers, which yielded polymorphism between three selected parental genotypes (8% polymorphism in P_1, 9% each in P_2 and P_3).

A total of 69 individual plant of wild mustard from 13 populations were scored for 60 RAPD markers using 40 primers of OPB and OPR kits (Moodie et al., 1997). Of these, 75% generated amplification products. A subset of six primers was selected that gave an average of 14 clear reproducible markers per primer (range - 8 to 22).

Weir and Pierre (1997) obtained a total of 98 DNA fragments using eight 9-mer primers for the characterisation of 16 saskatoon cultivars. The number of bands per primer ranged from 8 – 17 with an average of 12.3 .Out of the 98 bands, 29 (29.6%) bands were consistently amplified and used to generate a phenogram of the cultivars. 25 bands (25.5%)

were consistent but monomorphic and 44 (44.9%) were considered unstable and therefore excluded. Using two primers they could uniquely characterise the 16 cultivars.

In two different crosses involving four varieties of *Capsium*, Lefebvre *et al.* (1997) used 248 and 290 random primers out of which 96 (38.7%) and 65 (22.4%) primers revealed RAPDs respectively.

Out of a total of 126 random decamer primers screened for DNA amplification in rubber, Vargheese *et al.* (1997) reported that, 97 primers produced clearly distinguishable amplification products, out of which 57 (59%) showed polymorphism between the six clones. From these they selected 42 primers to profile 24 clones, which generated 220 fragments with 111 showing polymorphism. The number of polymorphic DNA fragments per primer ranged from 1-5 with an average of 2.6. Only 80 polymorphic bands, which were clearly and consistently scorable across all 24 clones, were considered for estimation of genetic distances. The scoring was done as discrete variables using 1 (presence) and 0 (absence).

Rival *et al.* (1998) used a series of 387 arbitrary decamer oligo nucleotides (Operon) to amplify four oil palm DNA samples out of which, 259 (67%) generated consistent bands. Among these, 73 (19%) produced polymorphic bands between clonal lines. Average band per primer was 5.4. From these 73, based on reproducibility and clarity, 24 primers were selected to profile 32 somatic embryogenic palms. A total of 8813 bands were scored from 68 palms with 24 primers.

Kumar *et al.* (1998) after a preliminary screening of 20 random primers selected 11 decamer primers to distinguish *Heliconia* species and cultivars. The number of bands varied from 4-20. They got distinct profile for 16 cultivars using a single primer, OPA 18. Screening 101 primers (decamer-Operon) using three DNA samples resulted in the selection of 47 primers, which were used to amplify DNAs from 32 plants. Based on

consistency, only 14 out of 47 were considered for cumulative analysis. These 14 primers produced a total of 357 bands.

2.4.5 Data Analysis and interpretation

The various investigators scored the different genotypes based on the presence or absence of RAPD fragments in the pattern revealed by the specific PCR carried out to generate data. This information was converted into matrix form and based on this matrix genetic analysis of the data was conducted.

For pair wise comparison of geneotypes, similarity coefficients (Jaccard, 1908) were worked out and phenograms (dendrograms) were constructed using various programmes such as Unweighted Pair Group Method with Arithmetical Averages (UPGMA), Polygenetic Analysis Using Parsimony (PAVP), NTSYS-pc (Vierling and Nguyen 1992; Halward et al., 1992; Neto et al., 1995; Khandka et al., 1996 and Das et al., 1999). Apart from these, Principal Co-ordinate Analysis was also performed using Genstat analysis system (Demeke et al., 1996; Moodie et al., 1997 or SYNTAX IV programme (Varghese et al., 1997).

In studies involving linkage of the RAPD marker with specific genes, linkage analysis was carried out using the programme MAPMAKER (Bennet et al., 1995). In certain other studies the genetic distance was calculated and cluster analysis was performed using SYSTAT package (Khandka et al., 1996). Teparkum and Veilleux (1998) and Goswami and Ranade (1999) generated dendrogram using UPGMA and Software 'RAPDistance' and neighbour joining trees were produced using NJTREE.

2.4.6 Draw backs

Williams *et al.* (1990) pointed out that as RAPD markers were typically dominant markers, they could not be efficiently mapped in F_2 populations. Back cross or recombinant inbred populations only will help to map these markers efficiently.

According to Vierling and Nguyen (1992), one limitation of RAPD analysis is that the complete sequence of amplification products is not known. A primer might produce identically sized products in two genotypes, but possible divergence within the internal sequence cannot be detected. They suggested that this could be overcome by using numerous primers. The second limitation is that very small difference in product size may be difficult or impossible to detect which was suggested to be overcome by using a higher concentration of agarose, using numerous primers, analysing products in question in adjacent lanes, or using acrylamide gels.

Liu and Furnier (1993) discussed in detail the possibility of over estimation of genetic variation obtained with RAPD and morphological markers. The dominant nature and the assumption that each band is a locus with two alleles were considered as the possible reasons for over estimation with RAPD markers.

The problem of errors in scoring bands is generally recognized in the RAPD technique, but Weeden *et al.* (1994) reported having reduced the error rate from eight per cent to zero per cent by systematically disregarding the faint staining fragments.

A number of different factors of the PCR reaction have been reported to be responsible for the artifacts affecting the reproducibility of the analysis. Reiter *et al.* (1992) suggested that obvious solution to this problem was to carry out replicate runs and discard all non-reproducible bands.

Halward *et al.* (1992) used RAPD technique in genetic studies of a diverse group of germplasm lines of *Arachis hypogaea* as well as some wild diploid *Arachis sp.* But they

could not distinguish among cultivars of *A. hypogaea* using RAPDs. Since RFLPs also revealed no polymorphism, they concluded that much of the morphological variation observed among germplasm lines of cultivated peanut might be controlled by only a few genes. Even though RAPD have been applied to mapping studies in many species, the complex banding pattern produced in the amplification reactions in peanut were reported to be undesirable for use in genetic mapping.

To be used for varietal identification there should be distinct inter-varietal variation and minimal intra-varietal variation, environmental stability and experimental reproducibility. RAPD technique usually satisfy these requirements, as mentioned before, but Newbury and Ford-Lloyd (1993) reported that in *Beta vulgaris*, RAPD fingerprinting has revealed extremely high levels of both inter- and intra- varietal variation so that varietal identification using RAPD was found difficult.

Taylor *et al.* (1995) found that RAPD analysis was suitable for detecting gross genetic changes occurring in sugarcane tissues subjected to prolonged *in vitro* culture, but was not sufficiently sensitive to detect smaller changes during genetic transformation.

Halden *et al.* (1996) has discussed limitations of RAPD markers such as the dominant nature, influence of reactions conditions on reproducibility, heteroduplex formation, competition for priming sites in the genome etc. and the possible reasons and solutions.

In oil palm, Rival *et al.* (1998) reported that RAPD analyses has failed to reveal any polymorphism associated with somaclonal variation in regenerants which were clearly identified in the field through their phenotypic characteristics. They found that, using 259 primers, the total size of amplification products scored for polymorphism averaged only roughly 0.04 per cent of the oil palm genome size of 3.6×10^9 bp. They concluded that, the probability of detecting polymorphisms resulting from macro events (deletion, insertion or substitution) at the DNA level within an amplified region was thus, rather low.

But there are ways to overcome these drawbacks and RAPD analysis still retain its position as one of the most important marker system in crop breeding experiments.

2.5 COMBINED USE OF MORE THAN ONE MARKER SYSTEM

Prabakaran *et al.* (1991) based on the karyo-morphological analysis of *Pennisetum americanum* concluded that, morphological closeness was accompanied by restricted karyotypic variations and recognisable morphological difference followed a marked karyotypic difference. Sreekumari and Mathew (1991) also reported differences in karyo-morphology among five morphotypes of Taro, which differed recognizably from one another with respect to several morphological features.

Torres *et al.* (1993) reported the linkage relationships of nine isozymes, one RFLP and 43 RAPD markers in *Vicia faba* to construct a preliminary linkage map for this species. They could identify at least 11 linkage groups in the same crop. Satovic *et al.* (1996) using 13 F_2 families from trisomics have revealed 48 linkage groups, six of which have been precisely assigned to specific chromosomes. They used morphological, isozyme and RAPD markers.

Beer *et al.* (1993) carried out a study in 177 accessions of wild oats, *Avena sterilis* to compare the RFLP and isozyme polymorphism and also, variation in qualitative and quantitative morphological traits. They assessed the correspondence between proximity matrices constructed from these data. Based on these, they have discussed the effectiveness of the use of one type of trait as well as combined use of different traits for sampling and management of plant germplasm collections. A correlation of – 0.35 was observed between Jaccard's similarity based on RFLPs and taxonomic distance based on morphological characters. Liu and Furnier (1993) reported higher estimates of genetic diversity for RAPD markers than isozymes in *Populus* sp.

MaaB and Klaas (1995) tested 300 clones of garlic with isozymes, out of which, 48 clones were also tested with RAPDs to compare the two marker systems. A total of 12 isozyme systems were tested which identified 22 loci, 10 of which were polymorhic and defined 16 isozyme groups. Predictably, the 125 RAPD markers allowed a finer distinction, but generally both markers allowed a good delimitation of varieties of *Allium sativum*.

Kongkiatngam *et al.* (1995) have compared morphological, isozyme and RAPD markers for estimating genetic variability within and between two heterogeneous red clover populations. They scored six monogenic morphological traits, 21 isozyme loci with 43 alleles using 12 enzyme systems and 133 RAPD bands (putative loci) using nine arbitrary primers. They reported that number of loci detected with RAPD markers was much higher than that detected with morphological traits and isozymes. Mean number of alleles per locus and percentage of polymorphic loci were higher with RAPD markers than with isozymes in both cultivars. The estimates of expected heterozygosity and the Shannon diversity measure obtained with morphological and RAPD markers were about the same in these two cultivars and almost doubled those detected with isozymes. The reason for this was attributed to the fact that RAPD markers can detect both coding and non-coding sequences in the genome. They have concluded that isozyme and RAPD markers could be used effectively to estimate genetic variability within and between cultivars of red clover. Compared with morphological characteristics, better estimates of genetic variation could be obtained using these two types of markers.

Tatineni *et al.* (1996) investigated genetic diversity among 16 elite cotton genotypes and compared the genetic distance estimated from RAPDs with taxonomic distance estimated from morphological characteristics. They have reported a correlation of 0.63 between the two.

Hoey *et al.* (1996) carried out a phylogenetic analysis of *Pisum* based on morphological, allozyme and RAPD markers. They could organise precisely the pea taxa into established taxonomic groupings using allozyme and RAPD markers. Parani *et al.* (1997) using protein, isozyme and RAPD markers to establish the hybridity of the F_1 produced by crossing *Sesamum alatum x S. indicum*. By observing the presence or absence of parental specific bands, they could identify the hybrids.

Papa *et al.* (1998) analysed the genetic diversity in land race populations of barley using RAPDs, isozymes and morphological traits. They found that the diversity level of the populations studied was often different for these three types of markers and even between isozymes and RAPDs, the low level of correlation was reported to be significant. They have explained this lack of correlation as due to the different genetic nature of these markers and suggested that care should be taken in extending the inferences on the level of diversity obtained with a given type of marker to the global genetic diversity, which can be found in a population. However, the partitioning of diversity between and within populations was reported to be similar for all types of markers.

MATERIALS AND METHODS...

3. MATERIALS AND METHODS

In the present investigation, characterisation of 40 TC clones and 20 conventionally propagated clones of four elite varieties of black pepper was aimed at, using four different marker systems. Accordingly the experiment has four different phases.

1. Morphological markers
   - 1.1 Quantitative characters
     - 1.1.1 Vegetative characters (22 numbers)
     - 1.1.2 Reproductive characters (19 numbers)
   - 1.2 Qualitative characters (39 numbers)
     - 1.2.1 Vegetative (30 numbers)
     - 1.2.2 Reproductive (9 numbers)
2. Cytological markers (somatic chromosome number)
3. Isoenzyme markers
   - 3.1 Peroxidase (PRX)
   - 3.2 Glutamate Oxaloacetate Transaminase (GOT)
4. RAPD markers

The morphological details were collected from the field established plants at the Pepper Research Station, Panniyur. The cytological, isozyme and RAPD analysis of the above vines were carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara and at College of Agriculture, Padannakkad during the period 1997-2000.

3. 1 MATERIALS

3.1.1 Plant material

Four most promising varieties of black pepper were used for the investigations. Conventionally, these are propagated through rooted cuttings. The standard protocol for

raising micropropagated black pepper plants through axillary bud culture method was developed and tested at the College of Horticulture, Kerala Agricultural University during 1995-96 (Joseph *et al.*, 1996). The suitable protocol thus developed was further tested and applied for mass multiplication of selected varieties and the plantlets were distributed to various regions including Pepper Research Station, Panniyur for field establishment and evaluation.

For the present investigation a total of 40 micropropagated plants produced at College of Horticulture, Vellanikkara and planted in the field at Pepper Research Station, Panniyur in 1995 were selected as TC group. For comparison of this group with conventional clones, 20 vines produced through rooted cuttings and planted in the same year at the station were also utilised. The conventional clones and TC clones of each variety were generated from different mother vines, but were of the same age (Plate 1a, 1b).

The Pepper Research Station, Panniyur receives an annual rainfall of 3500 mm with a hot humid climate. The experimental plot is a moderately sloping terrain and the soil is of lateritic nature with clay loam texture.

The vines were in the age group of 2 years when the investigations were started. Morphological observations were recorded for two consecutive years, 1997 and 1998 (2nd and 3rd year of growth). They were planted at a spacing of 3m X 3m and trailed on *Erythrina indica* standards. The management practices were uniform as per the recommended package of practices (KAU, 1996). The details of the experimental material selected are provided in Table 7.

Rooted cuttings from these vines were raised in polythene bags for taking root and leaf samples for cytological studies, enzyme extraction and DNA extraction.

Plate 1a

Experimental field

Plate 1b

Table 7. Details of the experimental material

Sl. No.	Varieties	Synonym	Number of parental clones	Number of TC plants
1	Panniyur 1 (P₁)	-	5	10
2	Panniyur 2 (P₂)	Krishna	5	10
3	Panniyur 4 (P₄)	Anjana	5	10
4	Subhakara (Su)	KS 27	5	10
	Total		20	40

3.1.2 Chemicals and equipments

The chemicals used were of reputed grade and the details are provided in Appendix I. The equipments and machinery available at CPBMB, College of Horticulture, Vellanikkara as well as biotechnology and the central instrument laboratories of the College of Agriculture, Padannakkad were used (Appendix II).

3.2 METHODS

3.2.1 Morphological scoring

Morphological details of individual plants were observed and recorded for 80 traits based on the descriptor for *Piper* sp provided by NBPGR Regional Station, Vellanikkara and earlier works in black pepper (Kanakamany, 1982; Sujatha and Namboodiri, 1995 and Anand, 1997).

The observations were recorded after grouping the different morphological characters into qualitative and quantitative attributes. Qualitative characters were scored by visual assessment and quantitative characters were measured according to the type. Data were recorded for two years, 1997 and 1998.

3.2.1.1 Quantitative characters

<u>Vegetative</u> (22 traits)

I. Young leaf - Length: Width ratio (average of 10 leaves)

II. Mature leaf - Petiole length, length: width ratio, leaf area

        (Average of 10 leaves of orthotrope and plageotrope)

III. Stem -Internodal length, thickness and nodal thickness (orthotrope and plageotrope)

        - Number and position of branch, direction of growth

<u>Reproductive</u> (19 traits)

IV. Flower - Number and length of spike, stamen and stigmatic lobe number

V. Fruit - Number of berries per spike, 100 berry weight and volume,

        Spike weight: berry weight, fresh berry weight: dry berry weight

VI. Seed - Weight and volume of 100 dried berries

VII. Yield / vine - Fresh berry yield

VII. Chemical composition - Oil, oleoresin and piperine content

3.2.1.2 Qualitative characters

<u>Vegetative</u> (30 traits)

I. Young leaf -Colour, shape, tip, base, colour of leaf sheath, stipule characters

II. Mature leaf - Petiole shape and hairiness

        - Leaf shape, texture, colour, venation, hairiness, phyllotaxy

III. Stem -Shape, colour, branching, adventitious roots (number & clinging ability)

<u>Reproductive</u> (9 traits)

IV. Flower - Season, sex form, spike orientation, texture, bract type

V. Fruit - Colour of berry, time taken from flowering to fruit maturity

The leaf and stem characters were recorded in centimetres from 10 samples each, selected at random and the mean worked out. The position of branch was measured as the angle subtended by the lateral with the main stem, in degrees and the direction of growth was recorded as drooping (if more than 80^0) and semi erect (if less than 80^0).

The reproductive characters were measured only for those vines, which yielded a minimum of 25 spikes per vine. The oleoresin content was estimated using the Soxhlet method of extraction (Horwitz, 1980). Oil content was estimated as difference in weight of the acetone extract before and after evaporation in a water bath at 100^0 C. Crude piperine was determined after estimation of nitrogen by micro-Kjeldahl method and multiplying the percentage nitrogen by a constant 20.36 (Horwitz, 1980).

3.2.2 Cytological analysis

Somatic chromosome number of the TC plants and corresponding parental clones were determined by root tip squash method. The technique was standardised with respect to season of collection, age of cutting, time of collection and slide preparation.

3.2.2.1 Season

The root samples were collected from January to December to identify the best season for root collection.

3.2.2.2 Age of cutting and collection of roots

Fleshy, short white roots with pearl-white tips were collected from three weeks to two months old two node cuttings, which were planted in polythene bags with potting mixture, after dipping in 1000 ppm Indole-3-butyric acid (IBA) for 45 seconds. Without

disturbing the roots, the cuttings were taken out, thoroughly washed with water and 1to2 cm long root-tips were cut. Then the base of the stem cutting was cut near the node, the IBA treatment was repeated and replanting was done in the polythene bag. Such bags were kept inside a mist chamber. For comparison, fleshy white climbing roots were also used whenever available.

3.2.2.3 Time of collection

Roots were collected from 10.30 am to 3.30 pm at one hour intervals.

3.2.2.4 Preparation of slides

The technique was standardised based on the cytological procedure suggested by Mathew (1958), Kao (1975) and Evans and Reed (1981) with suitable modifications as detailed below. The collected roots were thoroughly washed under running water using a soft brush and tea strainer, dried and transferred to glass vials for pre-treatment.

a) Pre-treatment

Several reports suggested the use of 8-hydroxyquinoline for pre-treatment. Two concentrations of 8-hydroxyquinoline were tried *viz.* 0.03 and 0.05 per cent. The chemical was dissolved in a small volume of distilled water in a beaker and the volume was then made up to 100ml in a volumetric flask and stored in a refrigerator at 4^0C.

The dried roots were placed in the cold pre-treatment solution and kept at 4^0C in a refrigerator for 2 to 3h. After treatment, the growing tip was clear white in colour and easily distinguishable from the rest of the root.

b) <u>Fixation</u>

The pre-treated roots were washed thoroughly under running water to remove all the traces of 8-hydroxyquinoline and dried between folds of blotting paper before fixation. The most common fixative used in earlier works was acetic alcohol in different proportions. Different fixatives tried were:

1. Acetic alcohol (1:3)

2. Carnoy's fixative - glacial acetic acid: chloroform: absolute alcohol (1:3:6)

3. Brown's solution- glacial acetic acid: formaldehyde (40%): picricacid (1:5:5)

4. CRAF solution - solution A: solution B (1:1), where,

<u>Solution A</u>		<u>Solution B</u>
chromic acid	- 1g	neutral formalin - 30ml
glacial acetic acid	- 7ml.	water - 70ml
water	- 92 ml	

The roots were kept in the fixative for one or two days. After that, they were taken out, washed and dried between folds of blotting paper.

c) <u>Hydrolysis</u>

The dried roots were hydrolysed in 1N HCl taken in a glass vial, which was suspended in a water bath. The time and temperature varied as follows:

$70\,^{0}C$ -	10 minutes, 8 minutes and 6 minutes
$65\,^{0}C$ -	10 minutes, 8 minutes and 6 minutes
$60\,^{0}C$ -	15 minutes, 10 minutes and 8 minutes
$55\,^{0}C$ -	20 minutes, 15 minutes and 10 minutes

After hydrolysis, the roots were placed in a petridish containing distilled water and washed 5 to 6 times to remove any traces of HCl, taking care not to damage the hydrolysed roots and were then air dried by spreading on filter paper.

d) <u>Staining</u>

Different stains were tested to select the easiest as well as the best staining method as per the procedure suggested by Armstrong (1995).

1. Aceto-carmine 2%:	Carmine powder	- 2g
	45% acetic acid	- 100 ml
2. Aceto-orcein 0.5%:	Orcein powder	- 0.5g
	45% acetic acid	- 100ml
3. Fuelgen stain 0.5%:	Basic Fuchsin	- 3g
	1N HCL	- 90ml
	K_2S_2O	- 9g
	Activated charcoal	- 3g
	Water	- 600 ml

The dried roots were kept in sufficient quantity of stain to completely immerse the roots. The time of staining varied from 30 minutes to 3 hours.

After staining, the roots were placed on microscopic slide with a drop of the stain. The tip, which was darkly stained, was separated, gently smashed with a glass rod and the larger debris was removed. The cover slip was placed over the cells taking care to avoid any air bubbles. The slide was gently warmed, thumb-pressed between folds of blotting paper and cooled. This was repeated 3 to 4 times. After the last hard pressing, the sides of cover slip were sealed.

The slides were observed under a Biomed microscope at a magnification of 100 x 10 and good cells were photographed at a magnification of 100 x 5.

3.2.3 Isozyme analysis

Precise standardisation of methods is essential to make the isozyme data fit for relative evaluation. This requires choosing the most suitable plant tissue, appropriate stage and method of sampling, suitable buffer systems and optimum electrophoretic and staining protocols.

In the present study, the black pepper plants were analysed with respect to two enzyme systems *viz.*, Glutamate Oxaloacetate Transaminase (Aspartate Amino-Transferase) and Peroxidase (PRX).

3.2.3.1 Choice of tissue and mode of extraction

A critical review of literature suggested leaf as the best choice for enzyme extraction and hence both the enzyme systems were analysed with leaves, collected from rooted cuttings taken from the 58 experimental pepper vines established in the field. Duplicate sets of cuttings were kept in both green house as well as open condition. Both tender and mature leaves were tried, as the age of the tissue was also critical for enzyme activity. The selection of the leaf type was based upon the method followed by Conklin and Smith (1971) and Visedo *et al.* (1990). First to third pale green leaves from tip were considered as the tender leaves and 5th to 7th dark green leaves from tip were considered as mature leaves. The extractions of both enzymes were done with fresh leaves immediately after collection and also after storage at 4^0 C for one hour as well as at -20^0C for one hour. The low temperature was provided to examine variations in enzyme activity, if any, due to storage. The various buffers tried for extraction of enzyme from the tissue are tabulated in Table 8.

Table 8. Type of the extraction buffers tried for isozyme analysis

No	Buffer	Molarity	pH	Adjusting pH
1	Tris Buffer	0.1M, 0.15M 0.35 M	7, 7.1, 7.3, 7.4, 7.5, 7.6 & 8	with HCl before adding the buffer additives
2	Tris Buffer	0.1M, 0.15M	7.5, 7,6 & 7.7	with NaOH after adding buffer additives
3	Tris Buffer –EDTA	0.1M-0.05M	8	with HCl
4	Tris HCl	0.35M	7.4	with NaOH
5	Tris HCl-EDTA	0.2M-0.01M	8	- do -
6	Na-Phosphate Buffer (0.1M Na_2HPO_4 + 0.1M NaH_2PO_4)	0.1M	7.4 & 7.5	by adjusting the relative proportion of the two solutions
7	Na Phosphate – EDTA	0.1M-0.005 M	7.5	- do -
8	K_2HPO_4 + KH_2PO_4	0.1M	7.5	- do -
9	Na-Phosphate (Na_2HPO_4)	0.1M	7.5 & 7.6	with NaOH after adding buffer additives

Grinding the leaves in the various extraction buffer solutions without any additives resulted in quick browning of the extract, which indicated the presence of high levels of phenolics. Hence, various buffer additives were tried which helped to retard or overcome the interference of phenolics (Table 9). In some treatments, for easy extraction, a pinch of acid washed sand was added while grinding mature leaf.

Different ratios of leaf tissue to extraction buffer was also tried, such as 1:1, 1:1.25, 1:1.5, 1:2, 1:3, 1:4, 1:10 and 4:5 (g/ml) in order to find the best proportion. Different modes of grinding were tried viz., (1) crushing the whole leaf in the buffer and squeezing out the juice for centrifugation and (2) grinding the whole leaf in the buffer and centrifuging the slurry obtained.

To get a clear enzyme extract the sample was centrifuged at -4⁰ C. The speed and time of centrifugation were changed from 5000 rpm to 22,000 rpm and 12 minutes to 30 minutes, to find out the appropriate sedimentation condition, in a Kubota high-speed refrigerated centrifuge.

Table 9. Extraction buffer additives used for enzyme extraction

No	Buffer additive	Concentration	Stage of addition
I. Phenol-complexing agents / phenol oxidase inhibitors			
1	PVP-40 (mg)	50, 75, 100, 130	while grinding
	-do-	250, 500, 1000	to the buffer stock (10ml)
2	PVPP (mg)	20, 40, 50, 200	while grinding
3	BSA (%)	0.1	-do-
4	Cysteine (mg)	20, 50, 55, 60	to the buffer stock (20ml)
II. Antioxidants / Reducing agents			
1	β mercaptoethanol (µl)	1, 2, 25,50	while grinding
2	DTT (mg)	10, 16, 25	to the buffer stock (10ml)
	L-Ascorbic acid (mg)	20, 55, 80, 90, 220, 440	-do-
3	L-Ascorbic acid (M)	0.1	to thebuffer stock (50ml)
4	Citric acid (mg)	100, 260	-do-
5	Sodium metabisufite (mg)	20, 40,50, 75, 100, 150, 200, 250, 270, 370, 400	while grinding or to the buffer stock (50ml)
	Sodium metabisufite (M)	0.1	to the buffer stock (50ml)
6	4% PMSF (ml)	5, 50	while grinding
III. Enzyme-stabilizing osmotica & detergents			
1	Sucrose (mg)	85, 170, 200, 220, 255, 340, 850, 500, 700	while grinding
	-do- (g)	1.25, 1.75	to the buffer stock (10ml)
2	Tryton x-100 (ml)	1, 2, 5, 10	while grinding

In certain samples, the supernatant was transferred into new vials and centrifuged again. Filtering the slurry through Whatman No.1 filter paper under cold conditions was also tried. The supernatant was collected in fresh vials and stored at - 20^0 C or directly loaded into the gel.

3.2.3.2 Electrophoretic media

Polyacrylamide gels were prepared using four different procedures, *viz.*, Type I (Perbal, 1988), Type II (Wendel and Weeden, 1989), Type III (Sebastian, 1995) and Type IV (modified from type III).

a) Reagents for different procedures

Type I

1. Monomer stock (30% T, 2.7% C):

    Acrylamide   - 30g

    N'N',methylene bis acrylamide   -0.8g

    Water to 100 ml (stored at 4^0C away from light)

2. 4 x resolving gel buffer (1.5M Tris - Cl pH 8.8)

    Tris base   - 36.3g

    pH adjusted to 8.8 with HCl.

    Water to 200 ml (stored at 4^0C)

3. 4 x Stacking gel buffer (0.5 M Tris - Cl pH 6.8)

    Tris base   - 3g

    pH adjusted to 6.8 with HCl

    Water to 50ml (stored at 4^0C)

4. Initiator (10% ammonium per sulphate) (freshly prepared)

5. TEMED (N N N' N'-tetramethylethylenediamine)

6. Electrophoresis buffer (0.025M Tris, 0.192M glycine, pH 8.3)

Tris base   - 12.1g

Glycine   -57.6g

Water to 4litre (Stored at 4^0C)

Type II

1. Monomer (30% T, 2.7% C): Same as above

2. 4 x resolving gel (Tris citrate 0.4 M pH 8.6)

Tris   -2.423g

Citric acid   - 0.05g

Water to 50 ml

3.   Initiator (10% ammonium per sulphate)   (freshly prepared)

4.   TEMED

5.   Electrode buffer (0. 3M Sodium Borate pH 8.3)

Boric acid   - 18.55 g

Water to 1 litre

pH adjusted to 8.3 with 5 N NaOH

Type III.

1.Stock solution A (8 x resolving buffer -Tris 3M)

Tris   -36.6g

TEMED   -0.23ml

1N HCl   -40 ml (To adjust the pH to 8. 9)

Water to 100 ml (stored at 4^0C in amber coloured bottles)

2. Stock solution B (30% monomer): Same as above

3. Stock solution C: Ammonium per sulphate   - 25mg

Water to 22.33ml   (Freshly prepared each time)

4. Stock solution D

      Acrylamide   - 18g

      Bis acrylamide   -0.47g

      Water to 100 ml (stored at 4^0C in amber coloured bottle)

5. Electrode buffer stock (0.05M Tris; 0.383M glycine)

      Tris   - 6g

      Glycine   - 28.8g

      Water to 1 litre   pH 8.3 (Diluted 1 volume to 10 volume before use)

Type-IV

1. Stock solution A* (8 x resolving buffer – Tris 0.3M) pH 8.9

      Tris   -3.66g

      TEMED   -0.200ml

      1N HCl   -40 ml (To adjust the pH to 8.9)

      Water to 100ml

2.   Stock solution B: Same as in Type III

3.   Stock solution C: Same as in Type III

4.   Stock solution D: Same as in Type III

5.   Stock solution E: Amonium per sulphate -25mg

                         Water to 5ml   (freshly prepared)

6.   Stock solution F :   (8 x stacking buffer – Tris 0.1M pH 7.00)

      Tris   - 0.61g

      TEMED   - 0.100ml

      IN HCl-1ml (to adjust pH to 7.00)

      Water to 50ml

7.   Electrode buffer stock same as Type III

    Modifications : 1) A* Reduced the molarity of stock Solution A of Type III

                      2) stock solutions E and F were absent in Type III

                      3)Different dilutions of 7th stock were tried: 1:2, 1:4 and 1:5

b) <u>Gel concentration</u>

For GOT, the different concentrations for resolving gel prepared include 7.5%, 8.5%, 9.5%, 10%, 10.5%, 11% and 12% whereas for peroxidase, it was 7.5%, 8%, 8.5%, 10% and 10.5%. The stacking gel concentration was 4 or 4.5% in both cases. The working solutions in the three procedures for a particular gel concentration were prepared as follows.

<u>Type I and II</u>

	Resolving gel (8%)	Stacking gel (4%)
30% monomer	8ml	1.06ml
4 x resolving buffer	7.5ml	-
4x Stacking buffer	-	4.48ml
10% APS	200µl	50µl
TEMED	10µl	10µl
Water	14.3ml	2.4ml
	30ml	8ml

<u>Type III</u>

	Resolving gel (8%)		Stacking gel (4%)	
Solution A	3.75ml	Solution A	1 ml
Solution B	8ml	Solution D	2ml
Solution C	18.25 ml	Solution C	5ml
	30ml		8ml

<u>Type IV</u>

	Resolving gel (8%)		Stacking gel (4%)	
Solution A*	3.75ml	Solution F	1ml
SolutionB	8ml	Solution D	2ml
Solution C	18.25ml	Solution E	5ml
	30ml		8ml

c) <u>Procedure</u>

The gel casting for standardisation of various steps during initial stages was carried out in Hoeffer Mighty small II unit (size – 10cm x 10.5cm) and later in vertical slab gel from Banglore Genei (size – 16 cm x 14cm). The working solution of resolving gel was gently poured between the glass plates kept on casting unit, taking care to avoid any air bubbles, till the meniscus reached about 1 to1.5cm below the bottom plane of the comb. About 1 to 2 ml of cold distilled water was slowly injected to the upper surface of the solution in order to avoid direct contact with oxygen and to get a straight upper meniscus. Then the entire unit was kept in a cold room for polymerisation. After about 30-45 minutes, the layer of water on top of the gel was blotted out and the stacking gel solution was poured with the comb in position and kept for 20 to 30 minutes in cold room. After the polymerisation was over, the unit was kept at 4^0C to cool the gel thoroughly.

3.2.3.3 Buffer system

After fixing the glass plates containing the gel on to the apparatus, any air bubbles that were trapped between the bottom portions of the glass plates were flushed out. Then the lower and the upper tanks of the electrophoresis unit were filled with cold electrophoresis buffer. Various combinations of pH and molarity of stacking and resolving gel buffers as well as electrode buffer were tried as detailed below.

<u>Stacking gel buffer (Tris)</u>	<u>Resolving gel buffer (Tris)</u>	<u>Electrode buffer (Tris- Glycine)</u>
S1 : 0.125M, pH 6.8	R1 : 0.375M, pH 8.9	E1 : 0.025-0.192, pH 8.3
S2 : 0.375M, pH 8.9	R2 : 0.0375M, pH 8.9	E2 : 0.0125-0.096, pH 8.3
S3 : 0.125M, pH 7		E3 : 0.01-0.0768, pH 8.3
S4 : 0.0125M, pH 6.8		E4 : 0.005-0.0383, pH 8.3

From among the 32 combinations, the most promising ones were tried with varying gel concentrations (8.5%, 10%) and extraction buffers (EB-2 and EB-3) as listed out in Table10.

Table 10. Gel buffer and electrode buffer combinations used for enzyme electrophoresis

Treatments	Stacking gel buffer	Resolving gel buffer	Electrode buffer
1.S1R1E1	Tris – 0.125 M pH 6.8 (with HCl)	Tris – 0.375 M pH 8.9 (with HCl)	Tris – 0.025M Glycine - 0.192M pH 8.3
2.S1R1E2	Tris – 0.125 M pH 6.8 (with HCl)	Tris – 0.375 M pH 8.9 (with HCl)	Tris – 0.0125M Glycine - 0.096M pH 8.3
3.S1R1E4	Tris – 0.125 M pH 6.8 (with HCl)	Tris – 0.375 M pH 8.9 (with HCl)	Tris – 0.005M Glycine - 0.0383M pH 8.3
4.S2R1E1	Tris – 0.375 M pH 8.9 (with HCl)	Tris – 0.375 M pH 8.9 (with HCl)	Tris – 0.005 M Glycine -0.0383M pH 8.3
5.S2R1E2	Tris – 0.375 M pH 8.9 (with HCl)	Tris – 0.375 M pH 8.9 (with HCl)	Tris – 0.025 M Glycine – 0.192M pH 8.3
6.S2R1E2	Tris – 0.375 M pH 8.9 (with HCl)	Tris – 0.375 M pH 8.9 (with HCl)	Tris – 0.0125 M Glycine – 0.096M pH 8.3
7.S2R1E3	Tris – 0.375 M pH 8.9 (with HCl)	Tris – 0.375 M pH 8.9 (with HCl)	Tris – 0.01 M Glycine - 0.0768M pH 8.3
8.S3R1E2	Tris –0.125M pH 7 (with HCl)	Tris – 0.375 M pH 8.9 (with HCl)	Tris – 0.0125M Glycine – 0.096M pH 8.3
9.S4R2E2	Tris – 0.0125M pH 7 (with HCl)	Tris- 0.0375 M pH 8.9 (with HCl)	Tris - 0.0125 M Glycine – 0.096 M pH 8.3

3.2.3.4 Sample loading

Loading of the sample into wells was done either by mixing the sample with a sample buffer (which contain the dye bromophenol blue) in different proportions or by adding first the sample buffer containing the dye into the wells followed by the sample. The sample buffers tried were prepared as follows.

1. 4 x stacking gel buffer   - 7ml

    (As in Type I procedure of gel preparation)

    Glycerol   - 3ml

    Bromophenol blue   - 1.2mg

    Water to   - 10ml

2. Tris buffer   - 2.99g

    1N HCl   - 24 ml (to adjust pH to 6.7)

    TEMED   - 0.23 ml

    Water to   - 50 ml

To 10 ml of this solution, 25mg of bromophenol blue was added.

In the first method, the proportions of mixing were 1:1, 1:2, 3:4 and 3:5. For mini gel 10 to 20μl was loaded and for slab gel, the loading volume varied from 40, 50, 60 and 70μl. In the second method ie, without any mixing, the wells were loaded first with 1 to 3μl of the sample buffer and then the sample extract was added in different volumes ranging from 60 to100μl per well.

3.2.3.5 Run conditions

The electrophoresis was carried out with an anodically migrating system. Gels were run both under constant voltage as well as constant amperage conditions. The current was

kept constant at milliamperes 10, 13, 15, 20, 25, 30, 40, 50 and 70 in different runs. Constant voltages were tried at 230, 250, and 300 volts. The run time varied from 1.30 h to 7h, depending upon the current or voltage applied, the gel size and the buffer systems (concentration and molarity) tried as detailed earlier. Throughout the experiment, extreme care was taken to keep the whole system at 4^0C.

3.2.3.6 Enzyme visualisation

After the dye front reached the bottom of the gel, power supply was cut off, gel was removed from the glass plates and put in cold distilled water to wash off the buffer. Then it was transferred to the substrate solution. For the staining of peroxidase, the composition of the substrate solution was as given below.

0.2M Sodium acetate pH 5.6 -100ml

Benzidine   -100mg

3% hydrogen peroxide (in varying volumes of 0.3ml, 0.4ml, 1ml, 1.5ml)

The benzidine was mixed with buffer, boiled, cooled and then the H_2O_2 solution was added. The gel was incubated at 35^0C for 1hour and de-stained in 7% acetic acid. Photographs were taken and the gel stored in polythene wrappers at 4^0c.

Modification of procedures suggested by Shaw and Koen (1968) and Wendel and Weeden (1989) was tried for staining GOT (Table 11). Based on the protocol thus derived, characterisation of TC as well as clonal plants of the four varieties were carried out with respect to the two enzyme systems.

Table 11. Different staining solutions used for GOT after PAGE

No.	Buffer (100ml)	L-aspartic acid (mg)	α-ketoglut-arate (mg)	Pyridoxal5-Phosphate(mg)	FVB* (mg)
1	0.1M KH$_2$PO$_4$ (pH 7 adjusted with 1M NaOH)	532	72	50	400
2	0.1M KH$_2$PO$_4$ + 0.1M K$_2$HPO$_4$ (pH 7)	532	72	50	400
3	0.2M NaH$_2$PO$_4$ + 0.2 Na$_2$HPO$_4$ (pH 7)	532	72	50	400
4	0.1M Na$_2$HPO$_4$ (pH 7 adjusted with NaH$_2$PO$_4$)	532	72	50	400
5	0.1M Na$_2$HPO$_4$ (pH 7 adjusted with NaH$_2$PO$_4$)	532	72	50	400
	pH adjusted with NaOH again after adding the substrates				
6	0.1M Na$_2$HPO$_4$ (pH 7.5)+ PVP 0.5g, EDTA 0.05g	134	36.5	---	100
	pH not adjusted. After adding the substrates, pH was 7.53				
7	0.1M Na$_2$HPO$_4$ (pH 7.5)	300	40	30	140
	pH adjusted with NaOH after adding the substrates				
8	0.1M Na$_2$HPO$_4$ (pH 7.5)	532	55	40	200
	pH adjusted with NaOH after adding the substrates				
9	0.2M Na$_2$HPO$_4$ (pH 7.5)	532	73	50	200
	pH adjusted with NaOH after adding the substrates				
10	0.1M Tris (pH 8.5 adjusted with HCl)	200	100	10	150
11	0.1M Tris (pH 8.5 adjusted with HCl)	532	72	50	400
12	0.1M Tris (pH 7.5 adjusted with HCl)	532	73	-	100

* FVB Fast Violet B salt

3.2.4 RAPD analysis

3.2.4.1 Sample for DNA isolation

Very young tender, pale green leaves (first or third from the tip) were collected on ice from individual plants in each accession grown in green house conditions. The surface was cleaned by wiping with 70 per cent alcohol. And the fresh leaves were ground into a fine powder in liquid nitrogen along with β- mercaptoethanol and $Na_2S_2O_5$ or PVP using ice-cold mortar and pestle in order to prevent browning due to phenol oxidase activity that is very high in black pepper.

3.2.4.2 DNA isolation protocol

From the various protocols reviewed, those suggested by Doyle and Doyle (1987) and Rogers and Bendich (1994) were tried for the extraction of genomic DNA.

<u>Protocol I: (Doyle and Doyle, 1987)</u>

<u>Reagents:</u>   I.Extraction Buffer (4x): 256g sorbitol

48g Tris

7.4g EDTA – sodium salt

80ml sterile Milli Q water

pH adjusted to 7.5 with HCl, water to 1 litre.

(Added 3.8g sodium metabisulfite (0.38%) prior to extraction)

II. Lysis buffer: 200ml 1M Tris – pH 8

200 ml of 250 mM   EDTA

200 ml Steile Milli Q

20 g CTAB, stirred to dissolve

400 ml of 5M NaCl

III. TE buffer: 10 mM Tris - pH 8

1m M EDTA

(Water to 100ml, autoclaved and stored at room temperature)

IV. Isopropanol

V. Chloroform : Isoamyl alcohol mixture (24 : 1) v /v

VI. Sarcosin 5%

VII. Ethanol 100% and 70%

Procedure: one gram of fresh leaf was macerated using a pre-chilled mortar and pestle after freezing with liquid nitrogen in presence of 10mM β-mercaptoehanol. To the powder, 6ml of 1x extraction buffer was added. The homogenate was then poured into a centrifuge tube (50ml) containing 15ml lysis buffer and 2.5 ml sarcosin. The content was mixed well by inversion. Equal volume of chloroform: isoamyl alcohol (24:1) mixture was added to the tube, mixed gently by inversion, and centrifuged at 10,000 rpm for 10 minutes at 4^0C. The upper aqueous phase was pipetted out and transferred to a 50ml centrifuge tube. Two third volume chilled isopropanol was added. The contents were mixed by gently inverting until the DNA precipitated. The DNA was pelletted by centrifuging at 10,000 rpm for 5 minutes at 4^0C. The isopropanol was poured off, drained well and the DNA pellet was washed with 70 per cent alcohol followed by absolute alcohol. The pellet was then air dried and re-suspended in 250μl of TE buffer.

Protocol II (Rogers and Bendich ,1994)

Reagents:

1.CTAB buffer (2x):   2% CTAB (w/v)

100mM Tris (pH8)

20mM EDTA (pH8)

1.4M NaCl

1% PVP

II. 10% CTAB solution: 10% CTAB (w/v)

                                 0.7M NaCl.

III. TE buffer: 10 mM Tris (pH8)

                                 1mM EDTA (pH8)

IV. Chloroform : Isoamyl alcohol (24 : 1) v/v

V. Isopropanol

VI. Ethanol 70% and 100%

Procedure: One gram leaf was ground in liquid nitrogen using a pre-chilled mortar and pestle in the presence of 10 mM β-mercaptoethanol and transferred to a sterile 50ml centrifuge tube containing 5ml hot 2x CTAB extraction buffer. The mixture was then incubated at 65^0 C for 15 to 20 minutes. Chloroform: isoamyl alcohol treatment was carried out as in protocol I. To the aqueous phase obtained after centrifugation, one-tenth volume of 10 per cent CTAB solution was added and mixed gently by inversion. A second treatment with chloroform: isoamyl alcohol mixture was given. The steps involving DNA pelletting and re-suspension were similar to those in protocol I.

Sodium chloride and CTAB separated the polysaccharides efficiently. The pigments and proteins were removed by the double treatment with chloroform-isopropanol mixture. The DNA samples thus isolated and re-suspended in TE buffer were stored at -80^0c in deep freezer. An aliquot of the same from each sample was mixed with tracking dye and loaded in an agarose gel for electrophoresis to check the quality.

The electrophoresis of the DNA sample was carried out using 1.2 percent agarose gel with TAE buffer system. The preparation of the buffer and gel was done as per the procedure given below.

TAE Buffer (50x):	Tris	-242g
	0.5M EDTA	-100ml (pH8)
	Glacial acetic acid	-57.1 ml
	Water to 1 litre	
Tracking dye:	Bromophenol blue	-0.5%
	Glycerol	-40ml
	TAE buffer	-50ml

Agarose was dissolved in TAE buffer by boiling, cooled to 65^0C and after adding 1 to 2µl of ethidium bromide, poured into a horizontal gel casting unit.. After 30 minutes, the solidified gel was placed in the horizontal electrophoresis unit, which was filled with 1x TAE buffer (enough to immerse the gel completely). The DNA sample was mixed with tracking dye in a ratio of 10µl: 5µl and loaded into the well at the cathode side, without overflowing. The electrophoresis was carried out at a constant voltage of 100 V till the dye front reached three fourth distance from the well (about 1.30 to 2h). The gel was then viewed under UV light in a transilluminater and the image was stored in the Alpha Imager gel documentation system.

3. 2.4.3 Purification and quantification of DNA

To remove RNA the DNA sample dissolved in 100 to 250µl TE buffer was incubated at 37^0C in a dry bath for 1h with RNase (one percent) at a proportion of 10µl for 100µl sample. The RNase solution was prepared by dissolving Ribonuclease A (Sigma, USA) in TE buffer at 100^0C for 15 minutes. The solution was then cooled, dispensed into aliquots and kept at -20^0C. The DNA was once again pelletted as before, using a micro centrifuge (10,000 rpm for 3min), resuspended in 50 to 100µl TE. Electrophoresis was

carried out on 1.2 percent agarose gel at a constant voltage of 100 V to test the quality and to find whether there was any shearing during RNase treatment.

The DNA sample thus obtained was checked for purity as well as quantified using a UV-VIS double beam spectrophotometer. The sample was diluted 25 times using sterile water and the absorbance ratio was calculated as Optical Density (OD) at 260 nm / OD at 280 nm. A ratio between 1.8 and 2 was considered to be pure. The quantity was determined from the equation,

$$OD = 1 \text{ at } 260 \text{ nm is equivalent to } 50 \text{ } \mu l \text{ ml}^{-1} \text{ DNA}$$

$$\text{So, quantity of DNA} = (OD \text{ at } 260 \times 50) \text{ } \mu g \text{ ml}^{-1}$$

According to the dilution of DNA, this quantity multiplied with 2.5 gives the approximate quantity of DNA as $\mu g \text{ g}^{-1}$ of leaf tissue. Pure samples with high DNA content were selected for PCR.

3.2.4.4 DNA amplification conditions

The PCR conditions required for effective amplifications in RAPD analysis include appropriate proportions of the components of the reaction mixture and optimum thermal profile. The reaction mixture consisted of the template DNA, assay buffer, $MgCl_2$, Taq DNA polymerase, dNTPs and random decamer primers taken in required quantities and made up to appropriate volume as a master mix for the required number of reactions. The aliquots of this master mix were dispensed into 0.5ml or 0.2ml PCR tubes. The PCR was carried out in a Peltier Thermal Cycler (PTC 200) from MJ Research, USA.

Another important factor, which affect amplification rate is the temperature profile of thermal cycle. One thermal cycle includes three steps *viz.*, the DNA denaturation step at 92^0C, annealing of primer to the template DNA at 37^0C followed by primer extension at

72^0C (Demeke *et al.*, 1992). Since very short (decamer) primers, which have less specificity are used in RAPD, the method is quite sensitive to small variations in the thermal cycle, particularly with respect to the annealing temperature. Most of earlier works suggested 37^0C as the best annealing temperature.

Based on this, the different thermal profiles tried were,

Step	Temperature (^{0}C)	Duration (minutes)	Number of cycles
1. Initial denaturation	93, 94	1, 1.30, 2	1
2. Denaturation	92, 93	1, 2	
3. Annealing	37	1, 1.30	40, 45
4. Extension	72, 74	1, 1.30, 2	
5. Final elongation	72	5, 10	1

Initially four primers were selected at random from OPP, OPF and OPE series of Operon Technology, USA. Using DNA sample from variety Anjana, PCR was carried out. From this, OPP1 was selected and using this primer and the same DNA, other reaction conditions were varied by changing $MgCl_2$ concentration in assay buffer (0.5mM, 1mM, 1.5mM, 2mM); molarity of dNTPs (100μM, 150μM, 250μM) and primer (3pmoles, 4pmoles); the enzyme concentration (0.3, 0.5, 0.6, 0.75 units) and quantity of genomic DNA which acts as the template (25ng, 50ng, 75 ng). Different combinations of these treatments were tried to identify the best one. The final volume of the reaction mixture was 25μl. It was overlaid with equal amount of mineral oil.

After selecting the best reaction mixture and thermal conditions, the 60 random primers (Appendix III) were screened for polymorphism. Those primers, which gave more

than five bands with good intensity and stability, were selected for screening the experimental material.

Using the best reaction mixture, selected primers and the best thermal profile, genomic DNA from the four varieties *viz.*, P_1, P_2, P_4 and Su were subjected to RAPD assay. The PCR products were separated by agarose gel electrophersis (1.5%), visualised and documented as explained under the DNA isolation step.

From the primers used for varietal screening those, which generated the polymorphic bands among varieties, were selected to characterise the clonally propagated as well as TC derived black pepper plants established in the field.

3.2.5 Statistical analysis

3.2.5.1 Biometric observations

The quantitative characters of the clones and TC plants have to be compared within the group in each variety as well as between the TC plants and corresponding parental clones. So, range, mean and variances of these characters were worked out for clones and TC plants of each variety. Bartlett's χ^2 test of homogeneity of variances (Bartlett, 1937) was carried out for all the 22 traits.

If they were found homogeneous, the means and variances of individuals in each group were pooled for that particular character to get overall mean and overall variance for the character. Then the comparison for the character was made between the pooled variance of conventional clones with the pooled variance of TC clones using 'F test'. Accordingly, the means of the two populations were also compared using 't test'. Coefficient of variation was also worked out for better comparison of variability.

If the χ^2 test for homogeneity was found significant for any of the characters, then the pooling of the data was not possible. In such cases, separate comparisons were made for clones and TC plants of each variety for a particular character using 'F test' and 't test' as described earlier.

3.2.5.2 Electrophoretic and RAPD data

The extent of polymorphism between the pairs of varieties as well as between individuals, if any, was worked out as Coefficient of Genetic Distance (GD) as per Demeke *et al.* (1996).

GD   =   1-F   where, F was the degree of similarity which is calculated as,

$$F = \frac{p}{n-a}$$

where,

  p = number of bands present in both varieties

  n = total number of bands

  a = number of bands absent in both varieties

1-F reflects the proportion of the isozymes or DNA fragments (RAPD markers), which distinguish the two varieties compared, relative to the total number of fragments occurring in this pair based on the electrophoretic as well as RAPD data generated. It is also used to have an understanding of the extent of polymorphism between the varieties of black pepper. Thus, if any polymorphism was detected within the TC clones, the genetic distance of the variant from the rest of the monomorphic vines was calculated.

RESULTS ...

4. RESULTS

One of the most crucial concerns of *in vitro* propagation is to retain genomic integrity of the micropropagated plants so that the advantages in the elite mother plant over the natural seedlings or other varieties are maintained. The real applicability of micropropagated plants would ultimately depend upon the comparative field performance with those of conventionally propagated plants. Several strategies can be used to assess the genetic integrity of *in vitro* derived clones. In the present study, the applicability and the reliability of morphological, cytological and molecular markers as indicators of variability in field grown tissue culture derived clones of black pepper was investigated along with the conventionally propagated clones. The data were recorded from 20 plants raised by rooted cuttings (referred as conventional clones -CC) and 40 tissue culture derived plants (referred as TC clones -TCC). (Since two TCC P_1 vines were lost due to quick wilt during 1997, the investigation for TCC group had to be confined to 38 vines). The results of the study are described below.

4.1 MORPHOLOGICAL MARKERS

The morphological markers recorded were of quantitative as well as qualitative nature.

4.1.1 Quantitative characters

The biometric observations recorded include 22 vegetative and 39 reproductive characters.

4.1.1.1 Vegetative characters

Measurements were made from the 22 vegetative characters relating to leaf and stem of the 20 conventional clones and 38 TC derived plants for two consecutive years (10

observations per vine per year). So each trait was represented by the average of these 20

observations per vine and was subjected to statistical analysis.

Range, Mean and Variance were estimated for each of the 58 plants and the

information is provided as Table 12.

Table 12.Variation for vegetative characters in conventional and TC clones

Character	Var-iety	Clone			TC		
		Range	Mean	Variance	Range	Mean	Variance
1. Length of young leaf	P_1	4.75-8.08	6.41	1.94	6.85-8.11	7.23	0.22
	P_2	3.65-6.52	5.45	2.29	6.74-12.9	12.9	9.71
	P_4	3.26-5.47	4.35	0.79	5.74-7.62	6.32	0.45
	Su	4.55-5.55	5.07	0.16	5.09-8.10	7.03	1.14
2. Width of young leaf	P_1	5.12-6.63	5.93	0.68	5.15-6.35	5.10	0.15
	P_2	2.28-3.59	2.98	0.35	4.06-9.25	6.57	4.11
	P_4	2.45-3.79	3.20	0.33	3.76-5.35	4.38	0.36
	Su	2.74-3.18	2.97	0.04	3.85-6.32	5.25	0.56
3. Area of young leaf	P_1	16.44-36.00	25.99	77.42*	24.36-34.61	27.71	10.46*
	P_2	6.50-15.73	11.24	23.80*	18.39-80.19	45.36	567.97*
	P_4	5.37-13.93	9.58	12.24*	14.78-26.99	18.78	21.16*
	Su	8.3811.38	10.15	1.84*	13.17-34.4	25.21	46.82*
4. L/W of young leaf	P_1	0.92-1.22	1.08	0.02	1.19-1.46	1.28	0.01
	P_2	1.58-2.12	1.85	0.05	1.33-1.69	1.52	0.02
	P_4	1.25-1.45	1.35	0.01	1.32-1.69	1.45	0.01
	Su	1.62-1.82	1.71	0.01	1.29-1.44	1.35	0.002
5. Length of petiole (o)	P_1	4.34-6-00	5.17	0.54	4.64-6.05	5.31	0.23
	P_2	3.83-6.12	4.64	0.96	3.47-6.57	4.56	0.85
	P_4	3.67-4.43	4.05	0.11	4.42-5.88	5.03	0.30
	Su	3.71-4.27	4.01	0.09	3.3-5.47	4.36	0.82
6. Length of petiole (p)	P_1	2.05-2.34	2.25	0.02	2.3-2.88	2.47	0.04
	P_2	1.23-2.41	1.82	0.26	1.45-2.75	2.07	0.18
	P_4	1.3-2.54	1.74	0.29	1.66-2.77	2.14	0.14
	Su	2.46-3.25	2.79	0.13	1.85-3.64	2.90	0.32

96

(Table 12 continued)						
7. Length of mature leaf (o)	P_1	11.71-16.16	13.15	4.02	11.34-15	12.41	1.96
	P_2	11.15- 14.08	12.30	1.79	12.31-16.28	14.33	1.51
	P_4	10.27-13.58	11.49	2.26	9.54-13.86	12.14	1.72
	Su	9.98-11.26	10.42	0.32	10.76-14.4	12.53	1.92
8. Width of mature leaf (o)	P_1	9.06-12.51	10.30	2.20	9.01-13	10.32	2.16
	P_2	8.78-11.48	9.57	1.61	9.16-12.94	10.90	1.76
	P_4	7.15-9.11	8.14	0.10	7.37-10.53	9.23	0.83
	Su	6.98-7.95	7.47	0.18	8.60-10.5	9.56	0.54
9. Area of mature leaf (o)	P_1	73.06-135.85	92.32	818.43*	69.08-131.04	87.07	539.90*
	P_2	65.94-108.62	79.75	384.45*	75.77-130.35	105.65	428.30*
	P_4	43.35-83.14	63.38	217.30*	47.25-95.47	75.82	202.22*
	Su	46.81-58.11	52.36	25.39*	62.18-99.38	80.96	210.17*
10. L/W of mature leaf (o)	P_1	1.23-1.34	1.28	0.003	1.16-1.3	1.22	0.004
	P_2	1.23-1.36	1.29	0.003	1.25-1.49	1.35	0.01
	P_4	1.34-1.49	1.41	0.004	1.16-1.43	1.32	0.01
	Su	1.32-1.47	1.40	0.01	1.24-1.44	1.31	0.01
11. Length of mature leaf (p)	P_1	14.95-16.87	16.22	0.77	12.64-17.4	14.95	3.24
	P_2	9.39-13.8	11.31	4.54	12.88-16.32	14.52	2.09
	P_4	11.52-14.92	13.59	2.15	11.74-15.05	13.51	1.39
	Su	6.74-8.63	7.60	0.57	12.50-14.58	13.49	0.60
12. Width of mature leaf (p)	P_1	10.74-12.28	11.64	0.45	8.38-12.0	10.40	1.97
	P_2	5.57-9.23	7.44	2.71	7.85-11.47	9.61	1.24
	P_4	7.7-10.53	9.20	1.28	8.42-10.66	9.34	0.52
	Su	3.85-5.60	4.70	0.51	9-10.43	9.50	0.17
13. Area of mature leaf (p)	P_1	107.90-137.65	127.08	186.67*	71.18-140.31	105.79	713.15*
	P_2	35.15-85.60	57.94	538.38*	69.11-125.79	94.53	385.85*
	P_4	59.61-105.58	84.74	280.06*	67.06-107.81	85.17	182.26*
	Su	17-44-32.48	24.21	36.99*	77.77-102.19	86.23	65.50*
14. L/W of mature leaf (p)	P_1	1.36-1.45	1.4	1.25	1.39-1.51	1.44	0.001
	P_2	1.35-1.69	1.54	0.02	1.37-1.67	1.52	0.01
	P_4	1.42-1.55	1.48	0.003	1.35-1.57	1.45	0.004
	Su	1.54-1.75	1.63	0.01	1.32-1.54	1.42	0.003

(Table 12 continued)						
15. Inter nodal length (o)	P_1	5.57-6.27	5.90	0.08	5.54-8.00	6.33	0.84
	P_2	5.37-6.67	6.09	0.28	5.12-6.90	5.85	0.38
	P_4	4.56-5.49	4.87	0.16	6.27-8.09	6.88	0.39
	Su	5.75-7.10	6.47	0.37	5.5-7.89	6.47	0.64
16.Inter nodal thickness (o)	P_1	5.57-6.27	5.90	0.08	5.54-8.00	6.33	0.84
	P_2	1.26-2.70	1.96	0.53	1.71-2.90	2.33	0.13
	P_4	1.17-1.89	1.55	0.10	1.59-2.67	1.94	0.12
	Su	1.31-1.48	1.43	0.01	1.69-2.57	2.13	0.11
17.Inter nodal length (p)	P_1	3.5-6.26	4.75	1.60	4.70-6.70	6.18	0.32
	P_2	5.05-7.23	5.97	0.79	4.65-7.47	5.76	0.95
	P_4	4.69-5.16	4.48	0.25	5.27-7.07	6.18	0.32
18.Inter nodal thickness (p)	P_1	1.43-2.16	1.76	0.13	1.31-1.99	1.53	0.05
	P_2	0.96-1.51	1.25	0.08	1.4-2.06	1.70	0.05
	P_4	1.10-1.45	1.30	0.02	1.16-1.46	1.30	0.01
	Su	1.0-1.21	1.09	0.01	1.39-1.65	1.53	0.01
19. Nodal thickness (o)	P_1	2.58-4.58	3.364	0.80	2.32-3.57	3.04	0.19
	P_2	2.06-3.41	2.80	0.52	1.53-4.46	3.01	0.92
	P_4	1.91-3.10	2.47	0.22	1.96-3.54	2.62	0.29
	Su	1.28-2.30	1.83	0.19	1.61-4.01	2.83	0.46
20. Nodal thickness (p)	P_1	1.68-2.73	2.10	0.29	1.79-3.010	2.32	0.23
	P_2	1.06-2.30	1.77	0.38	0.92-2.58	1.85	0.45
	P_4	1.29-1.72	1.51	0.03	1.34-3.06	2.05	0.44
	Su	0.95-1.29	1.13	0.02	1.89-3.09	2.49	0.19
21. No. of branches	P_1	6.00-27.50	14	95.94*	7.0-18.0	12.38	20.47*
	P_2	2.00-31.00	12.30	170.56*	3.5-28	12.40	93.01*
	P_4	6.00-21.00	13.20	40.88*	7.0-32.0	14.85	78.40*
	Su	6.50-12.00	9.50	6.41*	7.0-21.0	11.8	18.35*
22.Angle of insertion	P_1	50.2-73.5	61.50	96.96*	56.8-76.5	67.29	46.71*
	P_2	38.8-55.2	44.2	60.10*	41.4-77.5	59.24	167.6*
	P_4	43.3-76.8	66.94	237.86*	52.7-82	67.23	141.01*
	Su	56.9-77	65.04	71.27*	57.3-82	70.69	78.16*

* χ^2 significant at 0.05%,   o: orthotrope, p: plageotrope

a) <u>Intra group variability in conventional and TC clones</u>

Bartlett's χ^2 test for homogeneity of variances within the 20 CC group as well as within 38 TCC group helped to partition 22 vegetative traits into 2 groups as given below.

Classification chart

Vegetative traits in both CC and TCC (22 no.)

Within group variance homogenous	Within group variance heterogeneous
1. Length of young leaf	1.Area of young leaf
2. Width of young leaf	2.Area of mature leaf (o)
3. L/W ratio of young leaf	3. Area of mature leaf (p)
4. Length of petiole (o)	4.No. of branches
5.Length of petiole (p)	5.Angle of insertion of branches
6. Length of mature leaf (o)	
7.Width of mature leaf (o)	(o – orthotrope, p – plageotrope)
8. L/W ratio of mature leaf (o)	
9. Length of mature leaf (p)	
10. Width of mature leaf (p)	
11. L/W ratio of mature leaf (p)	
12. Internodal length (o)	
13. Internodal thickness (o)	
14. Internodal length (p)	
15. Internodal thickness (p)	
16. Nodal thickness (o)	
17. Nodal thickness (p)	

For these 17 characters, all the CC could be considered as a single group irrespective of variety and so for each trait the mean values were pooled over all the 20 CC. Similarly their variances were also pooled for each of the 17 traits. The same procedure was followed in the TCC group also. Then for each character the comparison was made between the pooled values of CC group with the pooled values of TCC group. For the remaining five characters, the CC group differed significantly with respect to the four varieties and hence, these could not be pooled. Similar was the case for TCC group also. Hence the comparison

between the two groups was made with variances and means of individual varieties separately for these characters.

b) Inter clonal comparison (CC vs. TCC) of mean values of traits

For the 17 characters for which χ^2 test showed homogeneity, factorial CRD with non-orthogonal data revealed that for these traits the comparison between the CC group and TCC group could be carried out irrespective of the varieties. The pooled means of the two groups were compared with respect to these 17 traits using 't' test and the mean values of TCC group was found significantly higher with respect to 11 out of 17 traits (Table 13). For the five characters for which within group variability was heterogeneous, this comparison was done separately for each variety.

Table 13. Mean, variance and coefficient of variation of pooled data in conventional and TC clones

	Character	Mean		Variance		C.V. (%)	
		CC	TCC	CC	TCC	CC	TCC
1	Length of young leaf	5.32	7.59*	1.45	3.34	22.63	24.08
2	Width of young leaf	3.77	5.46*	1.88	1.80	36.37	24.57
3	L/W ratio	1.50	1.41	0.11	0.02*	22.11	10.03
4	Length of petiole (o)	4.47	4.79	0.53	0.61	16.29	16.31
5	Length of petiole (p)	2.15	2.39	0.30	0.27	25.48	21.74
6	Length of mature leaf (o)	11.84	12.87*	2.48	2.26	13.30	11.68
7	Width of mature leaf (o)	8.87	9.99*	2.12	1.50	16.42	12.26
8	L/W (o)	1.35	1.30	0.01	0.01	7.41	7.69
9	Length of mature leaf (p)	12.18	14.07*	11.90	1.84*	28.32	9.64
10	Width of mature leaf (p)	8.25	9.68*	7.58	0.91	33.37	9.85
11	L/W (p)	1.51	1.46	0.014	0.005	7.84	4.84
12	Inter nodal length (o)	5.83	6.39*	0.52	0.59	12.37	42.02
13	Inter nodal thickness (o)	1.85	2.15*	0.35	0.15	31.98	18.10
14	Inter nodal length (p)	5.21	5.90*	0.96	0.60	18.81	13.13
15	Inter nodal thickness (p)	1.35	1.51*	0.11	0.05	24.57	14.81
16	Nodal thickness (o)	2.61	2.87	0.62	0.43	30.17	22.85
17	Nodal thickness (p)	1.63	2.17*	0.25	0.34	30.67	26.87

O: orthotrope   CC –Conventional clones
p: plageotrope   TCC- Tissue culture derived clones
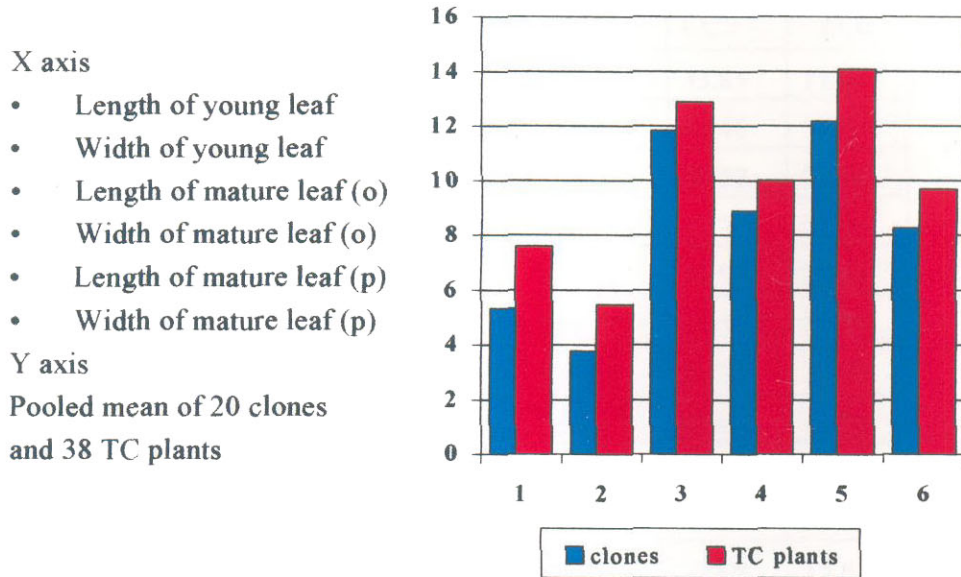* Significant at 5% level   C.V.- Coefficient of variation

(i) Leaf characters

The leaf lamina characters *viz.* length, width, L/W ratio and area were recorded for young leaf and mature leaf of orthotrope as well as plageotrope. Length and width of the lamina were measured and their ratio and area worked out for each vine. The pooled means of length and width of lamina in all the three types of leaves were found significantly higher in the case of TC plants compared to clones (Fig 1). When the length/width ratio was worked out, the mean ratio in young as well as mature leaves of ortho and plageotropes were on par between CC group and TCC group. For leaf area, the tests for comparing the means and variances of CC and TCC were done separately for each variety (Table 14).

The results revealed that, in general, the mean leaf area for all three types of leaves were higher in TC plants compared to that of clones, but the differences were significant in all the three types only for P_2. In P_1, both clones and TC plants were having almost uniform leaf area in all types of leaves. But in P_4 and Subhakara, the pattern was slightly different. In P_4, the mean leaf areas of mature leaves (ortho and plageotropes) were somewhat identical in clones and TC plants but were significantly higher in young leaves of TC plants. In Subhakara, the young leaves and mature leaves of plageotropes of TC plants showed significantly higher leaf area whereas in mature leaves of orthotropes, it was on par with conventional clones.

The length of petiole showed homogeneity in variance between the four varieties and hence, for this character, the conventional clones of all varieties were pooled to be considered as a single population. The same procedure was adopted for the TC pants also (Table 12). With respect to this character, the pooled mean values of TC plants were on par with that of conventional clones (Table 13).

Fig.1 Comparison of leaf lamina
(Clones vs. TC plants)

X axis
- Length of young leaf
- Width of young leaf
- Length of mature leaf (o)
- Width of mature leaf (o)
- Length of mature leaf (p)
- Width of mature leaf (p)

Y axis

Pooled mean of 20 clones
and 38 TC plants

clones ■ TC plants

Fig 2. Comparison of stem
(clones vs. TC plants)

X axis
1. Internodal length (o)
2. Internodal thickness (o)
3. Internodal length (p)
4. Internodal thickness (p)
5. Nodal thickness

Y axis

Pooled mean

clones ■ TC plants
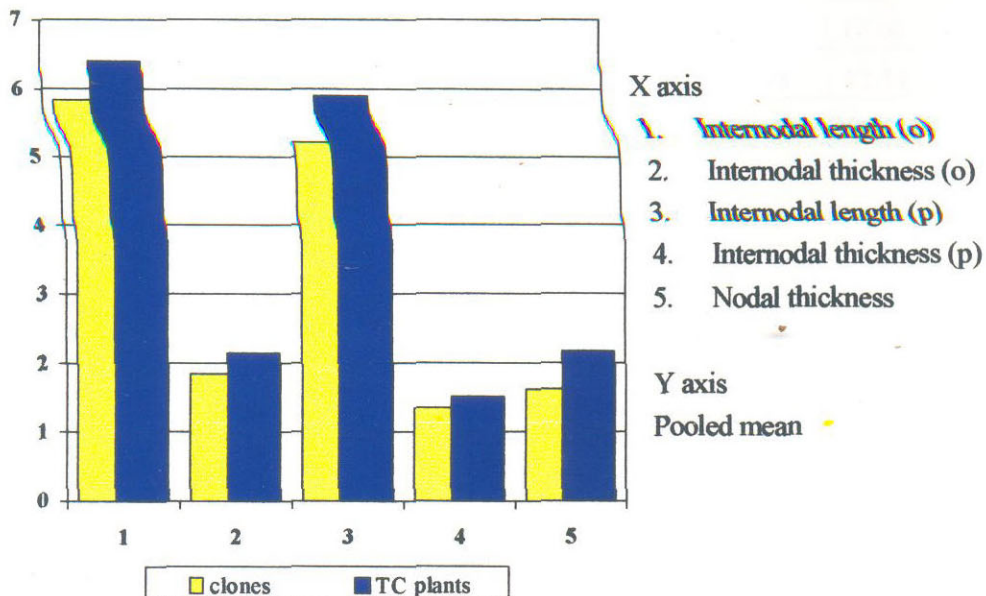
Table 14. Mean, variance and coefficient of variation of heterogeneous group of

traits in conventional and TC clones of black pepper

Character	Var	Mean		Variance		CV (%)	
		CC	TCC	CC	TCC	CC	TCC
1. Area of young leaf	P_1	25.99	27.71	10.46	77.42	33.85	11.67
	P_2	11.24	45.36*	567.97	23.80	43.40	52.54
	P_4	9.58	18.78*	21.16	12.24	36.52	24.50
	Su	10.15	25.21*	46.82	1.84	13.36	27.14
2. Area of mature leaf (O)	P_1	92.32	87.07	539.90	818.43	30.99	26.69
	P_2	79.75	105.65*	428.30	384.45	24.59	19.59
	P_4	63.38	75.82	202.22	217.30	23.26	18.75
	Su	52.36	80.96	210.17	25.39	9.60	17.91
3. Area of mature leaf (P)	P_1	127.08	105.79	713.15	186.67	10.75	25.24
	P_2	57.94	94.53*	385.85	538.38	40.05	20.78
	P_4	84.74	85.17	182.26	280.06	22.08	15.85
	Su	24.21	86.23*	65.50	36.99	26.12	9.39
4.Number of branches	P_1	14	12.38	20.47	95.94	69.96	36.55
	P_2	12.30	12.40	93.01	170.56	106.19	77.78
	P_4	13.20	14.85	78.40	40.88	48.44	59.63
	Su	9.50	11.8	18.35	6.41	26.65	36.3
5.Angle of insertion	P_1	61.50	67.29	46.71	96.96	16.01	10.16
	P_2	44.2	59.24*	167.6	60.10	17.45	21.85
	P_4	66.94	67.23	141.01	237.86	23.04	17.66
	Su	65.04	70.69	78.16	71.27	12.98	12.51

O: orthotrope   CC –Conventional clones

P: plageotrope   TCC- Tissue culture derived clones

* Significant at 5% level   C.V- Coefficient of variation

(ii) Stem characters

For the entire stem characters recorded, *viz.*, internodal length and thickness as well as nodal thickness in both ortho and plageotropes, the pooled means were compared between CC group and TCC group based on χ^2 test (Table 12). In all the characters relating

to internode and nodal thickness of plageotrope, the mean values were significantly greater in TCC group compared to CC group (Fig 2). However, for nodal thickness of orthotrope, the means were on par.

For the traits, number and angle of insertion of plageotropic branches, the results of χ^2 test showed that pooling of data over the varieties was not possible (Table 12). Hence, the means of CC group and TCC group were compared separately for each of the four varieties. The mean values for number of plageotropic branches were almost same in both CC group and TCC group. It was lowest (9.50) in CC Su and highest in TCC P_4 (14.85).

c) Inter clonal comparison of coefficient of variation of traits (CC vs. TCC)

In order to compare the extent of variability between conventional clones and TC clones, coefficient of variation (CV) was worked out. For the 17 homogeneous characters as per the classification chart, CV was computed using the pooled values of mean and variances (Table 13) and for the remaining five heterogeneous characters, it was done variety-wise (Table 14).

For length and width of young leaves and mature leaves of ortho and plageotropes, the CV was found higher in conventional clones compared to TC clones excepting length of young leaf (Table 13). For L/W ratio CV was on par only in mature leaves of orthotropes whereas in young leaves and mature leaves of plageotropes, it was higher in conventional clones.

The pattern of variability in leaf area was different in different varieties. For young leaf area conventional clones showed higher CV in P_1 and P_4 whereas it was greater in TC plants of P_2 and Su (Table 14). The area of mature leaves of orthotropes in CC group showed higher CV in P_1, P_2, and P_4 whereas in Su, TC plants had higher CV. At the same

time, the area of mature leaves of plageotropes showed higher CV in conventional clones of P_2, P_4 and Su but in P_1, TC plants had higher CV.

With respect to stem characters like length and thickness of internode as well as thickness of node of ortho and plageotrope, the conventional clones were having greater CV (Table 13). The CV for number of branches was higher in TC clones of Su only whereas for angle of insertion it was higher in TC clones of P_4 only (Table 14).

Thus, the relative distribution of variability was found higher in conventional clones with respect to the group of 17 traits (Fig.3). For the other group of five traits, though it differed with variety, in general, P_1, P_2 and P_4 showed higher variability in conventional clones whereas in Su, for three traits, it was higher in TC clones; for one trait, higher in conventional clones and for the remaining one trait, both groups were on par (Fig. 4).

4.1.1.2 Reproductive characters

Since the vines were at second and third year of planting at the time of recording the observations, all the vines had not started flowering. Even in many of the vines, which had flowered, the number of spikes produced was too low to record the various observations especially during the second year of growth. Hence, observations on reproductive characters were recorded only for those vines, which yielded more than 25 spikes. The vines thus selected for recording reproductive characters and the average observations recorded per vine are given in Table 15 and 16. Plates 2a, 2b, 2c, 2d, 2e, 2f and 2g represent the spike and berry characters of some of the TC vines.

a) Intra group variability in flowering habits of conventional and TC clones

In general, it was found that 53 percent of vines started flowering in TC derived plants whereas in conventional clones only 45 per cent started flowering. Only three conventional clones and eight TC clones produced more than 25 spikes.

Fig 3. Relative distribution of variability
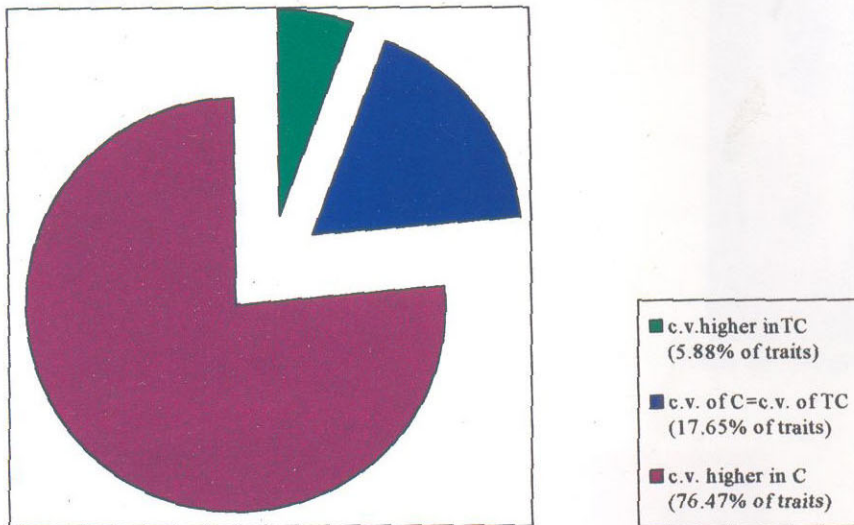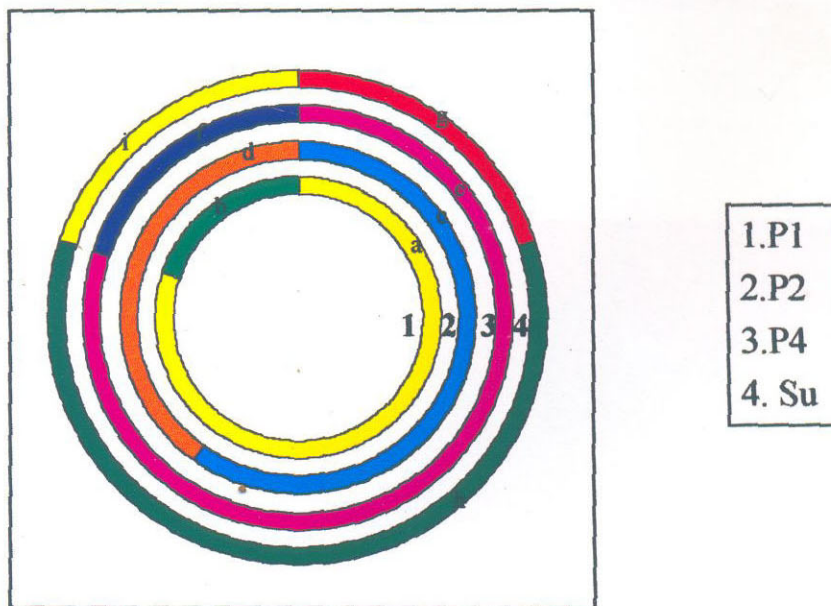(for the 17 homogeneous characters) (clones vs. TC plant)

- c.v.higher inTC
  (5.88% of traits)
- c.v. of C=c.v. of TC
  (17.65% of traits)
- c.v. higher in C
  (76.47% of traits)

Fig 4. Extent of variability
in different varieties (clones vs. TC plants)

1.P1
2.P2
3.P4
4. Su

a ,c,e,g - C.V higher in clones   b,d,f,h, - C.V higher in T.C.   i - C.V of clone = CV of TC
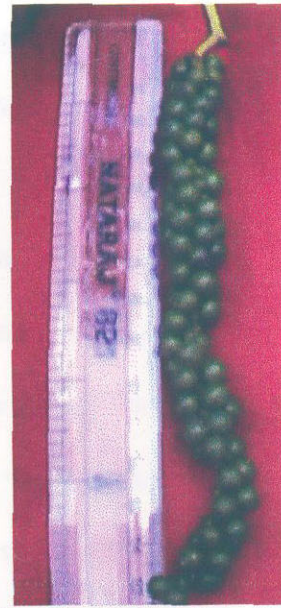
Plate 2a : Spikes of TCP₁

Plate 2b : Single spike of TCP₁

Plate 2c : Spikes of TCP₂
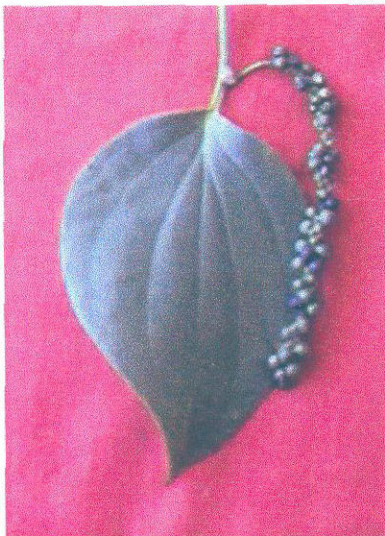
Plate 2d : Spike of TCSu

Plate 2e : Spikes of TCP₄

b) Inter clonal variability in flowering habits of conventional and TC clones

The time of first flowering was found generally earlier in TC plants than in conventional clones.

In variety P_1, only one conventional clone and one TC plant had produced more than 25 spikes per vine. CC P_1-1 had 45 spikes whereas TCC P_1-1 produced 56 spikes. The rate of production of spikes per branch was almost same in both. The average length of spikes in each vine was slightly higher in TC plant. But both of these vines were in the juvenile stage and had not attained the full potential for producing long spikes (18.14cm), which was characteristic of the variety. The floral characters like stamen and stigmatic lobe numbers were identical in both (Table 15).

Table 15. Floral characters in conventional and TC clones

Character	P_1		P_2		P_4				Su		
	CC1	TCC1	TCC1	TCC8	CC3	CC4	TCC1	TCC3	TCC1	TCC5	TCC6
Number of spikes per branch	2.8	2.6	3.5	3.7	3.1	2.7	2.6	2.2	2.8	1.6	2.8
Length of spike (cm)	14.1	16.9	11.3	12.6	11.7	9.4	9.9	10.5	8.6	8.2	9.9
Stamen number	2	2	2	2	2	2	2	2	2	2	2
Stigmatic lobe no.	4	5	4	4	3	3	4	3	3	3	3

Regarding of the fruit characters also, the performance of clones and TC plants were comparable (Table 16). The conventionally propagated vine showed slightly higher values of the berry characters in general except for 100-berry volume. Quality of berries as assessed from the chemical composition viz. oleoresin content was better in TC plant whereas essential oil and piperine contents were slightly higher in conventionally propagated clones.

Table 16. Fruit and seed characters in conventional and TC clones

Character	P_1		P_2		P_4				Su		
	CC1	TCC1	TCC1	TCC8	CC3	CC4	TCC1	TCC3	TCC1	TCC5	TCC6
Fruit Spike no. /vine	45	56	71	66	51	56	115	124	26	29	56
Well developed berries / spike (No)	41	31	44	49	30	34	36	63	46	46	60
Under developed berries / spike (No)	6	4	7	10	5	6	3	4	7	3	2
100 fresh berry weight (g)	17.5	18.6	12.3	12.8	11.5	11.2	11.9	12.2	13.8	15.3	14.9
100 dry berry weight (g)	5.8	5.6	4	4.1	4.1	4.2	4.8	4.7	4.9	5.2	5
Ratio (fresh/dry)	3.0	3.3	3.1	3.1	2.8	2.7	2.5	2.6	2.8	2.9	3.0
100 berry volume (cc) (fresh)	17	18	11.9	12	11	11.1	11.5	12	13	15	14
Wt. of 25 spikes (g)	158	116	173	142	113	138	130	153	142	155	248
Wt. of berries in 25 spikes (g)	133	94	150	125	103	122	120	143	104.8	125	224
Ratio (spike/berry)	1.2	1.2	1.2	1.1	1.1	1.1	1.1	1.1	1.4	1.2	1.1
Seed 100 dry seed wt (g)	4	4	3.5	3.7	4	3.9	4.2	4.1	4.5	4.8	4.8
Volume of 100 dry seeds (cc)	4	4	3	3.5	3.8	3	3.5	3.5	4	4	4
Green yield / vine (g)	260	272	425	360	230	310	550	707	105	145	502
Quality Oleoresin (%)	9.8	10.2	10.8	9.4	9.2	9	8.8	9.1	12	11.8	12.5
Oil (%)	3.4	3.2	2	2.9	4.1	4.2	4.5	4.1	5	4.5	4.3
Piperine (%)	4.8	4.5	6.1	5.8	5	5.4	5.1	5.2	3.8	4	3.7

In variety P_2 only two TC plants had more than 25 spikes, even though four conventional clones and six TC vines started flowering. Hence, comparison with conventionally propagated clones was not possible. In general, both TC plants showed uniformity in productive characters (Table 15 and Table 16). TCC P_2 -1 produced 71 spikes whereas TCC P_2-8 produced 66 spikes. The latter was comparatively better performing for most of the flower and fruit characters except for weight of 25 spikes and weight of the berries in them. Total yield was higher in TCC P_2 -1 and quality of berries was also better.

In P_4, two clones and two TC plants satisfied the criteria (more than 25spikes per vine) for recording data on reproductive characters *viz.*, CC P_4 – 3, CC P_4 – 4, TCC P_4 –1 and TCC P_4 – 3. The floral characters were more or less similar in CC and TCC plants (Table 15) whereas for fruit characters, the TC plants performed better. The total number of spikes produced by these vines were, 51, 56, 115 and 124 respectively (Table 16). The green spike yield as well as green berry yield per vine in TCC were almost double that of conventional clones. Quality of berries was comparable in both groups.

In Subhakara only one conventionally propagated vine started flowering and it did not have more than 25 spikes per vine. Three TC plants *viz.*, TCC Su_1, TCC Su_5 and TCC Su_6 produced 26, 29 and 56 spikes per vine respectively. The spike length was almost uniform in all the three TC vines (average length 9.2cm). Compared to the typical varietal character of Subhakara (7.7cm) as reported earlier, the spikes were longer in TC vines. The 100-berry weight and volume was generally high in all the three vines as well as the weight of spikes and weight of berries in them. The total yield varied between the three vines ranging from 104.8g to 501.4g (Table 16). The quality of berries with respect to oil and oleoresin was better compared to other varieties though piperine content was less.

4.1.2 Qualitative characters

<u>a) Intra group variability</u>

Qualitative parameters did not differ significantly within conventional clones or within TC clones in each variety. Even though slight but negligible vine to vine variation was observable with respect to certain characters such as the major type of leaf shape (Plate 3a, 3b), number of primary veins per leaf etc., they were not sufficiently significant to distinguish the vines within a variety from each other.

<u>b) Inter clonal variability (CC vs. TCC)</u>

With respect to qualitative traits, the conventional clones and TC clones of each variety showed uniformity. Here also vine-to-vine variation was observable but insignificant in varieties P_1, P_2 and P_4. But in Subhakara the leaf size and shape as well as the general growth habit of TC vines were found different from conventional clones. In the former the leaves were larger and almost ovate in shape with cuneate or slightly round base, whereas in the latter, the leaves were typically smaller with cuneate base. The general appearance of the TC vines also differed from the varietal character, as they resembled variety P_4 to a greater extent than variety Subhakara on visual assessment. The leaf shape, size, spike characters (Plate 4a) and overall appearance of vine (Plate 4b) of TC Su differed from CCSu. The conventional clones of Su resembled closely its parent, the local variety Karimunda (plates 4c, 4d).

4.1.3 Varietal polymorphism

Based on the observations recorded on biometric traits for the conventional clones and TC clones of the four varieties and their statistical analysis and also based on visual

Plate 3a. Intragroup variability in leaf shape of P_1

Plate 3b. Intragroup variability in leaf shape of P_2

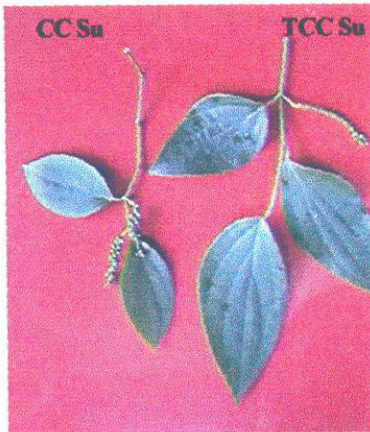Inter clonal variability of CC Su & TCC Su

Plate 4a

Plate 4b : Spikes of Karimunda

Plate 4c : Vine of TC Su

Plate 4d : Vine of Karimunda

assessment of qualitative data, certain morphological markers were identified which exhibited inter varietal polymorphism in black pepper.

4.1.3.1 Vegetative traits

Among the various vegetative biometric characters used for evaluation, the following five traits exhibited significant difference in variability between varieties.

1. Area of young leaf
2. Area of mature orthotropic leaf
3. Area of mature plageotropic leaf
4. Number of lateral branches
5. Direction of growth of lateral branches.

4.1.3.2 Reproductive traits

The reproductive characters, though not subjected to statistical analysis, showing varietal differences were the following.

1. Length of spike
2. Well developed berries per spike
3. Under developed berries per spike
4. Hundred berry weight
5. Hundred berry volume
6. Ratio of berry weight to volume
7. Chemical constituents

4.1.3.3 Qualitative traits

The qualitative characters were useful for testing inter varietal variability rather than inter clonal variability. The characters, which showed variation between varieties, are described below.

The shape, tip and base of young as well as mature leaves showed marked difference among varieties (Plate 5a). In P_1, the leaves were cordate with acuminate tip and cordate base. In P_2 they were ovate with acuminate tip and round base and in P_4, shape was mainly ovate with slightly elliptic nature, tip was acuminate and base round. In Subhakara the leaves were elliptic with cuneate base and acuminate tip in conventional clones.

P_1 and P_2 showed creamy green to pale green tender leaves with green leaf sheath. For P_4 and Subhakara, the tender leaves showed a purple tinge, lighter on upper surface but darker on lower surface. The petioles of these tender leaves as well as the leaf sheath were intensely purple coloured in both (Plate 5b).

The tender stem in P_1 and P_2 was without any anthocyanin pigmentation (Plate 5b). The mature stem in P_1 and P_2 was thick, fleshy and light to dark green when immature (Plate 5c), turning stout and dark green with brownish patches when older. Also, the adventitious roots produced from nodes of orthotrope were short, thick and strongly clinging to the standard (Plate 5d).

In P_4 and Subhakara, on the other hand, the tender stem was purple with anthocyanin pigments, elongated and slender (Plate 5b). When mature, brownish colour appears but the stem was not as stout as in the other group (Plate 5c). Also, the adventitious roots were longer, branched and the clinging ability varies from poor to strong (Plate 5e).

Fruit is bold and spherical in P_1 with light green colour when immature, turning red on maturity. In P_2, the berry was slightly smaller than P_1. In P_4 and Subhakara, the berries were somewhat oblong to spherical in shape, smaller sized and light green when immature turning reddish yellow on maturity. Also it was found that, compared to P_1 and P_2, P_4 and Subhakara were late maturing by about one week. The general pattern observed is provided as a descriptor for the four varieties.

Plate 5a. Varietal polymorphism in leaf shape (Ovate, elliptic & cordate)

Plate 5b. Varietal polymorphism
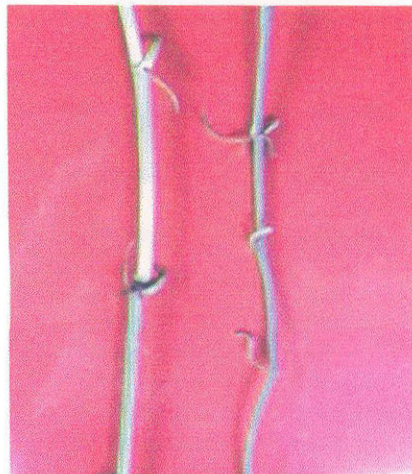in anthocyanin pigmentation (P_1 & P_4)

Plate 5c. Varietal polymorphism
in stem thickness (P_1 & P_4)

Varietal polymorphism in adventitious roots (P_1 & P_4)

Plate 5d

Plate 5e

Descriptor based on qualitative characters of TC clones of black pepper varieties

	Character		P_4	Su	P_1	P_2
I. Young leaf	1.colour	a. upper	Creamy with purple tinge	Creamy with purple tinge	Pale green	Pale green
		b. lower	Pale green with purple tinge	Pale green with purple tinge	Creamy green	Creamy green
	2.shape		Ovate	Elliptic/ovate	Cordate-ovate	Ovate
	3.tip		Acuminate	Acuminate	Acuminate	Acuminate
	4.base		Round	Cuneate/round	Cordate round	Round – oblique
	5.leaf sheath		Dark purple	Dark purple	Green	Green
	6.stipule	a. colour	Purple	Purple	Green	Green
		b. shape	Elongate	Elongate	Elongate	Elongate
		c. position	Adnate to petiole	Adnate to petiole	Adnate to petiole	Adnate to petiole
		d. texture	Membranous	Membranous	Membranous	Membranous
		e. hairiness	Absent	Absent	Absent	Absent
II. Mature leaf	1.petiole	a. hairiness	Absent	Absent	Absent	Absent
		b. shape	Rod shaped with groove on upper surface	Rod shaped with groove on upper surface	Rod shaped with groove on upper surface	Rod shaped with groove on upper surface
	2.lamina	a. shape	Ovate - elliptic	Elliptic/Ovate	Cordate	Ovate
		b. tip	Acuminate	Acuminate	Acuminate	Acuminate
		c. base	Round	Cuneate/round	Cordate	Round
		d. texture	Slightly-coriaceous	Slightly-coriaceous	Coriaceous	Coriaceous
		e. colour	Darkgreen	Darkgreen	Darkgreen	Darkgreen
		f. veins	5-7	5-7	5-7	5-7
		g. hairiness	Absent	Absent	Absent	Absent
		h. phyllotaxy	Alternate	Alternate	Alternate	Alternate
III. Stem	1.shape		Slender, elongate	Slender, elongate	Thick rod shaped	Thick rod shaped
	2.colour	a. tender	Purple	Purple	Light green	Light green
		b. mature	Dark green with brown patches	Dark green with brown patches	Dark green with brown patches	Dark green with brown patches
	3.laterals	direction	Horizontal	Horizontal	Semi erect to-horizontal	Semi erect
	4.node	a. appearance	Swollen	Swollen	Swollen	Swollen
		b. colour	Green/brown	Green/brown	Green/brown	Green/brown
	5.adventitious roots	a. appearance	Long, branched	Long, weak or branched	Short, thick	Short, thick
		b. number c. clinging ability	3 – 12 Poor / Strong	Very few /5-15 Poor /strong	10 – 15 Strong	5 –11, branched Strong
IV. Flower	1.time		June-July	June-July	June-July	June-July
	2.sex		Hermaphrodite-with few female-flowers	Hermaphrodite-with few female-flowers	Hermaphrodite	Hermaphrodite-with few female-flowers
	3.orientation		Pendulous	Pendulous	Pendulous	Pendulous
	4.spike texture		Glabrous	Glabrous	Glabrous	Glabrous
	5.bract		Adnate to rachis	Adnate to rachis	Adnate to rachis	Adnate to rachis
V. Fruit	1.shape		Oblong-spherical	Oblong-spherical	Spherical	Spherical
	2.colour	a. young	Light green	Light green	Light green	Light green
		b. mature	Dark-green-turning-yellowish red	Darkgreen-turning-yellowish red	Dark green with reddish tinch	Dark green
	3.maturity time		190-200 days	185-200 days	180-190 days	180-190 days

4.2 CYTOLOGICAL ANALYSIS

Cytological investigations in the genus *Piper* have been mainly confined to the determination of chromosome number of different species; may be because of the occurrence of large number of comparatively smaller chromosomes. *Piper nigrum* is believed to be a diploid of polyploid origin (2n = 4x) in which varying number of somatic chromosomes have been reported. For determination of somatic chromosome number, root tip squash technique is accepted as the best procedure in plants. Due to the specific features of the black pepper chromosomes, the preparation of slides by root-tip squash technique for cytological studies requires refinement of each and every step of the general procedure. In the present study, the technique is standardised as follows.

4.2.1 Standardisation of the protocol

4.2.1.1 Season of collection of sample

Root tip samples were collected from freshly rooted cuttings throughout the year to identify the best season for collection. It was found that good quality slides with dark and well spread chromosomes were obtained mainly during the period from June to July.

4.2.1.2 Age of cutting

Cuttings planted in potting mixture (1:1:1) after 1BA treatment (Pillai *et al.*, 1985) were used for collection of roots. It was found that fleshy, short, white roots were obtained when the cutting was about 3 to 4 weeks old. Before this stage, the number of roots would be very low. If it exceeds this stage considerably the roots become narrow, fibrous and elongated. It was observed that even though these had actively dividing root tips, the chances of obtaining good cells were less. Also, it was found that climbing roots were not as good as normal soil grown roots.

4.2.1.3 Time of Collection

Roots were collected at an interval of 1 hour starting from 10.30 am to 3.30 pm. The best time for collection based on the stage of cell division and spread of chromosomes was found to be between 1.30 to 2.30 pm. If collected earlier, the chromosomes were not clearly visible and if later the metaphase stage progressed further so that all chromosomes were grouped near the equatorial region and many cells were in anaphase or telophase stage (Plate 6a and 6b).

4.2.1.4 Protocol for slide preparation

The best concentration of 8-hydroxy quinoline for pre-treatment was found to be 0.05 percent for a period of 2 hours. Among the different fixatives tried, Carnoy's fixative with a composition of glacial acetic acid: chloroform: ethyl alcohol in 1:3:6 proportion was found ideal. Among the different temperatures and durations of hydrolysis tried with 1N HCl, immersing the glass vials containing roots along with 1N HCl in a water bath heated to 60°C for 15 minutes was found optimum.

The hydrolysed roots after thorough washing were stained with three different stains, among which 0.5 per cent aceto-orcein was the most efficient to stain the chromosomes properly. Staining of chromosomes with aceto-carmine produced less intensity of colour and so clear photographs were not obtained. Fuelgen stain, though specific for chromosome staining, was found to be very sensitive to the mode of preparation and a slight change resulted in poor result. Putting the roots in cold aceto-orcein for 1 to 2 hours gave sufficient intensity and specificity to chromosomes. The slides for microscopic observations were prepared using the root squash technique (Armstrong, 1995).

4.2.2 Somatic chromosome number

Using the protocol, 5 to 10 slides were prepared for each of the TC derived and conventional clones of the four varieties. The chromosomes were observed under a Biomed microscope at a magnification of 1000 times. In all the samples, there obtained one or two slides in which there were a large number of, enlarged, round and well separated cells at metaphase stage with darkly stained, well-spread chromosomes. There was not any variation in the chromosome number of tissue culture derived clones of any of the variety. In all the samples the chromosome number was found to be 2n = 52 without any variation (Plates 6c, 6d, 6e and 6f).

4.3. ISOZYME ANALYSIS

All the experimental vines were subjected to isozyme analysis with respect to two enzyme systems *viz.,* PRX and GOT.

4.3.1 Standardisation of protocol

Pepper, being a high phenol containing plant, required laborious enzyme extraction techniques and proper buffer solutions with various antioxidants, reducing agents and phenol binding or inhibiting substances for good enzyme activity. The standardisation of different steps is essential to get good results.

4.3.1.1 Extraction of enzyme

a) Choice of tissue

Leaves collected from plants kept in green house or controlled conditions as well as under open conditions were found to produce identical results. But the former was found better with respect to the handling of the sample, as the impurities adhering on the leaves were lesser.
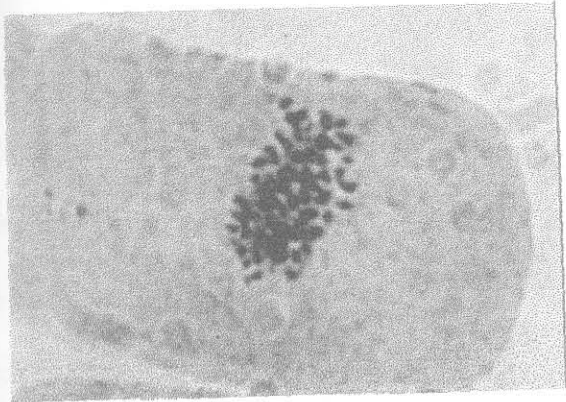
Plate 6c. Chromosomes in metaphase / early anaphase
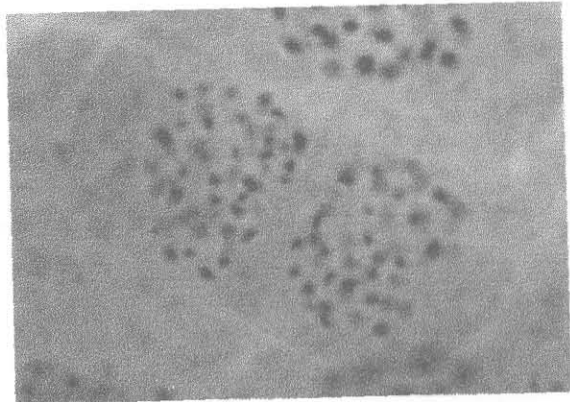
Plate 6d. Chromosomes in late anaphase / early telophase
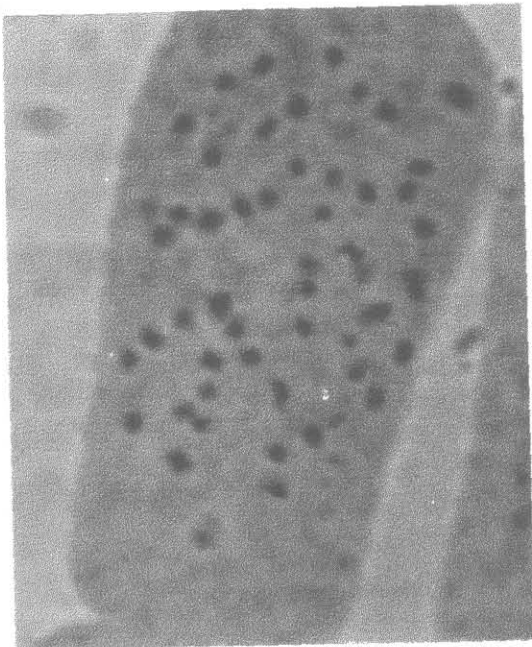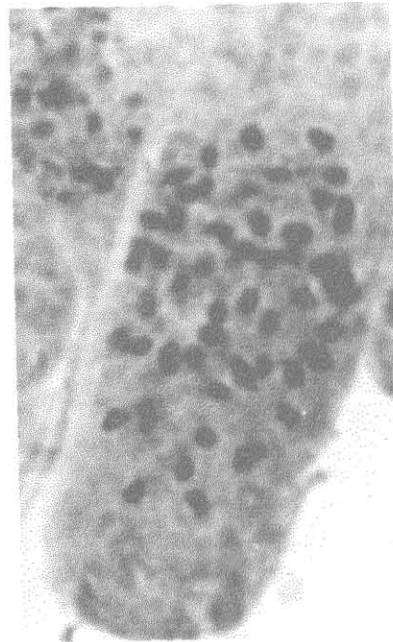

Plate 6c. Somatic chromosomes of P_1

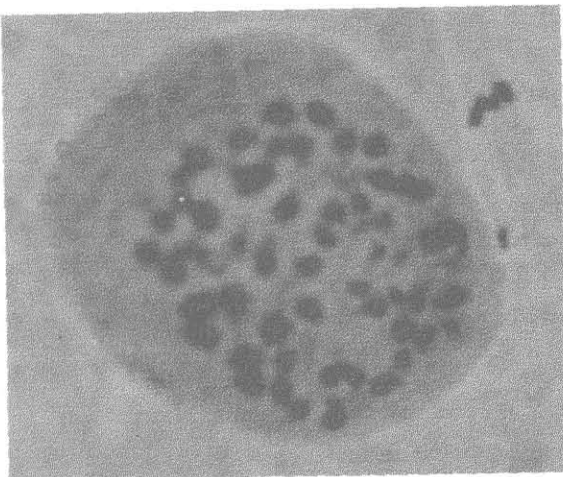Plate 6d. Somatic chromosomes of P_2

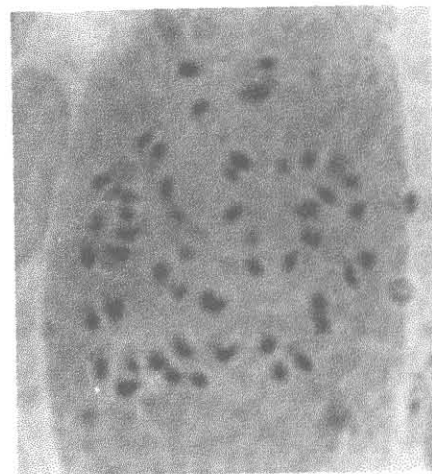Plate 6e. Somatic chromosomes of P_4

Plate 6f. Somatic chromosomes of Su

Leaves collected and kept immediately in cold conditions (4^0C) were subjected to three different treatments *viz.*, (1) subjecting to immediate extraction (2) storing at 4^0C for an hour and (3) storing at -20^0C for an hour. When fresh leaves collected in polythene bags were kept directly at -20^0C for 1 hour, the polyphenol oxidation occurred as indicated by the brown colour of the leaf and it also resulted in flaccidity of the leaves. The other two treatments were found equally good.

b) Stage of growth

Regarding the stage of growth, the mature leaves were more suitable for PRX isozyme, as the intensity of the colour in young leaf was found very low compared to that of mature leaf (Plate 7a). Here the first five wells were loaded with samples from tender leaves extracted with the five buffer systems EB1, EB2, EB3, EB4 and EB5 respectively and wells 6 to 10 were loaded with samples extracted from mature leaves, with the same buffers in that order. The result showed that, the tender leaves lacked the fast moving zone of PRX found in mature leaves extracted with the buffers EB2 and EB3. To confirm the results, the experiment was repeated by changing the buffer pH and buffer additives as well as by staining the same samples for another enzyme system *viz.*, esterase enzyme.

In Plate 7b the first five wells were loaded with tender leaf samples (extracted with EB3), which gave an isoperoxidase pattern of low intensity compared to the next five wells which were loaded with samples from mature leaves extracted with EB3. The same samples were loaded in the same order in another gel (Plate 7c) and stained for the enzyme esterase, which showed reverse effect. In this, the tender leaves exhibited presence of enzyme activity in greater intensity compared to mature leaves. For GOT, both tender and mature leaves produced identical results.
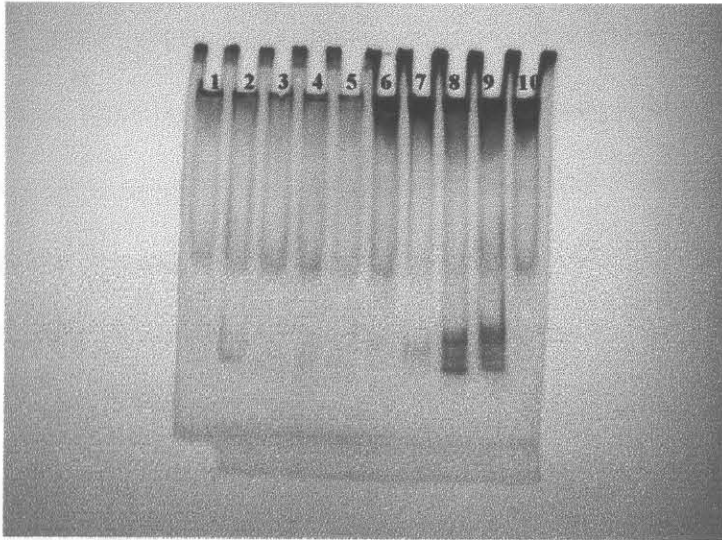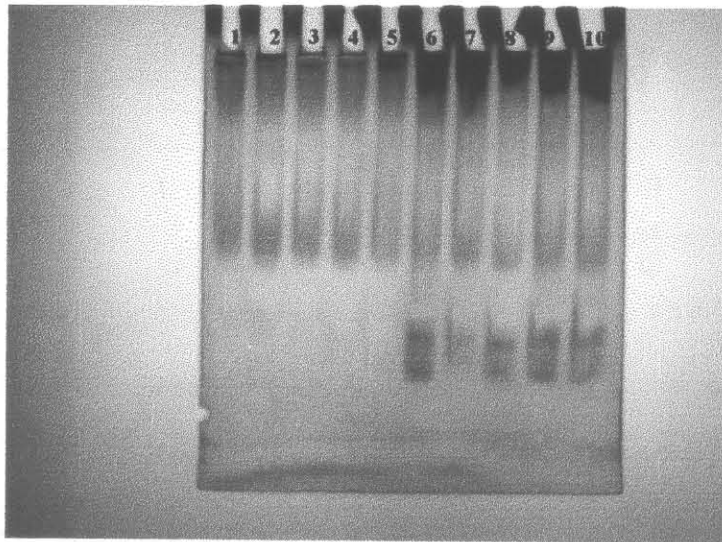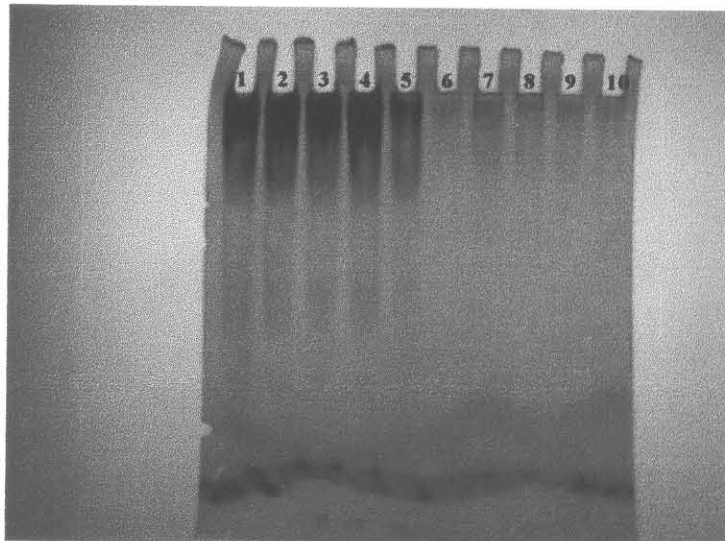
Plate 7

Effect of maturity of
leaf on enzyme activity
in black pepper

7a : Peroxidase.   1 to 5   - tender leaves (buffer - EB1 to EB5)
          6 to 10 - mature leaves (buffer - EB1 to EB5)

7b : Peroxidase. 1 to 5 - tender leaves, 6 to 10 - mature leaves
(buffer EB3)

7c : Esterase. 1 to 5 - tender leaves, 6 to 10 - mature leaves

c) Extraction buffer

Another important factor during extraction was the choice of buffer and buffer additives. Nine different buffers were tried at four different molarities and at eight different pH within a range of 7 to 8 (Table 8). From the 30 different combinations of these three factors, five were selected for tissue standardisation as given below. From these, EB2 and EB3 were selected based upon band formation and intensity of colour development (Plat 7a).

EB-1		**EB-2**		**EB-3**	
Tris buffer - 0.1M		Tris buffer - 0.15M		Sodium phosphate - 0.1M	
pH - 7.4		pH - 7.6		pH - 7.5	
EB-4			**EB-5**		
Tris buffer - 0.35M			Tris- HCl - 0.1M		
pH - 7			pH - 7.4		

Due to the high phenolic interference in black pepper, several phenol complexing agents, antioxidants and reducing agents were added to protect the enzymes from phenolic degradation (Table 9). These compounds were added directly during grinding and also to the buffer before adjusting the pH. Most of these were tried alone as well as in different combinations and concentrations to identify the best combination.

Initially, the extraction was carried out using simple buffer solutions such as 0.1M Tris (pH 7.5) with PVPP in varying concentrations. But this was found ineffective to prevent browning during grinding especially at lower concentrations of PVPP. Hence, addition of other antioxidants and complexing agents were tried. Among these, addition of β -mercaptoethanol (2 to 3µl), sodium metabisulfite (150 to 200mg while grinding or 0.1M in buffer solution) and L-ascorbic acid (0.1M in buffer solution) in different combinations were found powerful enough to prevent phenolic oxidation.

From the 70 different combinations of the buffer solutions and the additives at varying molarity and pH (Table 8 and Table 9), the best combination derived was Tris buffer (0.15M) solution, which contained sodium metabisulfite and L-ascorbic acid at a final molarity of 0.1M each and the pH was adjusted between 7.5 to 7.6. Better results were obtained when the extraction buffer was prepared fresh each time.

The tissue was ground in an ice-cold mortar and pestle with the extraction buffer kept at 0°C in different ratios. The optimum ratio of leaf tissue to buffer was 4:5. When lower quantities of tissue or buffer were used, the intensity of the enzyme was very low. Further increase in the quantity of tissue caused inconvenience in handling whereas increase in the buffer volume decreased the intensity and clarity of the zymogram.

For stabilizing the osmotic balance, addition of 0.5g of sucrose at the time of grinding was found effective. The cell debris was removed by centrifuging in a Kubota highspeed refrigerated centrifuge at 15000 rpm for 15 minutes at 4°C. The clear enzyme extract was stored in aliquots at -20°C for 3 to 4 days without any significant decrease in activity.

4.3.1.2 Electrophoresis

a) Electrophoretic media

Polyacrylamide gels were used as the supporting medium for electrophoresis due to its advantages over other media (Wilkinson, 1970 and Blackshear, 1984). Among the four protocols tried for the preparation of gel, the different steps and stock solutions were identical in Type I and Type II, the difference being in the use of two separate buffer systems viz., Tris-Cl - Tris-glycine in Type I and Tris-citrate - Sodium-borate in Type II. Similarly, the steps were identical in Type III and Type IV, as the latter is only a modification of the former. In Type IV, the gel buffer molarity was considerably reduced whereas the electrode buffer molarity was increased and a separate stacking buffer was

used. In Type I and II the polymerisation was very fast (within 15 to 20 minutes) but sometimes the gel got distorted in the case of vertical slab gel of size 16cm x 14cm whereas in Type III and IV the polymerisation was slow (30 to 45 minutes), but the gel formation was perfect. For PRX, 8 percent resolving gel and for GOT, 10 percent resolving gel were found suitable. For stacking, 4 percent gel was used for both enzymes. The polymerization was more perfect when the gel casting was carried out inside a cool chamber.

b) Buffer system for electrophoresis

The proper combination of gel buffer and electrode buffer at optimum pH and molarity determines the rate of migration, separation and resolution of isozymes. The two buffer systems tried in the present study involved Tris-Cl – Tris-glycine system and Tris-citrate – Sodium-borate system. Among these, the former was found more suitable for pepper. For the standardisation of the various steps as detailed earlier, the Type I and Type III gel and buffer systems were used. But the results showed that there was no satisfactory resolution of the bands. Instead, only certain zones of activity could be identified especially in the case of PRX (Plate 7a, 7b and 7c).

Through a process of elimination, the various factors affecting the rate of migration and extent of resolution were tested. Thus, the possible factors like the concentration of gel, gel buffer pH, extraction buffer as well as the type and the concentration of buffer additives were modified and tested alone and also in different combinations (Tables 8, 9 and 10) but without any positive result. The only factor remaining was the molarity of the gel and electrode buffer system.

Different molarities of electrode buffer tried were 0.005M, 0.01M, 0.0125M, 0.025M of Tris, along with the Type III procedure for gel casting (Table 10). The results showed that among these four, the lower concentrations were better but even then, the resolution

was not proper. In further experiments the molarity of the gel buffer was also reduced. The final results showed that the Type IV procedure of gel casting with treatment $S_4R_2E_2$ (Table 10) was the best combination at the selected gel concentration and extraction procedure (Plate 8).

c) Sample loading

For sample loading, the first method tried, i.e., mixing the sample with the tracking dye solution in different ratios, gave fainter bands. Hence, the second method was followed where the samples were loaded after loading the wells with 1 to 3μl of dye prepared in sample buffer 2 at the cathodal end. The optimum loading volume was 70μl per well so that there was good intensity of colour at the regions showing enzyme activity. When volumes below this were tried, the bands were fainter and above this, there occurred overflowing of the sample to adjacent wells.

d) Run conditions

Satisfactory separation was obtained by keeping current constant at 20mA with voltage at 250 to 350v for about 3 to 4 hrs at 4°C. When the bromophenol blue dye-front moved 12 to 14cm from the origin towards the anode, the system was disconnected and the gel was taken out for staining. Some times the gel was disconnected only after the dye-front reached the bottom of the gel or entered the lower buffer tank for proper separation of the different zones of activity.

4.3.1.3 Enzyme visualisation

For peroxidase, staining procedure suggested by Shaw and Koen (1968) was employed. The incubation of the gel at 35°C in the dark for about 1 hour was sufficient for the enzymatic reaction to take place, which resulted in very dark blue bands in the regions

where isozymes were present. The reaction was arrested and the gel fixed in 7 percent acetic acid, the bands turning intense dark brown.

For GOT, the number, separation and resolution of bands were not perfect even after 30 to 40 runs with varying combinations of all the above factors as detailed earlier. Also while staining, several combinations of substrate concentrations, buffer type, pH, as well as molarity were tried (Table 11). The modifications of the procedure suggested by Shaw and Koen (1968) were tried based on reports by Brewbaker *et al*. (1968), Oliver and Martinez-Zapater (1985), Wendel and Weeden (1989) and Sadasivam and Manickam (1996). From the several modifications of staining solutions tried, combination number 9 (Table 11) was found better compared to the rest. The stain solution consisted of 100ml 0.2M Na_2HPO_4 to which 532 mg L-aspartic acid, 73mg α-ketoglutaric acid and 50mg pyridoxal-5-phosphate were added and the pH was adjusted to 7.5 using NaOH. The dye, Fast Violet Blue (200mg), was added just before use.

It was also observed that the GOT activity as indicated by the band intensity was very low when samples were collected from older cuttings (more than one year old) compared to that from younger ones (2 to 3 months old).

4.3.2 Nomenclature

The electrophoretic pattern showed that for a particular enzyme there exist certain major zones of activity in the gel. Within each zone, there were several bands. Hence, the method of nomenclature followed was based upon that suggested by Oliver and Martinez-Zapater (1985), but instead of capital and small alphabets, Roman numbers were used to designate the major zones of activity and Arabic numbers, for bands within a zone. Thus the zone with fastest anodal migration, when all the four varieties were considered together, was represented as PRX-I and the remaining ones as PRX-II and PRX-III according to the
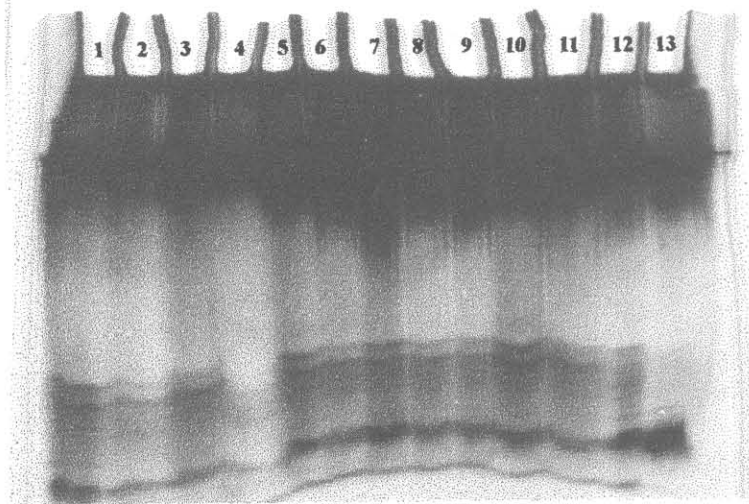
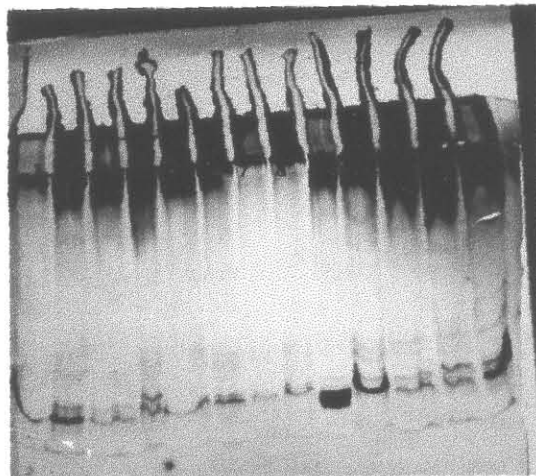Plate 8. Effect of buffer molarity on resolution of peroxidase bands

Plate 9a. Peroxidase in *Piper* sp.
1,8-Su (tender) ; 2,3,11,13- Su (mature)
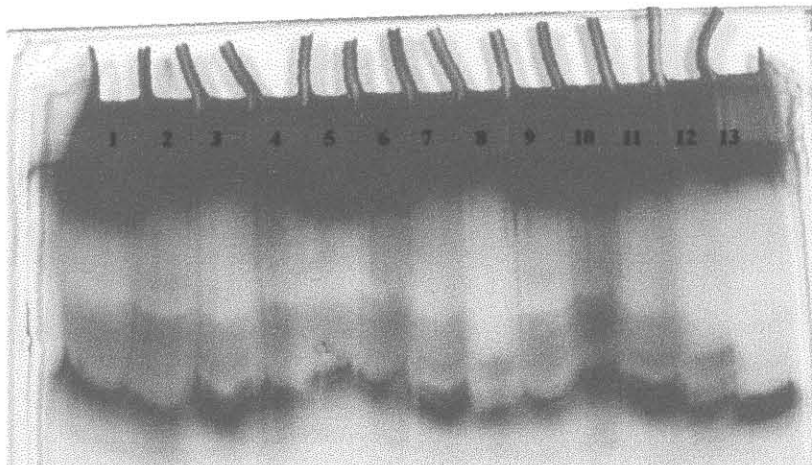4,6,12- P_1 (tender) ; 5,7- P_1 (mature)
9- *P. colubrinum* ; 10 - *P.longum*

Plate 9b. Varietal polymorphism for peroxidase
1 to 4 - TC P_1, P_2, P_4 and Su ; 5 to 8 & 9 to 12 - CC P_1, P_2, P_4 and Su
13 - *P. colubrinum*

rate of migration. Within each zone of activity of an enzyme, the bands from all the varieties were pooled and the band with fastest anodal migration within that zone was numbered as PRX-I (1) and the slower ones by the subsequent numbers PRX-I (2), PRX-I (3) etc. Similarly for the 2nd zone, the numbering was PRX-II (1) and so on.

4.3.3 Isozymes of PRX for characterisation

Plate 9a shows the trial run of the final protocol where, the wells were loaded with samples of different varieties of *Piper nigrum* as well as two related species, *P. colubrinum* and *P. longum* as a check. Duplicates of samples were loaded randomly in different wells to avoid any possible effects of malformations in the gel that may affect the band resolution. The darkest bands belonged to *P. colubrinum*, which consisted of three closely spaced bands with high intensity and fast migration rate towards the anodal end (well no. 9). Zymograms of the experimental vines showed that there were three zones of activity, the fastest zone PRX-I followed by PRX-II, and the slowest zone being PRX-III. But the separation of these zones were clearer if the run time was extended until the dye front moved out into the lower tank buffer. Hence, *P. colubrinum* sample was loaded in the last well of every gel in order to act as an internal marker for comparison and to work out the relative mobility (Rm) values of individual isozymes of *P. nigrum*.

In *P. longum* there were two zones (well no. 10), one with fast anodal migration rate and the other, with very slow rate of migration. The fast zone had seven isozymes among which one was very intensely coloured and the rest were faint. Some of the bands in this zone were monomorphic with corresponding bands in *P. colubrinum*. The slow moving zone consisted of two bands and these were almost near the origin, but well resolved.

The sample for black pepper included Subhakara (TC) representing the variety with red leaf sheath and Panniyur 1 (TC) representing the variety with green leaf sheath. Both

tender and mature leaves were subjected to electrophoresis. The result showed that, the number of bands was more in samples extracted from mature leaves in both types of varieties. Rm values were calculated by measuring the distance travelled by the bands relative to that of the *P. colubrinum* band. Since the resolution and intensity of bands were satisfactory with the protocol developed, the TC and conventional clones were characterised with respect to PRX isozymes the results of which are explained below.

4.3.3.1 Intra group characterisation

All the 20 conventional clones and the 38 TC clones were subjected to peroxidase assay to get the characteristic zymogram of the isoperoxidases present. Pooling the number of bands from all the 58 vines, there were a total of 16 bands distributed in the three zones. The details of the zones and the relative mobility of the different bands in the zones are provided as Table 17.

Table17. Number, distribution and relative mobility of isoperoxidases in black pepper

Zone of activity	No. of bands	Band	Relative mobility
PRX- I	7	PRX-I (1)	1.12
		PRX-I (2)	1.10
		PRX-I (3)	1.05
		PRX-I (4)	1.03
		PRX-I (5)	1.00
		PRX-I (6)	0.96
		PRX-I (7)	0.94
PRX- II	8	PRX-II (1)	0.92
		PRX-II (2)	0.88
		PRX-II (3)	0.84
		PRX-II (4)	0.82
		PRX-II (5)	0.80
		PRX-II (6)	0.76
		PRX-II (7)	0.72
		PRX-II (8)	0.68
PRX- III	1	PRX-III (1)	0.45
Total	16		

a) Characterisation of conventional vines

No polymorphism was detected within clones of each variety showing the true - to - type nature. The zymogram of one sample each from each variety coming under CC group and TCC group is given as Plate 9b. The distribution of bands in the different zones for the conventional clone group was as detailed below.

Zone	CC P_1	CC P_2	CC P_4	CC Su
PRX-I	(1), (4), (5)	(1), (2), (3), (4), (5), (6)	(1), (5), (6), (7)	(1), (5)
PRX-II	(1), (2), (3), (5), (6)	(1), (2), (3), (5), (6), (7), (8)	(3), (5), (6), (8)	(2), (3), (4), (5)
PRX-III	-	-	(1)	-
Total	8	13	9	6

b) Characterisation of TC vines

Unlike the clones, one or two TC plants in each variety exhibited some polymorphism with respect to the presence or absence of some bands.

In TC clones of P_1 the banding pattern showed two fast moving zones PRX-I and PRX-II, the former with three bands and the latter with five bands as in the case of conventional clones. But one of the TC plants, P_1-1, showed the presence of two additional bands, one each in zones PRX-I and PRX-II, both slowest in the respective zones. As the vine TC P_1-1 was observed to be a variant, to confirm the result, the electrophoresis was repeated along with samples from one normal TC vine, TC P_1-3 and one conventional clone, CC P_1, repeating in that order (Plate 10a). All other TC vines showed uniform pattern. The distribution of the bands in the different zones was as detailed below. The bands showing polymorphism are given as bold numbers.

Zone	General (TC P_1)	Variant (TC P_1-1)
PRX-I	(1), (4), (5)	(1), (4), (5), **(6)**
PRX-II	(1), (2), (3), (5), (6)	(1), (2), (3), (5), (6), **(8)**
PRX-III	-	-
Total	8	10

In variety P_2, the bands appeared continuously without demarcation into different zones, as the fast migrating zone had not completely moved towards the anode. There were a total of 13 bands in all the TC as well as clonal samples except TC P_2-7 (Plate 10b). In this gel the samples were loaded in the reverse order i.e., first well contain TC P_2-10 and tenth well, TC P_2-1 in that order. For comparing with conventional clones, 11th and 12th wells were loaded with CC P_2. TC P_2-7 differed from the rest with respect to both PRX-I and PRX-II zones. In PRX-I, two isozymes PRX-I (4) and PRX-I (6) were absent, whereas in the second zone, the slowest bands PRX-II (8) as well as the fast ones PRX-II (1), PRX-II (2) and PRX-II (3) were absent. There were only seven bands here and the intensity was also less compared to others. The distribution of the bands in the different zones was as detailed below. The bands showing polymorphism are given as bold numbers.

Zone	General (TC P_2)	Variant (TC P_2-7)
PRX-I	(1), (2), (3), **(4)**, (5), **(6)**	(1), (2), (3), (5)
PRX-II	**(1)**, **(2)**, **(3)**, (5), (6), (7), **(8)**	(5), (6), (7)
PRX-III	-	-
Total	13	7

In variety P_4, electrophoresis revealed that apart from the fast migrating zones PRX-I and PRX-II there was an additional slow moving zone, PRX-III (Plate 10c). There were
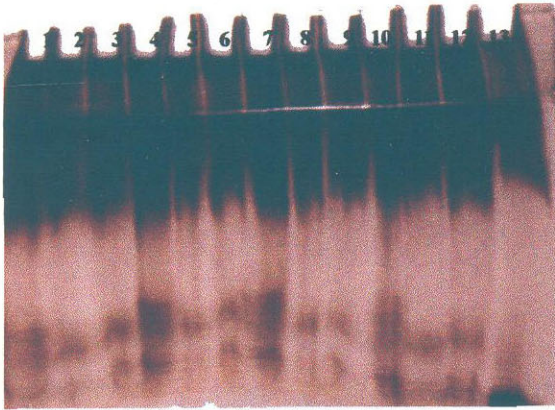
Plate 10a. Peroxidase zymogram of P_1
(Variant TC P_1-1: well no. 1, 4, 7, 10)
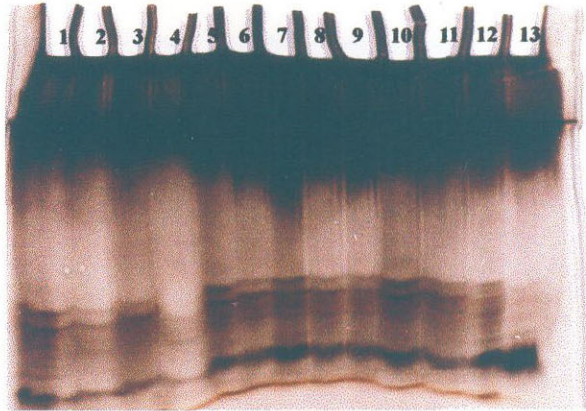2,5,8,11 - TC P_1-3 ; 3,6,9,12 - CC P_1
13- *P.cloubrinum*

Plate 10b. Peroxidase zymogram of P_2
(Variant - TC P_2-7 : well no. 4)
1-10 - TC P_2-10 to TC P_2-1 ; 11,12 - CC P_2
13- *P.cloubrinum*

Plate 10c. Peroxidase zymogram of P_4
(Variant TC P_4-4 & TC P_4-8 : well no. 4 &8)
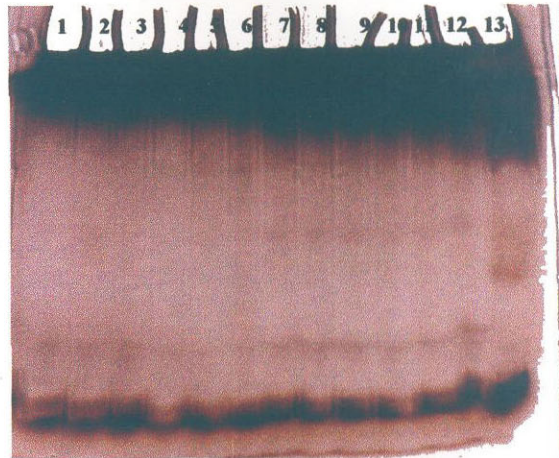1-10 - TC P_4-1 to TC P_4-10 ; 11,12 - CC P_4
13- *P.cloubrinum*

Plate 10d. Peroxidase zymogram of Su
(Uniform)
1-12 - TC Su1 to TC Su 10
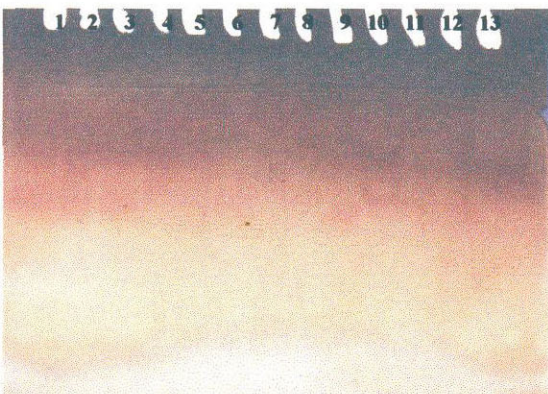13- *P.cloubrinum*

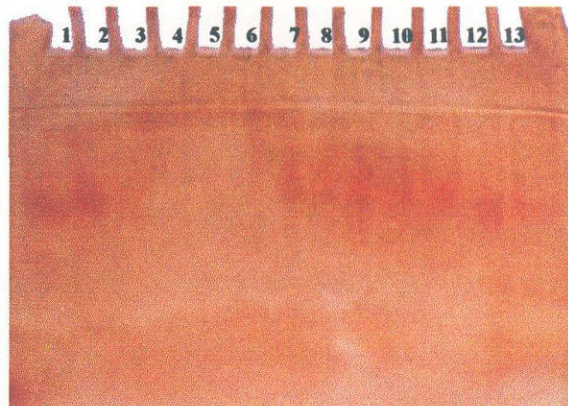Plate 11a. GOT zymogram of P_1
(Well no. 1-10 : TC, 11-13 : clones)

Plate 11b. GOT zymogram of P_2
(Well no. 1-10 : TC, 11-13 : clones)

nine bands in total, but in TC P_4-4 and TC P_4-8 four bands were absent. The distribution of the bands in the different zones was as detailed below. The bands showing polymorphism are given as bold numbers.

Zone	General (TC P_4)	Variants (TCP$_4$-4 & TC P_4-8)
PRX-I	(1), (5), **(6), (7)**	(1), (5)
PRX-II	(3), (5), **(6), (8)**	(3), (5)
PRX-III	(1)	(1)
Total	9	5

In TC clones of Subhakara, all vines exhibited uniform pattern for isoperoxidases. No variants were detected among these (Plate 10d). There were a total of 11 bands but some of the bands in PRX-II zone were too faint to be photographed, especially PRX-II (3), PRX-II (4), PRX-II (5) and PRX-II (6). Even in repeated runs, the intensity was very low. The distribution of the bands in the different zones was as detailed below.

Zone	General (TC Su)	Variant
PRX-I	(1), (5), (6), (7)	NIL
PRX-II	(2), (3), (4), (5), (6), (7)	
PRX-III	(1)	
Total	11	

4.3.3.2 Inter clonal polymorphism (CC Vs. TCC)

Except for one variant in P_1, the rest of the TC and all the parental clones were having the same pattern. In all the TC as well as clones of this variety, the bands corresponding to the marker bands, PRX-I (5) and PRX-I (6), which were usually very thick, intensely

stained and most prominent among all the isozymes were found to be with less intensity (Plate 9b, 10a).

In P_2, the marker bands and corresponding PRX-I (5) and PRX-I (6) isozymes were with almost equal intensity. Also, PRX-I (2) and PRX-I (3) that were characteristic of this variety were present in all vines including the variant, TC P_2-7. Here also, there was no polymorphism between the remaining TC vines and conventional clones (Plate 9b, 10b).

For the variety P_4 there were nine monomorphic bands in all the conventional and TC clones except for the variants, though the intensity of PRX-I (6) varied slightly in certain vines. The isozyme PRX-I (7) that was characteristic of the variety was present in all, excepting the two variants. But the third zone PRX-III was present in all the vines (Plate 9b, 10d).

Unlike the other varieties, there was difference in banding pattern between the conventional Subhakara clones and TC Subhakara with respect to the presence of additional slow moving isozymes in the later as shown below.

Zone	General (TC Su)	Conventional clone (CCSu)
PRX-I	(1), (5), **(6), (7)**	(1), (5)
PRX-II	(2), (3), (4), (5), **(6), (7)**	(2), (3), (4), (5)
PRX-III	**(1)**	-
Total	11	6

Here, the TC samples showed more number of bands compared to conventional clones; both fast moving as well as slow moving bands. There were four bands PRX-I (1), PRX-I (5), PRX-I (6) and PRX-I (7) in the faster zone and seven bands [PRX-II (2) to PRX-II (7) and PRX-III (1)] in the slower zone in TC sample, with a total of 11 isozymes. On the other hand, there were only 6 isozymes in conventional clones. When P_4 and TC

Subhakara were compared, there found certain similarity in pattern of isoperoxidases. In the former, though PRX-I (6) had higher intensity, the other three, the PRX-I (1), PRX-I (5) and PRX-I (7) were monomorphic with those of TC Subhakara. But in the second zone, some additional faint bands were present in TC Subhakara.

4.3.4 Isozymes of GOT for characterisation

As there was no proper band resolution and intensity, the characterisation of conventional and TC clones of each variety using this enzyme system was impossible and so, the Rm values and genetic distance were not worked out with respect to this enzyme (Plates 11a, 11b).

One sample each from the conventional and TC clones of each of the four varieties P_1, P_2, P_4 and Subhakara were subjected to PAGE using the protocol developed and the gel was stained for the enzyme GOT (Plates 11c, 11d). The result showed three main regions of activity for this enzyme also. Distributed among these three regions, there were a total of eight bands. Within the region GOT-I, there were three isozymes showing polymorphism between varieties. The fastest GOT-I (1) and the next GOT-I (2) were present only in variety Subhakara. The slow moving isozyme of the zone viz., GOT-I (3) was present in all the varieties.

The second region GOT-II had four isozymes but the separation was not clear. All the four varieties showed similar isozymes in this zone. GOT-III had only one band, which was very close to the well and it was present only in Subhakara. This was the slowest moving, when all the eight isozymes were considered together.

To check the efficiency of the protocol to detect GOT activity on the gel, samples extracted from *Piper nigrum* (P_1 and P_4), *P. colubrinum*, *P. longum* and nutmeg, a high phenol containing crop were loaded together in a single gel and electrophoresis was carried
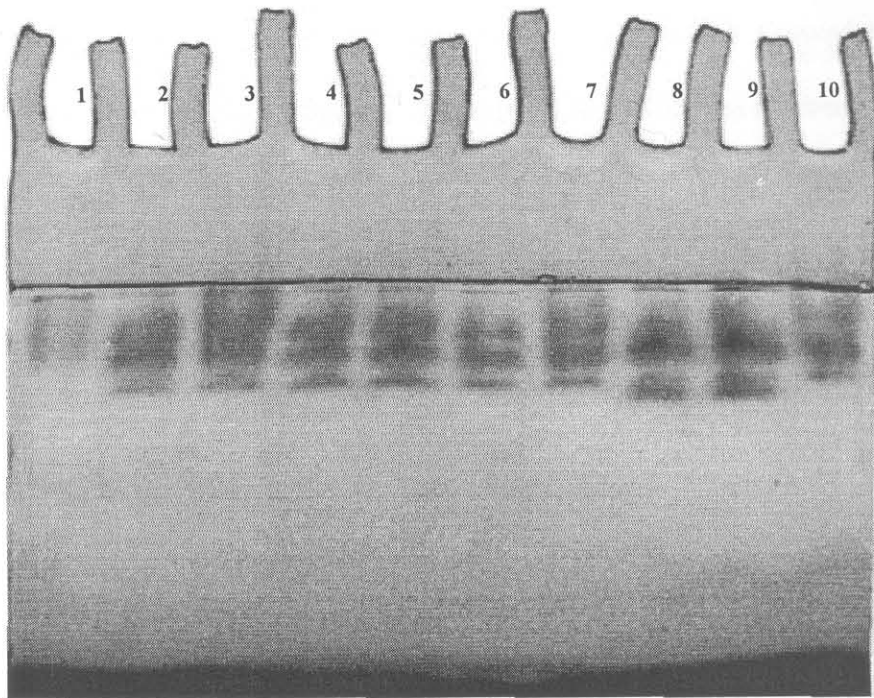
Plate 11c. GOT in *Piper nigrum*
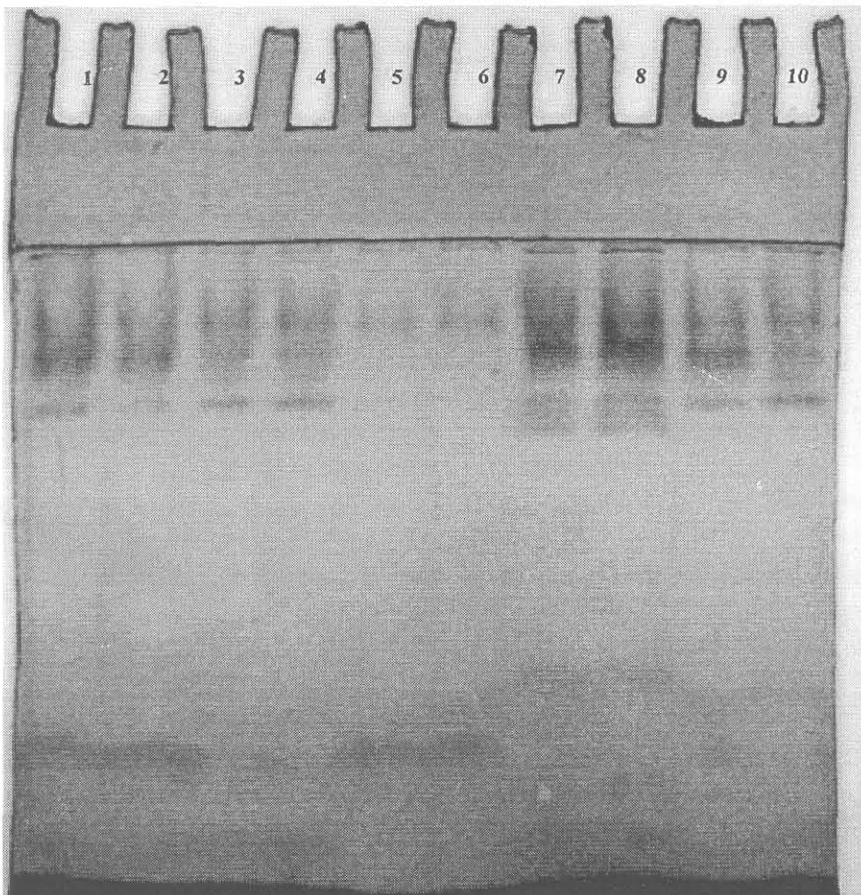1,2,3 - P$_1$;   4,5- P$_2$;   6,7 -P$_4$;   8,9,10 - Su

Plate 11d. GOT in *P. nigrum* (1,2 - P$_1$; 3,4- P$_2$; 5, 6 -blank ; 7, 8 -Su; 9,10- P$_4$)

out (Plate 12a). It was found that, within five minutes in the stain solution, the wells loaded with nutmeg (well no. 11,12,13) turned dark reddish in colour, which showed that the protocol was suitable for GOT isozyme studies. To confirm the result, the run was repeated once more with new samples, which gave the same result (Plate 12b).

4.3.5.Varietal polymorphism

As additional information from the investigation, the varietal polymorphism with respect to the peroxidase isozymes was also recorded. Along with varietal comparison a comparison of mature and tender leaves of TC Su and TC P_1 was made from the zymogram (Plate 9a).

Mature leaves of Subhakara had four bands in the fast moving zone with Rm values ranging from 1.12 to 0.94 and six bands in the second zone with Rm values 0.88 to 0.72, but the last two bands PRX-II (6) and PRX-II (7) were very faint. In tender leaves, the fast moving zone PRX-I was present but the zone PRX-II had only 2 faint bands with Rm values 0.8 [PRX-II (5)] and 0.76 [PRX-II (6)].

For P_1, the samples from mature leaves had three bands in zone PRX-I with Rm values 1.12 to 1. These two varieties differ with respect to PRX-I (4) present in P_1 and PRX-I (6) and PRX-I (7) present in TC Subhakara. In the zone PRX-II, there were five bands with Rm values 0.92 to 0.76. It differed from that of Subhakara with respect to presence of PRX-II (1) as well as absence of PRX-II (4) and PRX-II (7). The tender leaves had three bands in the zone PRX-I (Rm = 1.12 to 1) but the band corresponding to the standard band of *P. colubrinum viz.*, PRX-I (5) was very faint, though present, whereas in the zone PRX -II, there were only two bands.

In a separate electrophoresis, in order to have a comparison of all four varieties samples from TCC P_1, P_2, P_4 and Subhakara were loaded in the first four wells and CC P_1,

Plate 12a. GOT in *Piper* sp. & Nutmeg
(Well no. 1-6 : P$_1$, 7-8 : P$_4$, 9-10 : *P. colubrinum* , 11-13 : nutmeg)

Plate 12b. GOT in *Piper* sp. & Nutmeg
(Well no. 1-6 : P$_1$, 7-8 : P$_4$, 9-10 : *P. colubrinum* , 11-13 : nutmeg)

P_2, P_4 and Subhakara in 5th to 8th wells. A duplicate set of CC group was loaded in the remaining 9th to 12th wells (Plate 9b). The detailed electrophoretic phenotypes of the four varieties of clonal as well as TC plants are provided as Table 18. The presence of a band is represented as '+' and absence as '-'.

Table 18. Varietal polymorphism for isoperoxidases

Zone of activity	Isozyme	Varietal polymorphism (TCC)				Varietal polymorphism (CC)			
		P_1	P_2	P_4	Su	P_1	P_2	P_4	Su
PRX- I	PRX-I (1)	+	+	+	+	+	+	+	+
	PRX-I (2)	-	+	-	-	-	+	-	-
	PRX-I (3)	-	+	-	-	-	+	-	-
	PRX-I (4)	+	+	-	-	+	+	+	-
	PRX-I (5)	+	+	+	+	+	+	+	+
	PRX-I (6)	-	+	+	+	-	+	+	-
	PRX-I (7)	-	-	+	+	-	-	+	-
PRX- II	PRX-II (1)	+	+	-	-	+	+	-	-
	PRX-II (2)	+	+	-	+	+	+	-	+
	PRX-II (3)	+	+	+	+	+	+	+	+
	PRX-II (4)	-	-	-	+	-	+	-	+
	PRX-II (5)	+	+	+	+	+	+	+	+
	PRX-II (6)	+	+	+	+	+	+	+	-
	PRX-II (7)	-	+	-	+	-	+	-	-
	PRX-II (8)	-	+	+	-	-	+	+	-
PRX- III	PRX-III (1)	-	-	+	+	-	-	+	-
Total		8	13	9	11	8	13	9	6

When all the four varieties were considered together, in the faster zone PRX-I, there were three monomorphic isozymes PRX-I (1), PRX-I (5) and PRX-I (6) with Rm values 1.12, 1 and 0.96 respectively. Among these, the PRX-I (5) was corresponding to the marker band of *P. colubrinum* that was very dark and prominent in all followed by PRX-I (6), which was closely spaced to the former. PRX-I (1) was the fastest among all the isozymes

considering all the three zones. This was comparatively with lesser intensity but was clear and well resolved in almost all the gels. Polymorphism in this zone was exhibited by PRX-I (2) and PRX-I (3) that were characteristic of P_2. P_1 and P_2 shared three bands in this zone and P_4 and Subhakara exhibited similar pattern.

In the second zone, PRX-II, there were a total of eight bands, out of which three were monomorphic and five polymorphic in TC plants. Here also, TCC Su differed from CC Su with the presence of two additional bands, PRX-II (6) and PRX-II (7). For the other varieties, the patterns were same in both TCC and CC groups.

The general pattern of P_1 and P_2 were identical with respect to this zone also except for isozyme PRX-II (7) and PRX-II (8), which were absent in P_1. P_4 and Subhakara differed with respect to four bands in this region, viz. PRX-II (2), PRX-II (4), PRX-II (7) and PRX-II (8). In the third zone, which was present only in TCC P_4, CC P_4 and TC Subhakara, there was only one band.

4.3.6 Genetic diversity analysis

Pooling all the bands, there were 16 isozymes out of which, five were monomorphic and eleven, polymorphic. Genetic diversity analysis (Demeke et al., 1996) of the data generated from the zymogram showed that P_1 and P_2 were genetically more similar (GD = 0.23) whereas P_4 and Subhakara were closer (GD = 0.33) (Table 22). The extent of similarity was more in the former group. Comparison between the groups indicated that from Subhakara, both P_1 and P_2 had equal distance (GD = 0.5) whereas from P_4, P_2 was more distant (GD = 0.53) than P_1 (GD = 0.42).

4.4 RAPD ANALYSIS

The technique of molecular fingerprinting in characterisation of germplasm as well as varietal screening to assess the extent of inter and intra-specific variability has met with extensive acceptance in the research programmes of various crops.

4.4.1 Standardisation of protocol

As the reproducibility of RAPD analysis depended greatly on the various factors involved in DNA isolation as well as the quality and quantity of the reaction mixture for PCR and the thermal profile applied, the standardisation of each and every step is the most important pre-requisite for using the technique in a specific crop. After the stability and reproducibility of the bands are assured, they can be screened for polymorphisms in the required field.

4.4.1.1 Source of DNA

As reported in many other crops, leaves were selected as the ideal part for extraction of total genomic DNA of the plant. In black pepper, very tender, fresh, pale green leaves (0.5 to 1g) yielded good quality DNA in sufficient quantity. There was no browning of the extract if suitable antioxidants were added.

4.4.1.2 DNA isolation

From the various protocols reviewed, the one suggested by Doyle and Doyle (1987) and Rogers and Bendich (1994) were selected since the former has been used in many taxa whereas the latter involved the use of PVP and CTAB which were reported to reduce the polyphenol inhibition. Between these two methods, the quality of DNA was found good in samples extracted with both the protocols (Table 19). But comparatively, protocol II, a

modified CTAB method based on that suggested by Rogers and Bendich (1994) was found to yield better and also, the procedure was easier. Hence, protocol II was selected for further experiments. The agarose gel electrophoresis of the sample showed presence of RNA (Plate 13a). For use in amplification reactions, RNA must be eliminated.

4.4.1.3. Purification and quantification of DNA

The treatment of the sample with RNase A (Bovine pancreatic RNase) was successful in removing the RNA completely. This was ensured by electrophoresis of the RNase treated sample once again. Under UV light, the DNA was observed as a single band on the agarose gel without any smear below (Plate 13b).

Table 19. The quality and yield of DNA samples isolated using different protocols

Variety	Proto -col	OD at 260	OD at 280	Absorbance Ratio (260/280)	Quality	Quantity $\mu g\ ml^{-1}$	$\mu g\ g^{-1}$ of fresh leaf
P_1	I	0.163	0.079	2.1	-	8.15	40.75
	II	0.566	0.324	1.8	Good	28.30	84.90
P_2	I	0.103	0.053	1.9	Good	5.15	25.75
	II	0.354	0.197	1.8	Good	17.70	44.25
P_4	I	0.250	0.148	1.8	Good	12.95	64.75
	II	0.507	0.242	2.1	-	25.35	63.38
Subhakara	II	0.520	0.252	2.1	-	26.00	65.00
Karimunda	II	1.848	0.972	1.9	Good	92.40	231.00
KS27 (mother clone)	II	1.421	0.670	2.1	-	71.10	177.25

The absorbance ratio calculated for the various samples also indicated that, in 62 per cent of the samples ratio was between 1.8 and 2, in 21 per cent, between 1.7 and 1.8 and in 8 percent, more than 2. The remaining 9 per cent were having a low ratio (less than 1.7). The samples in which the quality was poor were not used for the amplification and DNA

was isolated from fresh samples. The quantity of DNA isolated ranged between $44.25\mu g$ to

231 μg per gram of fresh leaf weight.

4.4.1.4 DNA amplification conditions

Based on the review of literature, initially a few primers were screened and OPP1 was

selected for standardisation of other conditions. Using OPP1 and 50ng DNA from variety

P_4, various combinations of reaction conditions were tried and a best set was selected from

them, which was suitable to PTC-200 model Thermal Cycler (MJ Research, USA).

a) Reaction mixture

It was observed that variation in the $MgCl_2$ concentration in the buffer considerably

affected the amplification pattern. When the amount of $MgCl_2$ was below 1mM, the

amplification was very feeble and inconsistent. A concentration of 1.5mM was optimum

for the given conditions. Two concentrations of the enzyme, 0.5U and 0.6U of Taq DNA

polymerase supplied by Bangalore Genei Pvt. Ltd were found to give good results.

Concentrations below and above this failed to give consistent and clear bands. Changing the

molarity of the dNTP solutions as well as the primer solution helped to improve the number

and intensify of bands. A concentration of $150\mu M$ each of the four dNTP solutions was

found optimum whereas for primers, 4 pmoles was the optimum concentration. With

respect to the amount of template DNA, there was no significant variation in the banding

pattern, and hence 50ng was used in all the reactions.

b) Thermal profile

Among the different temperatures tried using the best set of reaction mixture, two

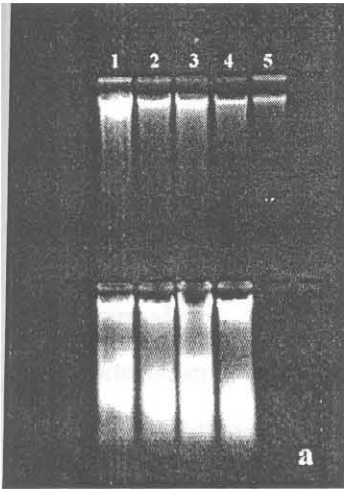profiles were found good giving almost identical amplifications.

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Plate 13
Quality test for DNA by agarose gel electrophoresis

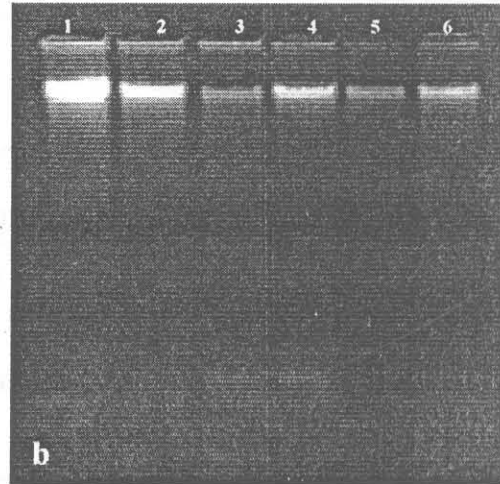a : before RNase treatment
b : after RNase treatment

Plate 14
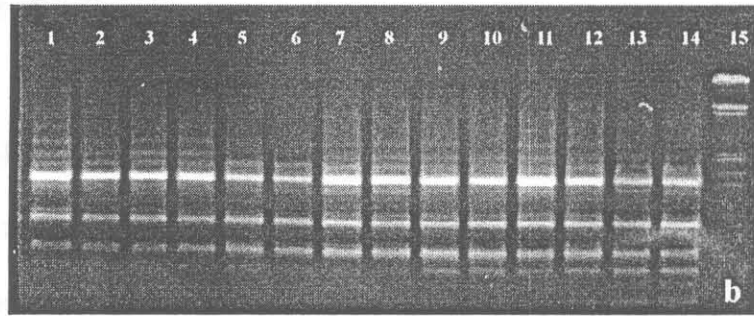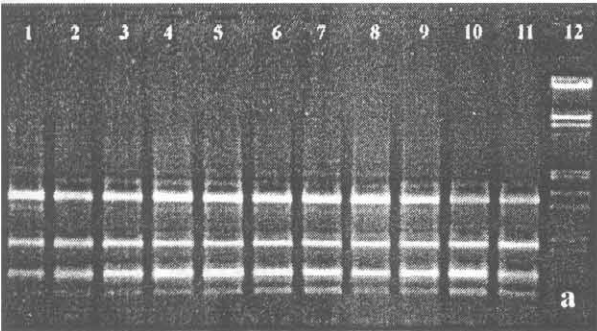Standardisation of thermal profile

a : TP I
b : TP II

Plate 15
Primer screening (Variety P₄)

Upper gel
1-7 : OPP
8-13 : OPF

Lower gel
1-7 : OPE

Steps	Thermal Profile I (TP I)		Thermal Profile II (TP II)	
	Temp.	Time	Temp.	Time
1. Initial denaturation	$94^{0}c$	2 min	$94^{0}c$	1.3 min
2. Denaturation	$92^{0}c$	2 min	$92^{0}c$	1 min
3. Annealing ⟶ 45 cycles	$37^{0}c$	1 min	$37^{0}c$	1 min
4. Extension	$72^{0}c$	1.3 min	$72^{0}c$	1.3 min
5. Final extension	$72^{0}c$	10 min	$72^{0}c$	10 min

Plate 14a and Plate 14b shows the amplification of DNA from P_1, under TP I and TP II, using primer OPP1 with all other reaction conditions remaining the same. Even though the patterns were almost identical, the intensity was better for PCR with TP II.

c) Primer screening

Using the selected levels of various reagents in the reaction mix and the ideal thermal profile, the OPP 1-20, OPF 1-20 and OPE 1-20 series of primers were screened for amplification using the template DNA from P_2. From these 60 primers, a total of 37 numbers were selected in the second round with 15 OPP, 12 OPF and 10 OPE, using DNA of P_4. In the third round 20 out of 37 were once again subjected to PCR (Plate 15). The number of bands generated by 7 OPP primers varied from 1 to 8, that by 6 OPF primers varied from 1 to 7 and that by 7 OPE primers varied from 0 to 4. Based upon the number, intensity and consistency of the bands produced by the above 4 PCR reactions, 11 numbers of OPP and 2 numbers of OPF were selected for screening the varieties. Based on the extent of polymorphism exhibited by the different varieties with respect to these primers, four were ultimately selected for characterisation of the experimental vines.

4.4.2 Characterisation using RAPD

For the characterisation of the parental clones and the TC plants of these four varieties, four primers *viz.*, OPP 1, OPP 8, OPP 14 and OPF 13 were selected. In each variety there were 5 clonal and 10 TC derived vines to be characterised. The number of samples that could be loaded in the agarose gel at a time in the electrophoresis unit used was only 15. Since one well had to be loaded with a molecular weight marker, for comparison of the clonal as well as TC derived plants together in a single PCR, only the first four wells were loaded with clonal DNA, the next 5 to 14 wells with TC derived DNA and the 15th well was loaded with the molecular weight maker (Hind III digested λ DNA).

4.4.2.1 Intra group characterisation

a) Characterisation of conventional clones

The RAPD products generated by each of the four decamer primer gave characteristic pattern for the conventionally propagated vines and were uniform within the clones of each variety. Using the four primers, the clones of P_1 produced the highest number of products (38 bands) and Subhakara produced the lowest (26 bands). The distribution of bands with respect to the different   primers for each of the four varieties was as given below.

Primer	CC P_1	CC P_2	CC P_4	CC Su
OPP 1	9	6	9	8
OPP 8	12	9	12	6
OPP 14	9	9	10	9
OPF 13	7	7	6	4
Total	**37**	**31**	**37**	**27**

b) Characterisation of TC clones

The electrophoresis of the RAPD products of TC clones exhibited some polymorphic bands in certain vines. With respect to each of the four decamer primers used, TC vines of each variety showed characteristic pattern. In all the four varieties there existed one or two variants with respect to the presence of additional bands or absence of bands found in the other vines.

(i) TC clones of P_1

In P_1 since there were only eight tissue culture derived plants of the variety, the 13th and 14th wells were also loaded with TCC P_1-1and TCC P_1 -2 respectively. The amplification patterns of the TC plants of the variety with respect to the different primers were as detailed below.

Primer	General (TCC)	Variants (TC P_1-1, TC P_1-2)
OPP 1	9	7
OPP 8	12	10
OPP 14	9	8
OPF 13	7	7
Total	37	20

The amplification pattern of primer OPP1 revealed monomorphic bands in all the TC derived clones except in TCC P_1-1 and TCC P_1-2 (Plate 16a). The total number of bands were nine in which seven bands were monomorphic in all the samples. The first two high molecular weight bands present in all the other TC derived plants were absent in TCC P_1-1 and TCC P_1-2.
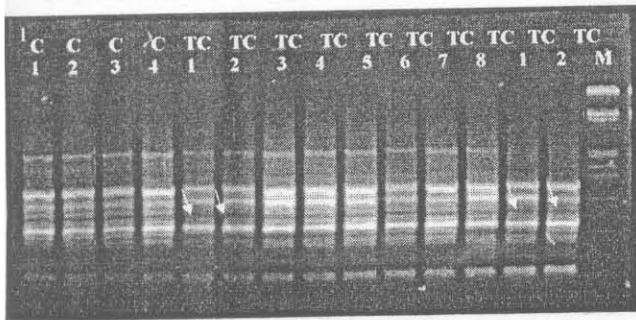
Plate 16. RAPD banding pattern of P_1
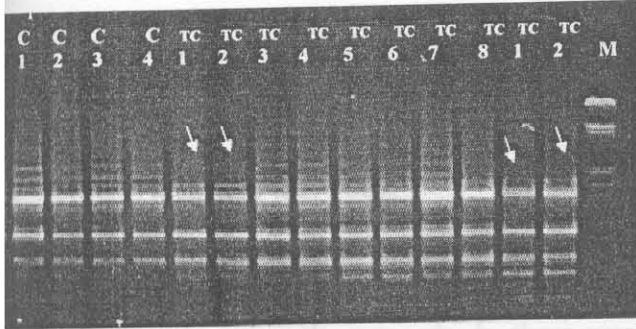
16a. Primer OPP1

Monomorphic
Clones : 9
TC (3 to 8) : 9

Polymorphic
TC P_1-1 : 7
TC P_1-2 : 7

16b. Primer OPP8

Monomorphic
Clones : 12
TC (3 to 8) : 12

Polymorphic
TC P_1-1 : 10
TC P_1-2 : 10

16c. Primer OPP14

Monomorphic
Clones : 9
TC (3 to 8) : 9

Polymorphic
TC P_1-1 : 8
TC P_1-2 : 8

16d. Primer OPF13

Monomorphic
Clones : 7
TC (1 to 6) : 7

Polymorphic
TC P_1-7 : 8
TC P_1-8 : 4

For OPP 8 there were 12 bands in general among the TC clones (Plate 16b). Here also, TCC P_1-1 and TCC P_1-2 were different from the rest with respect to the absence of two high molecular weight bands.

In the case of OPP 14 there were nine bands in all except in well number 5,6,13 and 14 where TCC P_1-1 and TCC P_1-2 were loaded. In these, one of the products with medium size was absent (Plate 16c).

The amplification products from OPF 13 primer showed a different pattern with respect to the other three in which, there were seven monomorphic bands in TC plants and TCC P_1-1 and TCC P_1-2 were having identical amplification products with the rest of the TC plants (Plate 16d). TCC P_1-7 had one additional high molecular weight band and TCC P_1-8 lacked the first, second and sixth bands present in all the rest.

Pooling the RAPD products from all these four primers, there were a total of 37 bands out of which, the variant vines TCC P_1-1 and TCC P_1-2 exhibited polymorphism with respect to five bands.

(ii) TC clones of P_2

Primer	General (TCC)	Variants (TC P_2-7, TC P_2-10)
OPP 1	6	8
OPP 8	9	6
OPP 14	9	9
OPF 13	7	7
Total	**31**	**30**

The gel with PCR samples of OPP1 revealed six bands in TC plants except TCC P_2-7 and TCC P_2-10 (Plate 17a). In these two, the 1st band present in others was very faint.

Plate 17. RAPD banding pattern of P_2

17a. Primer OPP1

Monomorphic
Clones : 6
TC (1 to 6,8,9) : 6

Polymorphic
TC P_2-7 : 8
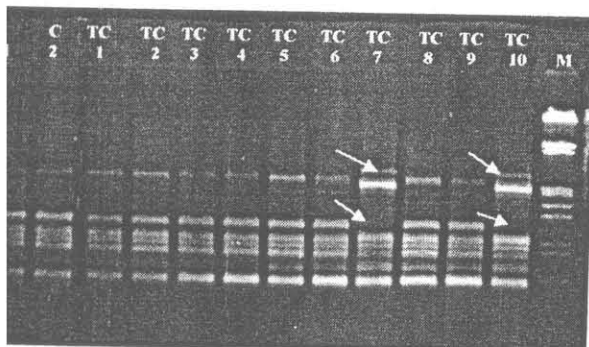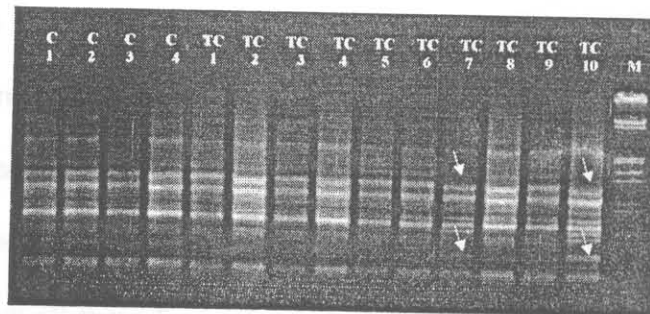TC P_2-10 : 8
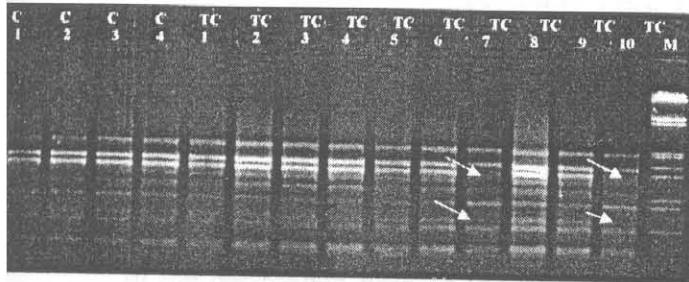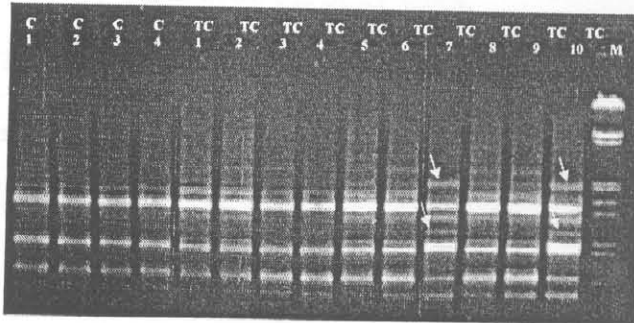
17b. Primer OPP8

Monomorphic
Clones   : 9
TC (1 to 6,8,9) : 9

Polymorphic
TC P_2-7   : 6
TC P_2-10 : 6

17c. Primer OPP14

Monomorphic
Clones : 9
TC (1 to 6,8,9) : 9

Polymorphic
TC P_2-7 : 9
TC P_2-10 : 9

17d. Primer OPF13

Monomorphic
Clones   : 7
TC (1 to 6,8,9) : 7

Polymorphic
TC P_2-7 : 7
TC P_2-10 : 7

Also there were two additional bands, one with high molecular weight and the other with medium molecular weight. So, there were a total of eight bands in TCC P_2-7 and TCC P_2-10 including the six monomorphic bands present in all the other vines.

For OPP 8 also, except in TCC P_2-7 and TCC P_2-10 there were a total of nine bands (Plate 17b). The two variant TC cones differed from others in that they lacked two high molecular weight and one low molecular weight bands present in others, the total bands in these being six.

There were nine monomorphic bands for the primer OPP 14 in all the TC plants except TCC P_2-7 and TCC P_2-10 (Plate 17c). These two variants exhibited polymorphism compared to the rest of the plants with respect to the absence of one high molecular weight band (which was present in others) as well as presence of one additional low molecular weight band.

In the gel loaded with PCR samples of OPF 13 there were only 13 wells and hence, only first two wells were loaded with DNA samples from clonal plants (Plate 17d). Here also, the polymorphism of TC plants TCC P_2-7 and TCC P_2-10 were very clear. In all the samples, there were a total of seven bands. But the difference was that, there were two additional high molecular weight bands in TCC P_2-7 and TCC P_2-10 but these vines lacked one high molecular weight and one medium molecular weight products present in others.

Pooling the RAPD products from all these four primers in TCC and CC, there were a total of 36 bands (31+additional 5 in variants) out of which, the variant vines TCC P_2-7 and TCC P_2-10 exhibited polymorphism with respect to 11 bands.

(iii) TC clones of P_4

The pattern on agarose gel of this variety using primer OPP 1 indicated no difference between samples except in lane number 11 (Plate 18a) which represented TCC P_4-9. In the

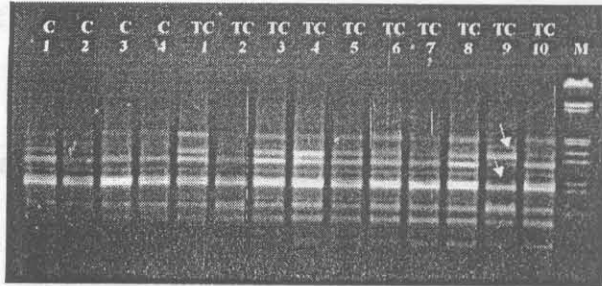# Plate 18. RAPD banding pattern of P_4

18a. Primer OPP1

Monomorphic
Clones : 9
TC (1 to 8,10) : 9

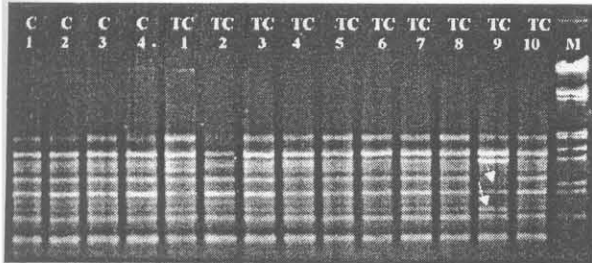Polymorphic
TC P_4-9 : 8

18b. Primer OPP8

Monomorphic
Clones : 12
TC (1 to 8,10) : 12
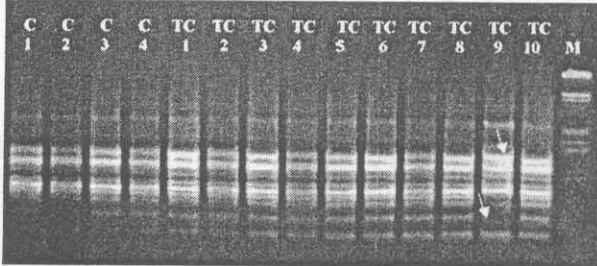
Polymorphic
TC P_4-9 : 10

18c. Primer OPP14

Monomorphic
Clones : 10
TC (1 to 8,10) : 10
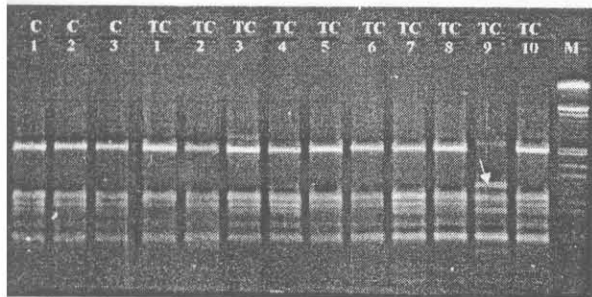
Polymorphic
TC P_4-9 : 10

18d. Primer OPF13

Monomorphic
Clones : 6
TC (1 to 8,10) : 6
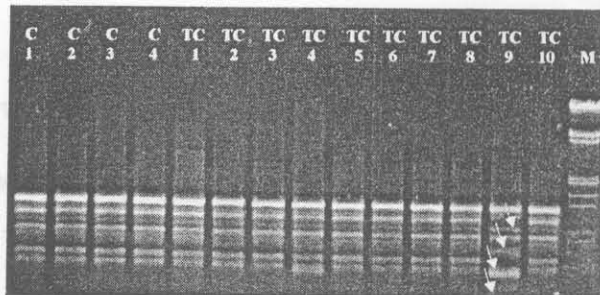
Polymorphic
TC P_4-9 : 7

18e. Primer OPP12

Monomorphic
Clones : 8
TC (1 to 8,10) : 8

Polymorphic
TC P_4-9 : 7

general pattern, there were nine bands whereas in TCC P$_4$-9, there were only eight. The polymorphism was exhibited as the presence of an additional high molecular weight band in between the first and second bands of others and absence of the medium sized fourth and fifth bands present in others.

Primer	General (TCC)	Variant (TC P$_4$-9)
OPP 1	9	8
OPP 8	12	10
OPP 14	10	10
OPF 13	6	7
OPP12	8	7
Total	**45**	**42**

With respect to primer OPP 8 the general pattern showed a total of 12 bands. Here also TCC P$_4$-9 exhibited variation (Plate 18b) in which 7th and 9th bands were absent.

There were 10 PCR products produced by primer OPP 14 in the samples in general (Plate 18c). In TCC P$_4$-9 there was clear polymorphism. Band numbers 1 to 6 found in the general pattern were all present in TCC P$_4$-9 but there was one additional band each between 1st and 2nd as well as between 2nd and 3rd pairs of bands. Also, the 7th and 9th bands were absent. Thus, the total number of bands was 10 in all, but the sizes of some of the fragments were different.

All the samples had six bands each when PCR was carried out with primer OPP13 (Plate18d). In TCC P$_4$-9 one additional band was found which clearly distinguished TCC P$_4$-9 from the rest of the vines.

As an additional test, this variety was tested with one more primer, OPP 12 (Plate 18e). Here also, all the samples were exactly similar with eight bands in general except

TCC P_4-9. In this variant, band number 3, 6 and 7 of the general pattern were absent whereas two additional bands were present, one each between 6th and 7th and after 8th band.

Pooling the RAPD products from all the five primers, there were a total of 51 bands (45+ additional 6 in variant). Out of this the variant vine exhibited polymorphism with respect to 15 bands.

(iv) TC clones of Subhakara

Primer	General (TCC)	Variant (TC Su-9, TC Su-10)
OPP 1	10	7
OPP 8	8	6
OPP 14	10	7
OPF 13	6	5
Total	**34**	**25**

There were 10 bands in total in the different TC plants for OPP1. Among these, TCC Su_9 and TCC Su_{10} were having one additional low molecular weight band, whereas, the four high molecular weight bands present in other TC plants were absent (Plate 19a).

In PCR of TC plants with OPP 8 there were a total of eight bands in general. Among these, TCC Su_9 and TCC Su_{10} were different from the rest by the absence of 1st and 4th bands (Plate 19b).

Among the TC plants the general pattern showed 10 bands for the primer OPP 14 but TCC Su_9 and TCC Su_{10} were different from the rest in that, the first high molecular weight and the fourth medium sized bands present in all the TC plants were absent in these two (Plate 19c). Instead, one low molecular weight band was present just above the last monomorphic band present in the gel.

Plate 19. RAPD banding pattern of Su

19a. Primer OPP1
Monomorphic
TC (1 to 8) : 10
Polymorphic
TC Su-9 : 7
TC Su-10 : 7
Clones : 8
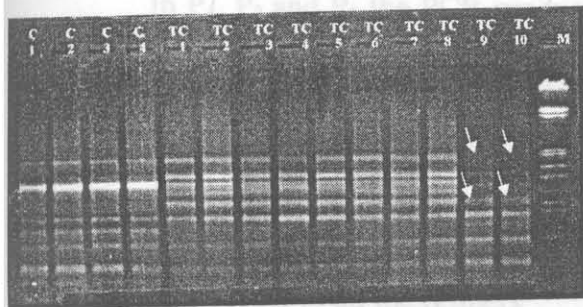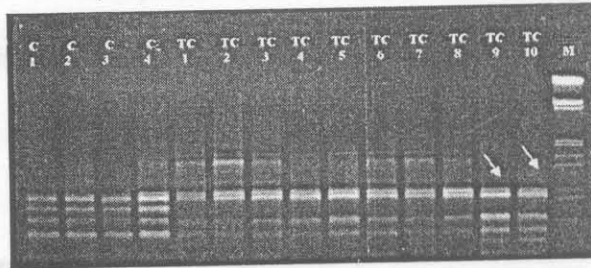
19b. Primer OPP8
Monomorphic
TC (1 to 8) : 8
Polymorphic
TC Su-9 : 6
TC Su-10 : 6
Clones : 6

19c. Primer OPP14
Monomorphic
TC (1 to 8) : 10
Polymorphic
TC Su-9 : 7
TC Su-10 : 7
Clones : 9

19d. Primer OPF13
Monomorphic
TC (1 to 8) : 6
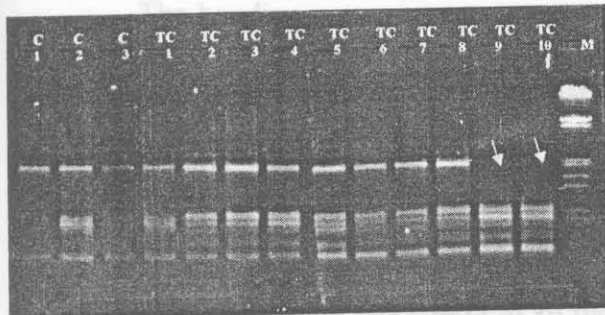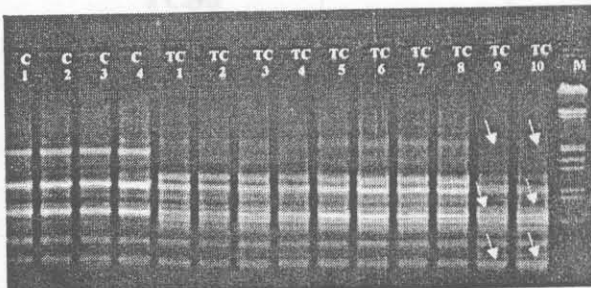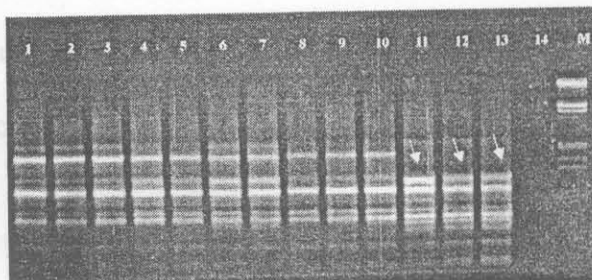Polymorphic
TC Su-9 : 5
TC Su-10 : 5
Clones : 4

19e. Karimunda & Subhakara
(Primer OPP14)
1-3 : CCSu1
4-5 : Karimunda
6-7 : TCKm
8-10 : KS27
11-12 : TCCSu1,TCCSu2
13 : TCCP$_4$-8

The TCC Su$_9$ and TCC Su$_{10}$ were different from the rest of the TC plants with respect to OPF 13 also (Plate 19d). They lacked the first high molecular weight band in others.

Pooling the RAPD products from all the four primers, there were a total of 36 bands (34+ additional 2 in variants). Out of this the variant vines exhibited polymorphism with respect to 11 bands.

4.4.2.2 Inter clonal polymorphism (CC vs. TCC)

In P$_1$, P$_2$ and P$_4$ the PCR products of the CC group as well as TCC group were all monomorphic with respect to the different primers used for characterisation though in P$_1$, a single product of OPF 13 showed polymorphism between CC and TCC group. But in Subhakara, there was profound difference in the banding pattern of CC group and TCC group with respect to all the primers.

Primer	CCSu	TCSu
OPP 1	8	10
OPP 8	5	8
OPP 14	9	10
OPF 13	4	6
Total	26	34

The banding pattern produced by OPP 1 was different in CC group from that of TC samples (Plate 19a). There were five distinct bands and 2 to 3 faint high molecular weight bands in conventional clones. In TC plants, the high molecular weight bands were clearer and 2 additional bands were also there before the 1st clear band in conventional clones. The 3rd clear band in clones was absent in all the TC plants.

For OPP 8 also the conventional clones were found different from TC plants (Plate 19b). There were five monomorphic bands in all. The polymorhism was exhibited

as the presence of one high molecular weight (2nd) band and two medium sized bands (4th and 5th) in TC plants, which were absent in CC group. But for CC group, there was an additional band between 5th and 6th bands of TCC group.

For OPP 14 there were nine bands in conventional clones whereas in TC plants there were 10 (Plate 19c). Out of these, eight were monomorphic in both groups. One high molecular weight band present in all the CC showed polymorphism, as it was absent in all the TCC. Instead, TC plants had a high molecular weight band below this, which was absent in conventional clones. Further down in the gel, there was one more additional band in TC plants between the 6th and 7th bands of conventional clones, which also distinguished the TC plants from CC group.

For OPF 13 the total number of bands in conventional clones was four (in well no.1 and 3 the amplification was not proper) whereas in TC plants, there were six bands (Plate 19d). The extra two bands distinguished the TC plants from the conventional clones.

Since it was clear from these four PCR of Subhakara that there existed some difference between the CC group and TCC group, another PCR was performed using primer OPP 14 and DNA samples collected from one of the conventional clones under the experiment (CC Su_1), Karimunda in field (Km), as it was the parent of Subhakara, TC Karimunda in field (TC Km) and the mother clone of Subhakara (KS27) maintained in the glass house of College of Horticulture, Vellanikkara, along with TCC Su_1, TCC Su_2 and TCC P_4-8 (Plate 19e). The banding pattern clearly showed monomorphic bands in CC Su_1, Km, TC Km and KS27. In TCC Su_1 and TCC Su_2 as well as TCC P_4-8, the second high molecular weight high intensity band was absent and two additional bands were present which were identical in all these three. Comparison of well number 1 to 10 as one group and well no 11 to 13 as the second group unequivocally showed that the two groups differ from each other.

4.4.3 Inter varietal polymorphism

For identifying varietal polymorphism, fingerprinting of the four varieties under study, was done using the 13 selected primers. The varieties were scored based upon the presence (+) or absence (-) of individual polymorphic bands.

Using 13 primers, a total of 89 DNA amplification products (bands) were generated. The bands generated by each primer varied in number and size. The number of bands ranged from 5 to 9 with an average of 6.85. Out of these 89, the polymorphic bands were 40 in number based upon which the four varieties were scored. The numbers of bands that differed in all possible pairs of 4 varieties are presented in Table 20.

Table 20. Extent of RAPD polymorphism between pairs of varieties

Variety	Total bands (13 primers) Mono + Poly = Total			P1	K	A	Su
P_1	49	+ 24	= 73	-			
P_2	49	+ 17	= 66	7	-		
P_4	49	+ 20	= 69	26	27	-	
Su	49	+ 18	= 67	30	31	16	-

They were further subjected to genetic diversity analysis as per Demeke *et al.* (1996). The results showed that maximum similarity was between P_1 and P_2 (GD = 0.1) followed by P_4 and Subhakara (GD = 0.21). P_2 and Subhakara were widely separated with maximum genetic distance (GD = 0.38). The genetic distance between P_1 and Subhakara (0.36) was only slightly lesser than that between P_2 and Subhakara whereas P_1 and P_4 were closer (GD = 0.31).

When the average GD values of RAPD and electrophoretic data of PRX were worked out it was seen that, P_1 and P_2 were genetically more similar among the four varieties compared to any other pair, followed by P_4 and Subhakara (Table 21).

Table 21. Average GD values based on RAPD and PRX data

Marker	Pairs of varieties					
	$P_1 - P_2$	$P_1 - P_4$	P_1 - Su	$P_2 - P_4$	P_2 - Su	P_4 - Su
RAPD	0.10	0.31	0.36	0.33	0.38	0.21
PRX	0.23	0.42	0.50	0.53	0.50	0.33
$\overline{GD}$	0.17	0.37	0.43	0.43	0.44	0.27

Hence these four varieties may be grouped into two with, P_1 and P_2 forming one group and P_4 and Subhakara the other. Between group comparison showed that, P_1 and P_2 were genetically closer than P_4 and Subhakara. The most widely separated pair was P_2 and Subhakara.

4.5 Comparison of efficiency of the different techniques used for characterisation

Depending upon the extent of polymorphism revealed, the different marker systems could be arranged as follows in the increasing order of efficiency

1. Cytological marker (chromosome no.): All had uniform chromosome number

2. Morphological markers: Detected inter clonal and inter varietal polymorphism but the discrimination was not fool proof.

3. Isozyme markers: Detected variations within and between conventional and TC clones as well as between varieties but the zymogram was intricate.

4. RAPD markers: Characterisation of individual vines was very effective and discrimination of the variants was distinct and uncomplicated.

DISCUSSION...

5. DISCUSSION

In the present investigation, attempts were made to screen the TC derived clones of four elite varieties of black pepper for their genetic integrity and trueness to type by simultaneously comparing them with the conventional clones of the corresponding varieties.

Instability from plant tissue culture has been documented in several plant species (Adkins *et al.*, 1995; Bregitzer and Poulson, 1995; Rani *et al.*, 1995). Somaclonal variation is an important tool for crop improvement (Karp, 1995) but is unwanted in mass propagation programmes especially in perennial crops. Several strategies can be used to assess the genetic integrity of *in vitro* raised plants, but most of them have limitations.

For many years, the principles of genetics have been applied to crop variety improvement with great success. Until recently, virtually all procedures for both breeding and model genetic systems have relied upon phenotypic assay of genotype. Classical phenotypic features, such as morphological traits are still extremely useful. But the morphological data are influenced by environmental conditions.

Variation in chromosome number has been reported as one of the reasons for somaclonal variation (Karp and Maddock, 1984). Hence documentation of the karyological state of the *in vitro* raised plants provides valuable information. But it cannot reveal other specific alterations within the chromosomes such as DNA mutations, transpositional changes etc.

The use of molecular markers is becoming widespread for the identification of somaclonal variants and the assessment of micro propagation or *in vitro* regeneration protocols (Heinze and Schmidt, 1995; Wallner *et al.*, 1996). Individual proteins or enzymes are used as markers since they are the direct products of individual genes. But the range of

proteins/ enzymes that can be used as markers is constrained by the number of resolvable proteins/ enzymes that can be visualised as clear bands in an extract from a particular plant organ. Again, environmental effects can influence results to some extent.

RAPD appears particularly suitable for the evaluation of genetic integrity during tissue culture (Isabel *et al.*, 1993; Taylor *et al.*, 1995), the identification of clonal plant material (Castiglione *et al.*, 1993, Rani *et al.*, 1995) and detection of somaclonal variants (Munthali *et al.*, 1996).

Many of the reports include a combination of two or more of the above methods for overcoming the drawbacks of individual systems and thus to generate a wholesome information about the material under study (Prabakaran *et al.*, 1991; Beer *et al.*, 1993; Kongkiatngam *et al.*, 1995; Tatineni *et al.*, 1996; Parani *et al.*, 1997 and Papa *et al.*, 1998) In the present investigation a combination of four different marker systems *viz.*, morphological, cytological, isoenzyme and RAPD analysis were used to characterise the conventionally propagated and micro propagated vines of black pepper.

5.1 MORPHOLOGICAL MARKERS

As this is a perennial crop it was not possible to manipulate the prevailing conditions beyond a particular limit. Care was taken as far as possible to see that both groups received identical management practices in order to reduce the component of environmental influence in the expression of the various morphologic characters.

The clones and micropropagated plants of each variety compared in the present investigation were not originated from the same mother vine as they were generated at two different places. Rival *et al.* (1998) also compared the mother palm and regenerants originated from the same variety but different clones in oil palm. Also the plot from which conventional clones were selected for comparison existed at a distance of about 20 to 25m

from the TC plot. These may affect the expression of the morphological features of individual vines to a certain extent as vine-to-vine variation of the same variety is observed in the crop even under vegetative propagation through cuttings (Ratnambal *et al.*, 1985; Pillai *et al.*, 1985; Geetha and Aravindakshan, 1993; Pillai, 1997). The present investigation also supported this fact as several morphological features of conventionally propagated and TC propagated clones exhibited variation. The conservation of the initial heterozygosity in the vine through several years of vegetative propagation and the action of natural selection upon this genetic constitution may be the reason for such variation. In cranberry also evolution of natural clones has been reported by Novy and Vorsa (1995).

The investigations were carried out using 58 pepper vines among which, 20 were conventionally propagated and 38 were TC derived. Actual sample size for floral characters was slightly lower than this because some vines did not start bearing. Identical studies with comparable sample size were also reported by Isabel *et al.* (1993) for testing the genetic integrity of somatic embryogenic populations of *Picea mariana* using RAPD assay (108 samples), Rani *et al.* (1995) for genetic analysis of micropropagated plants of *Populus deltoids* (23 samples from a batch of about 500 plants) and Kongkiatngam *et al.* (1995) for detecting genetic variation within and between cultivars of red clover (40 plants for RAPD analysis).

The experimental vines were compared with each other with respect to 22 vegetative traits and 19 reproductive traits of quantitative nature. The descriptor of *Piper* sp. developed by NBPGR Regional Station, Vellanikkara as well as earlier research works in black pepper such as that of Nambiar *et al.* (1978), Kanakamany (1982), Ibrahim *et al.* (1985a, 1985b), Sujatha and Namboodiri (1995) and Anand (1997) identified these traits as useful to measure the extent of variability between different varieties of black pepper as well as different *Piper* spp.

5.1.1 Quantitative characters

5.1.1.1 Vegetative characters

The range and mean values of 22 vegetative traits showed that, within each variety, the different conventional clones as well as the different TC propagated clones exhibited certain differences with respect to each other.

a) Intra group variability in conventional and TC clones

When the variances for each of the characters in all the clonal plants were subjected to Bartlets's χ^2 test of homogeneity, it was observed that, except for five characters, the variances of the clonal plants were homogeneous for the vegetative biometric characters irrespective of the different varieties. Similar pattern was seen in TC plants also. So, for these 17 characters, all the 20 clones could be considered as a single population and all the 38 TC plants, as the second population.

b) Inter clonal comparison of mean values of traits (CC vs. TCC)

By pooling the data for 17 vegetative traits, the means and coefficient of variation of CC population for each of these characters could be compared to those of TC population to check whether there is any uniformity between these two populations with respect to these biometric observations.

(i) Leaf characters

From the results of the comparison of mean values of CC group and TCC group, the following inferences could be arrived at. In general, the TC plants had larger leaves than clones. This increased vigour of micro propagated plants is an accepted fact and has been reported in many crops (Swartz *et al.*, 1983, Zimmerman, 1986, Zaman *et al.*, 1997).

During the evaluation of the field performance of one-year-old micropropagated mulberry plants, Zaman *et al.*, (1997) also found significant difference between micropropagated and cutting derived plants with respect to morphological characters.

The variation in leaf length was proportionate to that of leaf width in both populations as indicated by the mean values of L/W ratio. Ibrahim *et al.*, (1985a) also reported the relation between length and width of leaf in black pepper.

Leaf area is basic to many investigations in assessing the potentiality of plant production. Within each variety, the conventional clones and TC plants had comparable leaf area in P_1 and mature leaves of P_4 (the difference was negligible) whereas it was significantly higher in TC plants of P_2 and Subhakara than that of corresponding conventional clones. The increased vigour of TC plants for length and width of leaf was not fully expressed for leaf area. This may be because; the leaf area in black pepper is rather a function of length and length/width ratio rather than its width (Ibrahim *et al.*, 1985). The mean L/W ratio was uniform in TC and conventional clones of all varieties. The petiole length was found more or less uniform in the CC and TCC populations.

(ii) Stem characters

The various stem characters such as length and thickness of internodes and nodes in ortho and plageotropic stems as well as the number and position of branches also indicated an increased vigour in TC clones though not significant. For angle of insertion, when the conventional clones and TC plants of each variety were compared, the mean values were found significantly higher in TC P_2 compared to clones whereas, in all the other three varieties, the means were comparable.

c) Inter clonal comparison of coefficient of variation (CC vs. TCC)

In general, the CV was found higher in CC group for most of the leaf and stem characters (thirteen out of seventeen traits) indicating that, in the micropropagation method the variability was comparatively lower than that in conventional method. For the five heterogenous traits also, the varieties P_1, P_2 and P_4 showed higher variability in conventional clones than TC clones. But for Subhakara, CV of various traits in TC plants was higher compared to conventional clones. When TC Subhakara were compared with the rest of the TC plants, the CV in the former was equivalent to or lower than that in the later group. So, the difference in conventional clones and TC clones of Subhakara must be an indication of either of them being a different variety, which was further tested with molecular markers.

5.1.1.2 Reproductive characters

Since all the experimental vines were only two and three years old at the time of recording the yield characters, these observations provided only a comparison of the rate of growth of the conventional clones and TC plants. These observations may change when the vines reach their full maturity and yield potential.

a) Intra group variability in flowering habits of conventional and TC clones

Among the 58 experimental vines, only around half of the vines flowered in CC group as well as TCC group. This may be because of comparatively sub optimal soil and agroclimatic condition at Panniyur, which restrict the full expression of the yield potential of vines (KAU, 1996). The different varieties were reported to have early bearing and significantly higher yield in areas of Wynad district compared to Panniyur

(Ramankutty, 1977). Apart from this, the extent of shade in the TC plots was found higher during the initial years of establishment of the vines. This may be another reason for the delayed flowering in most of the vines. This also caused incidence of stem borer attack, which slightly affected the vegetative growth of the vines during this period.

b) Interclonal variability in flowering habits of conventional and TC clones

In variety P_1, TC clone showed slightly higher yield. Even though the rate of production of spikes was the same in both, the higher yield in TC clone was due to the higher number of productive laterals. This indicated the increased vigour of TC clone. But this was compensated by better berry characters of conventional clone. Also, water content in berries of TC clones was higher and hence the dry berry weight was lower. But in general, the performances of the two vines were comparable with each other as well as with earlier reports about the general performance of the variety (Ramankutty, 1977; Pillai et al., 1987, 1989; Menon and Nair, 1989 and Babu and Ravindran, 1992).

As there were no clones to be compared in variety P_2, the general characters of the two TC plants were compared between themselves as well as with earlier reports (Sujatha, 1991 and Babu and Ravindran, 1992). The results showed that there was not much variation in the various data though there was vine-to-vine difference. Both of the TC plants had more or less uniform performance and the different observations recorded were typical of the variety as recorded earlier. While spike yield and berry quality were better in TCC P_2–1, other berry characters showed higher values for TCC P_2–8.

Maximum number of vines with more than 25 spikes was recorded in variety P_4 with two conventional clones and two TC clones yielding 51 to 124 spikes per vine. In general, the TC plants were found to have better fruit characters and correspondingly better

yield than the clones. Quality of the berries with respect to oleoresin, piperin and oil was uniform in both. The difference in yield may be attributed to the increased vigour in TC plants, though further studies will be required after stabilization of yield. But in general, the data agree with the corresponding values reported in the variety (Babu and Ravindran, 1992; Sujatha, 1991; Pillai *et al.*, 1989).

In Subhakara, the three vines, which yielded more than 25 spikes, were all belonging to TC plants. In general, these vines had vigorous vegetative growth than conventional clones. Also, the yield characters were found higher when compared to the earlier reports (Babu and Ravindran, 1992), such as spike length, 100 berry weight and volume, weight of 25 spikes and weight of berries in them etc., which were correspondingly reflected in the final green yield per vine. Even though the number of spikes per vine was lesser compared to other varieties, the yield was comparatively high especially in TCC Su_6. The quality of berries was also generally good.

When compared with the earlier reports of varietal characters (Babu and Ravindran, 1992) the values for various fruit and spike characters were found higher in TC clones. But this may not be due to the difference in method of propagation or due to somaclonal variation. All the TC clones exhibited difference from the conventional clones of the variety as discussed earlier with respect to vegetative growth also. Thus the TCC group as a whole was found to differ from the CC group, which was further tested with the molecular markers.

5.1.2 Qualitative characters

These were recorded mainly based on the descriptor developed by NBPGR, with slight modifications. The qualitative traits were found more suitable to distinguish between

varieties rather than between clones of a single variety. These traits could not differentiate between vines within a variety and for P_1, P_2 and P_4, both CC and TCC groups were uniform with respect to these traits. But for Subhakara the two groups differed especially with respect to leaf shape, which is a typical varietal character (Ibrahim *et al.*, 1985a and Sujatha and Namboothiri, 1995). The clones and TC plants of P_1, P_2 and P_4 showed leaf shapes corresponding to the characteristic feature of the respective variety. The conventional Subhakara clones showed the typical varietal character i.e., small leaves with elliptic shape and cuneate base, whereas in TC Subhakara the leaf shape resembled that of P_4. This indicates a deviation from the general pattern and a doubtful genetic identity that was further tested with molecular markers.

5.1.3 Varietal polymorphism

Several vegetative, reproductive and qualitative characters were identified as varietal characters. Among the vegetative characters leaf area as well as number and direction of growth of lateral branches were found efficient to distinguish the varieties.

5.1.3.1 Vegetative traits

In black pepper, leaf area is identified as an important varietal character (Kanakamany, 1982; Ibrahim *et al.*, 1985a; Sujatha and Namboodiri, 1995). The present investigation also provided an additional proof for this fact, because in the χ^2 tests, both among conventional clones and among TC plants, the inter-varietal variability for this character was significantly different. In different varieties, even though leaf area was computed as a function of length and width of the leaf and a constant as suggested by Ibrahim *et al.* (1985a), they also proposed a partial regression model of leaf area based on leaf length and length/width ratio.

The intensity of lateral branch production was reported to be a varietal character, which was influenced by many agroclimatic factors (Chandy and Pillai, 1979). The vines were in their initial stage of growth and had not reached their full growth. Hence, the number of branches produced may vary considerably at this stage. Since this variation was uniform in clones and TC plants, in each of the varieties, it may be considered as the varietal character.

Similarly, Chandy and Pillai (1979) and Pillai *et al.* (1985) also pointed out that, in black pepper, the angle of insertion of laterals on main shoot vary from variety to variety, with some varieties having drooping branches and others with somewhat horizontal or semi-erect or almost erect branches. The present study also supported the fact. This morphological character was reported to have a direct bearing on photosynthetic efficiency of the plant (Sujatha and Namboothiri, 1995) since the intensity of sunlight falling on the leaves varies accordingly. The mean values showed that the variety P_2 had semi erect branches whereas in others, the angle was greater, giving a horizontal arrangement of laterals.

5.1.3.2 Reproductive traits

Reproductive traits related to spike and berries were also found to vary with variety. The typical long spike with bold, round berries of P_1 was reported earlier by several workers (Pillai *et al.*, 1987; Babu and Ravindran, 1992; Ramankutty, 1977 and Ibrahim *et al* 1986a). Similarly the varietal difference with respect to these traits was also reported in P_2, P_4 and Subhakara (Pillai *et al.*, 1989; Babu and Ravindran, 1992).

5.1.3.3 Qualitative characters

Based upon the descriptor each of the variety was characterised for the qualitative traits also. It was found that many characters were more or less identical even between varieties but there were some, which exhibited variation. Characters such as anthocyanin pigmentation on various parts (leaf sheath, tender leaves, tender stem, petiole and stipules), leaf shape, leaf base, stoutness of stem, clinging ability of adventitious roots, sex form in flower, berry shape, berry colour on maturity and time taken for maturity etc., were found to vary between varieties.

The leaf shape was identified as a varietal character and in P_1 the large cordate leaves were characteristic of the variety whereas in typical Subhakara, like its parent Karimunda, small, elliptic leaves helped in identification of the variety (Kanakamany, 1982 and Sujatha, 1991).

Anthocyanin pigmentation of stipules was reported in many varieties of black pepper and was found to have a dominant-recessive intra allelic interaction wherein; the allele favouring pigmentation was dominant (Ibrahim et al., 1986b). P_1 and P_2 were reported to be devoid of such pigmentation while P_4 and Subhakara were pigmented varieties. The present study also supported this observation.

The vigorous nature of vine with strong stout stem, bold and spherical berries, 99.9% hermaphrodite flowers etc. were characteristic of P_1, whereas P_2 was with ovate leaves, moderately sized berries, 96.7% hermaphrodite flowers. These observations are in agreement with earlier reports (Pillai et al., 1985, Ibrahim et al., 1985b, Sujatha, 1991, Babu and Ravindran, 1992). The late maturity of P_4 was reported as the characteristic of the variety (Pillai et al., 1989). Based upon the descriptor, it was found that, P_1 and P_2 shared some of the characters while Subhakara and P_4 were closer to each other.

5.2 CYTOLOGICAL ANALYSIS

Chromosomal instability and resulting variation during *in vitro* culture, which leads to somaclonal variation especially in regenerants from callus or cell suspension cultures has been reported to be a frequent phenomenon (Larkin and Scowcroft, 1981; review by Evans and Reed 1981; Karp *et al.*, 1989a and 1989b; Karp,1995).

Among the various methods developed to micropropagate plants, enhanced axillary branching culture has become the most important propagation method due to its simplicity, high rate of multiplication and low risk of genetic instability. Notwithstanding these considerations, there are numerous reports on the incidence of somaclonal variation among micropropagated plants as reviewed by Rani *et al.* (1995).

The loss or gain in a chromosome will normally be exhibited by a drastic change in morphology. In the present investigation, the vines under evaluation did not show any such unusual phenotypes. Even then in order to have a systematic and scrupulous characterization, the somatic chromosome number of all the vines were also checked.

5.2.1 Standardisation of protocol

Jose (1981) and Rahiman and Nair (1983) in their review about cytological investigations in *Piper* pointed out that, reports on chromosome morphology in the genus have been limited, probably due to the large number of small sized chromosomes. Most of the reports pointed out that somatic chromosome number in black pepper is 52 with their length varying from 1 to 3µ (Mathew, 1958 and 1973), 0.7 to 3.4µ (Sharma and Bhattacharya, 1959) and 0.77 to 2.3µ (Dasgupta and Datta, 1976). Mathew (1973) also reported that the chromosomes were characterised by indistinct centromere positions and he found that the absolute length of 52 chromosomes in different varieties vary from 62 to 74µm. These features make the cytological analysis in black pepper, a tedious process. The

present investigation resulted in the refinement of previous procedures suggested starting from the time and stage of collection of roots up to slide preparation, in order to suit the *Piper nigrum* karyology.

5.2.1.1 Season of collection

Standardisation of the protocol for root-tip squash technique in black pepper revealed that, season of collection of the roots play an important role in obtaining good quality slides. It was found that the best period was during June to July, which is the southwest monsoon season with heavy rains, high humidity and low temperature. It is a well-known fact that the best period of planting pepper is during *Thiruvathira njattuvela* since there is profuse rooting during this period (Pillai *et al.*, 1985). This may be the reason for obtaining better slides during this period. Since the pepper chromosomes were comparatively smaller in size and more in number, obtaining cells with well spread chromosomes was very difficult. As there were copious fresh, fleshy roots available during this period, the chances of obtaining such cells were more. Such effect of season of collection on the quality of slides prepared is being reported for the first time.

5.2.1.2 Age of cutting

Rooted cuttings of 3 to 4 weeks old were found ideal for collection of roots. At this stage a large number of short, white, fleshy and robust roots were obtained from the cuttings. If younger, the number of good quality roots obtained were less and if older, the roots started elongating, became narrow and brown coloured, even though with white tips. The chances of obtaining large number of good, dividing cells from such roots were observed to be less. This may be because, in such roots due to aging the rate of cell division may be comparatively less. Mathew (1958 and 1973), Dasgupta and Datta (1976),

Jose (1981) and Nair *et al.* (1991) also reported using rooted cuttings for collection of roots but they have not specified the age of the cutting.

5.2.1.3 Time of collection

The time between 1.30 and 2.30 pm. was found good for the collection of root tips so that there were numerous dividing cells with most of them being at the metaphase stage. After 2.30pm, most of the cells were found in the anaphase or telophase stage. Earlier periods (between 11 and 12.30pm) were reported in black pepper by Nair *et al.* (1991) and Anand (1997) and also in *Pennisetum* by Prabakaran *et al.* (1991). The difference in the optimum time may be due to the influence of sunshine hours on the time of active division.

5.2.1.4 Root tip squash technique

Pre-treating the root tips in 0.05 per cent 8-hydroxiquinoline at 4^0C followed by fixation in Carnoy's fluid for 1 to 2 days was found ideal for getting maximum number of cells in metaphase stage and for efficient killing and fixing of the cells in their life-like condition. The efficiency of these chemicals was reported earlier by many scientists (Mathew 1958, 1973; Dasgupta and Datta, 1976; Jose, 1981; Mathew and Mathew, 1982; StellaBai and Subramanian, 1985; Nair *et al.*, 1991 and Versha and Ravi, 1995. Mathew (1958) also reported better spread of chromosomes by cooling prior to fixation.

Hydrolysis of roots was carried out with 1N HCl. From the 12 different combinations of temperature and time of hydrolysis experimented with; the best combination obtained was 60^0C for 15 minutes. This helped to dissolve the middle lamella and thus to separate the cells, so that individual cells could be studied. Also the separation from the compact tissue structure, helped in the expansion of cell slightly, thus giving more space for the chromosomes to spread. The advantageous effect of dilute acids as

hydrolysing agents has been reported by Sharma (1991), Prabakaran *et al.* (1991), Nair *et al.* (1991) and Versha and Ravi (1995).

Among the various stains specific to chromosomes, aceto orcein was found better as it gave intensely dark-meroon colour to the chromosomes, with clear cytoplasm. Samuel and Bavappa (1981) and Anand (1997) also reported that aceto-orcein gave better results in *Piper* spp. than aceto carmine. There are several other reports also stating the use of orcein as the stain for karyo-morphological studies in pepper and various other crops (Dasgupta and Datta 1976; Jose, 1981; StellaBai and Subramanian, 1985 and Prabakaran *et al.*, 1991).

5.2.2 Somatic chromosome number

The slides were prepared for microscopic examination by root tip squash technique for each of the TC as well as conventional clones of the four varieties and the chromosome number was counted as 2n = 52 in all. There was no variation in the chromosome number of the vines under study due to the difference in mode of propagation such as by vegetative propagation through cuttings or by micro-propagation using single noded cuttings. This is in agreement with earlier reports about somatic chromosome number in *Piper nigrum* being 2n = 52 (Mathew, 1958 and 1973; Martin and Gregory, 1962; Samuel and Bavappa, 1981; Mathew and Mathew 1982; Jose and Sharma, 1983; Samuel, 1986 and Anand, 1997).

5.3 ISOZYME ANALYSIS

The use of genetic information expressed in isozyme form has enhanced our understanding of heritable variations within and among plant populations. For over four decades now, protein electrophoresis coupled with histochemical staining has provided data on the concordance of observable features in plants such as morphology, cytology, ecological adaptation etc., with the isozyme phenotypes.

In electophoretic analysis, the physical properties of enzymes or proteins such as number, spacing and intensity of the bands are compared based on the hypothesis that these properties, to a large extent, are the record of the accumulated mutations that have taken place in the gene specifying that particular enzyme or protein (Gottlieb, 1977).

In vegetatively propagated crops, characterisation of vines through isozyme analysis is possible mainly by band-to-band comparison of enzyme profiles. Accordingly, the isozyme banding pattern obtained for each vine for each enzyme system could be treated as 'phenotypes' based on which characterisation is possible (DeWald et al., 1988). The use of isozyme technique for finger printing requires high level of clearly recognizable polymorphism, stable enzyme phenotype over a wide variety of environmental and developmental conditions and a simple and efficient method to characterise the enzyme phenotype.

The black pepper appears to be an ideal candidate for electrophoretic characterization using isozyme polymorphisms due to the high level of intraspecific genetic variation. The conventional and TC clones of the four varieties were characterized using isozymes of PRX and GOT and the enzyme profiles were compared to detect any polymorphism within and between the two groups.

5.3.1 Standardisation of protocol

So many variables were found to influence the preparation and analysis of gels for isozyme polymorphism. Interference of compounds such as polyphenols often prevented clear resolution of isozyme bands. Arulsekhar et al. (1985) and Kephart (1990) also reported the limited use of isozyme studies in perennial crops and in plants of various ploidy levels and phylogenetic histories due to the difficulties in producing good enzyme activity in gels.

5.3.1.1 Extraction of enzyme

a) Choice of tissue

In black pepper fresh leaves collected under cold conditions were found to be the best material for enzyme extraction. Leaves stored at -20°C in a deep freezer were unsuitable for extraction as they became brownish due to polyphenol oxidation. This was due to the rupturing of the cell by the formation of ice. During this process the phenolics were released and get oxidised to quinones, which produced the brown colour. Once the quinones are formed, it will inhibit the enzymes and hence such leaves are unsuitable. Storing at 4°C by keeping in a refrigerator did not show such cell rupturing and were similar to fresh leaves even after one or two hours. Many previous reports suggested the use of fresh leaves as the best material for the enzyme extraction (Wendel and Weeden, 1989; Kephart, 1990; Bhat *et al.*, 1992a, 1992b and Reyes *et al.*, 1998).

b) Stage of growth

The stage of growth of the leaf was determined as per the procedure followed by Conklin and Smith (1971) and Sebastian (1995). 1st to 3rd tender pale green leaves from the tip as well as 5th to 7th dark green mature leaves were found suitable for extraction of GOT in black pepper. Sebastian (1995) also reported that both tender and mature leaves were suitable for GOT extraction in this crop. For peroxidase, the tender leaves were found unsuitable for extraction as the band intensity was very low and also, certain zones of activity were completely absent. This is in conformation with earlier reports (Scandalios 1969; Racusen and Foote, 1966; Hart and Bhatia, 1967; Siegel and Galston, 1967; Upadhyaya and Yee, 1968; Bhatia and Nilson, 1969; Conklin and Smith, 1971; Sebastian, 1995 and Joseph, 1999). As the age of plant part increases, there is an increase in lignification process. In mature leaves this is needed to strengthen the cell wall and middle

lamella. The role of PRX in lignification has been reported by Gasper *et al.* (1975) and Thorpe *et al.* (1978), which explain its increased activity in mature leaves.

The tender leaves had the advantage of low interference due to chlorophyll and were easy to grind. For mature leaves addition of a pinch of acid washed sand while grinding helped in easy rupturing of the tissue. Addition of Tryton X-100 in extremely low volume (below 5µl) improved the intensity of colour but if the volume of Tryton increased, the chlorophyll interference was very high especially in mature leaves. Use of TrytonX-100 for release of enzymes from subcellular compartments and organelles have been reported by Wendel and Weeden (1989) and Kephart (1990). Even after double centrifugation at 15,000 rpm for 10 minutes each, the extract was found intensely green.

Both green house and glass house grown plants as well as field grown plants did not show any difference in pattern proving the absence of influence of environment on enzymatic activity. Conklin and Smith (1971) also had identical results.

c) Extraction buffer

Kelley and Adams (1977) has summed up the importance of standardisation of extraction buffer and buffer additives for isozyme assay in different tissues in one sentence, 'the optimal extraction buffer that yields the sharpest bands for the broadest spectrum of isozymes vary for any given tissue for all enzymes or, any given enzyme from all taxa'. In the present investigation also, the type, molarity and pH of the extraction buffer as well as the quantity and type of the phenol complexing agents added along with the buffer were found to have tremendous effect on the intensity and clarity of the isozyme bands in the gel.

The initial screening of 30 different combinations of buffer type, molarity and pH showed that Tris buffer at 0.1M concentration and pH 7.6 as well as Sodium phosphate buffer at 0.1M concentration and pH 7.5 could be used for enzyme extraction in black

pepper. Comparison between Tris system and Phosphate system showed that Tris buffer was more suitable. Many earlier reports identified Tris-Cl system as most suitable for a large number of taxa (Nisselbaum and Kopelovich, 1975; Weeden *et al.*, 1988; Garvin *et al.*, 1989; Visedo *et al.*, 1990; Arunachalam *et al.*, 1996 and Reyes *et al.*, 1998). This was further refined by increasing the molarity of Tris to 0.15M from 0.1M. The increase in molarity helped to increase the buffer capacity.

These simple buffers alone were not sufficient to prevent the phenolic degradation of the enzyme systems in black pepper, it necessitated the addition of several buffer ingredients (Van Sumere *et al.*, 1975; Kelley and Adams, 1977 and Kephart, 1990). Wendel and Weeden (1989) and Kephart (1990), in their review also pointed out the use of complex buffer systems in crops with high phenolic activity. Based on these reports, several buffer additives were tried at different concentrations. Among these, sodium metabisulfite was found most effective to prevent browning during cellular disruption. It acted as a powerful reducing agent and could be added either directly while grinding or after dissolving in the extraction buffer, the final concentration being 0.1M. The latter method was found more suitable because, the pH of the buffer could be checked after addition and corrected, if required. Addition of L-ascorbic acid, another powerful antioxidant (0.1M) before adjusting the pH was also useful for better quality of the extract. Arulsekar *et al.* (1985), Wickneswari (1990) and Arunachalam *et al.* (1996) also reported the use of ascorbic acid in extraction buffers. Other antioxidants like mercaptoethanol, DTT and PMSF were found effective in preventing browning of the extract. But sodium metabisulfite and ascorbic acid gave good results and also, they were not as hazardous and costly as the former group. Apart from these reasons, Bergmeyer *et al.*, (1974) opined that low activities of enzymes might not only be due to too mild extracting agents, but also too extensive extraction.

The ratio of tissue to buffer was also found critical, the optimum being 4:5. If the amount of tissue or the volume of buffer was below this, the enzyme extracted was not sufficient to produce clear zones of activity. This may be either due to low enzymatic activity per volume of tissue of black pepper or insufficient buffering capacity due to low volume of the buffer or both. If the buffer volume increased beyond this optimum, the clarity of zymogram reduced due to excessive dilution. These conclusions were also supported by the review of the aspect by Wendel and Weeden (1989) and Kephart (1990) as well as the report by Sebastian (1995) in *Piper* spp.

Centrifugation of the extract at 15000rpm for 15 minutes in a refrigerated centrifuge provided a clear extract without any cellular debris, which when present, resulted in streaking of the enzyme bands. Such streaking effects were also reported by Wendel and Weeden (1989) and Kephart (1990).

The storing of the supernatant at -20°C after centrifugation did not produce any decrease in activity. This is in agreement with the reports of Stuber and Goodman (1983), Wendel and Weeden (1989) and Kephart (1990).

5.3.1.2 Electrophoresis

a) Electrophoretic media

Polyacrylamide gel was selected based upon the reports by Wilkinson (1970) and Blackshear (1984). The protocols of gel preparation as per procedure Type III and Type IV was found more suitable for vertical slab gel (16cm x 14cm) as the gel distortion was less compared to that of Type I and Type II. This may be due to the difference in the addition of TEMED and APS. In the former, these chemicals were much diluted before mixing the different working solutions so that their distribution was more uniform. In the latter, these

were added directly as low volume concentrated solutions. So polymerisation might have started immediately at the point of contact and if there was no thorough mixing (especially when the volume is more), the polymerisation may not be uniform resulting in distortion of gel. This seems to be the reason for the advantageous effect of procedure Type III and IV.

Peroxidase isozyme molecules required a gel with larger pore size (8%) whereas GOT isozymes required closer sieve (10%) for better separation. This is in agreement with the results reported by Sebastian (1995) even though the concentrations reported were different. The gel casting was done in a cool chamber as polymerisation is an exothermic reaction and the gel was kept at 4^0C throughout the experiment as suggested by Wendel and Weeden (1989).

b) Buffer system for electrophoresis

The molarity of the buffer system (including both gel buffer as well as electrode buffer) was found to be the most crucial factor among all others. There were many reports that stressed the importance of this aspect in the migration and separation of the enzyme (Wilkinson, 1970; Andrews, 1981; Pasteur et al., 1988 and Wendel and Weeden, 1989). Even with the best combinations of the various factors like extraction buffer, phenol complexing or reducing agents, gel concentration, electrophoretic conditions and staining procedure, the migration and separation of the isozymes were not proper. This necessitated experimenting with the molarity of buffer system. This was based upon the principle that, at low ionic strength of the buffer in gel, the proportion of current carried by the buffer will decrease and the share of the current carried by the sample will increase, thus increasing its rate of migration and separation. Also, a low ionic strength of buffer reduces the overall current and results in less heat production.

As a result, considerable reduction of the molarity of gel buffer and slight increase in the molarity of electrode buffer was found advantageous. The best combination of various factors identified was Type IV gel polymerisation procedure using 0.0375M resolving gel buffer (pH 8.9), 0.0125M stacking gel buffer (pH 7) and 0.0125M – 0.096M Tris-Glycine electrode buffer (pH 8.3). Many of the earlier reports with varying buffer systems were also found to have low molarity of gel and electrode buffer (Everette *et al.*, 1985; Arulsekar *et al.*, 1985; Weeden and Marx 1987; Weeden *et al.*, 1988; Visedo *et al.*, 1990 and Kertadikara and Pratt, 1995).

c) Sample loading

Electrophoresis was carried out in a vertical electrophoresis slab gel unit with polyacrylamide gel of size 16 cm x 14 cm and 1.5 mm thickness. Sample was loaded in each well after the loading 1 to 3µl of the tracking dye, bromophenolblue, dissolved in Tris-Cl buffer at pH 6.7, at the cathode end. When the quantity of the sample loaded per well was low, the intensity of the enzyme band in the gel decreased. Also the method of mixing the sample with the dye solution in different ratios before loading resulted in further dilution of the sample and consequently, reduction in band intensity. Wilkinson (1970) and Kephart (1990) also reported such effects and suggested to increase the amount of sample loaded to the gel or stained (by using a thicker gel) for improving the band intensity. The optimum gel thickness for practical purpose was reported to be 1.5mm (Wiem, 1974).

d) Run condition

At a constant current of 20mA and 250 to 350v for 3 to 4 hours the dye front reached 12 to 14cm from the origin of the gel and the system was put off. In certain cases for better separation of the zones the elecrophoresis was continued till the dye front entered into the

lower tank buffer. For dissipation of the heat generated during the electrophoresis, the system was kept in a refrigerated chamber as suggested by Wieme (1974) and Wendel and Weeden (1989).

5.3.1.3 Enzyme visualisation

The zymograms developed using peroxidase staining technique (Shaw and Koen, 1968) was clear and with several well separated bands. The complex zymogram of PRX has also been reported by Liu (1975). In GOT even after experimenting with a variety of combinations of various factors such as extraction buffer, additives to prevent phenol oxidase activity, pH, molarity and type of buffer system (extraction, gel, electrode and stain buffers), gel concentration, molarity of the substrates for staining etc., only certain zones could be identified with no clear separation of bands in these zones. Also the enzyme activity zones were comparatively clearer in the zymograms developed using samples collected from 2 to 3 months old rooted cuttings from glasshouse. After 1year, the samples collected from the same cuttings were found to produce only fainter zones of activity. This may be due to the influence of stage of growth of the plant. Scandalios (1969) based on a study in maize catalase concluded that, an isozyme might be absent at a specific developmental stage or occur in such a low concentration that it cannot be detected.

The physiological and ontogenic condition of the sample has been reported to be crucial for isozyme studies by Hart and Bhatia (1967), Conklin and Smith (1971) and Wendel and Weeden (1989). Also it was reported that, the electrophoretic phenotypes of the same tissue may vary in intensity of bands or appearance or disappearance of bands which were correlated with factors such as leaf position / developmental stage, season etc (Endo, 1981 and Tyson et al., 1985).

Another reason for the fewer zones of activity for GOT may be the lesser number of structural genes, which code for GOT compared to that of PRX. Scandalios (1969), Rick *et al.*, (1974) Smith and Conklin (1975) and Gottlieb (1977) pointed out that for peroxidases, single individuals displayed complex patterns with as many as 10 to 15 bands because, they possessed numerous gene loci that code different molecular forms. In contrast, other enzymes were specified by a single or few bands following electrophoresis.

5.3.2 Nomenclature

It was observed that, there were certain major zones of activity for PRX, which again were resolved into bands. These zones may be representing the individual locus coded by single structural gene and the bands within each locus may be coded by different alleles of the same gene. Such zones of activity with bands within were also reported by Gottlieb (1977) and Oliver and Martinez-Zapater (1985) based on genetic analysis these zones and bands were differentiated as isozymes and allozymes respectively. Such differentiation into isozymes and allozymes require detailed genetic analysis using parental and progeny populations, which is not within the purview of this study as pepper is a perennial crop. Hence, these regions of enzyme activity were designated as the major zones only and the bands within each zone, as isozymes of the enzyme.

5.3.3 Isozymes of PRX for characterisation

The final protocol developed with considerable reduction in the molarity of gel buffer was found efficient to resolve the different bands present in the three zones of PRX activity detected in the gel. When all the isozymes from the four different varieties were pooled, there were 16 isoperoxidases in total. Sebastian (1995) reported 14 isoperoxidases in an experiment involving different *Piper* species. Occurrence of several zones of activity for

peroxidase was also reported by Brewbaker and Hasegava (1975), Oliver and Martinez–Zapater (1985), Weeden and Marx (1987), Weeden *et al.* (1988), Wickneswari (1990), Reyes *et al.* (1998) and Alarmelu *et al.* (1999).

During the electrophoresis for the standardization of the final protocol, two other species of *Piper* were also included as checks, *viz., P. colubrinum* and *P. longum.* For characterisation of individual vines, the relative mobility (Rm) of the bands were calculated based on one of the prominent fast moving band with high intensity in *P. colubrinum.* The reason to follow this method was the improper separation of bands between the fastest zone PRX - I and the next zone PRX - II, when the run was limited to 3 to 4 hours. Only under longer run periods (up to 5h), the PRX - I moved further down to have a clear separation of the two zones. But as the dye front moves out of the gel by this time, there was no standard band to compare the Rm values of the different bands during the different electrophoresis. The calculation of relative mobility based on a related variety included as a marker uniformly in all the gels was reported earlier (Oliver and Martinez-Zapater, 1985).

The Rm values of the 16 bands ranged from 1.12 to 0.45. The zone with maximum anodal migration rate PRX – I was distributed between the Rm values 1.12 and 0.94, with seven bands. In the second zone PRX-II, there were eight isozymes distributed between the Rm values of 0.92 and 0.68. In general, the isozymes in this zone were fainter. The third zone was present only in vines of variety P_4 and TC Su with a single band (Rm = 0.45).

It was found that the younger leaves lacked 4 to 5 bands present in the mature leaves and also, in the former the intensity of bands in general, was less. Using these isozymes, the conventional and TC clones were characterized by subjecting them to electrophoresis separately for each variety.

5.3.3.1 Intra group characterisation

<u>a) Characterisation of conventional clones</u>

The conventional clones under each variety were having identical zymograms. Hence the variations exhibited by the morphological traits may be neglected, as they have no genetic basis. The statistical analysis of the vegetative traits also supported this as for 17 out of 22 characters, the variances between all the 20 clones were homogeneous irrespective of the varieties. Only the most specific varietal characters like leaf area and branching habits showed heterogeneity. Price *et al.* (1984) and Beer *et al.* (1993) reported that, the level of isozyme diversity was a useful predictor of the relative level of morphological diversity even though the correlation between the two was not very high.

<u>b) Characterisation of TC clones</u>

Unlike in the case of conventional clones zymogram patterns of TC clones under each variety exhibited certain variants. There was one variant each in TC clones of variety P_1 and P_2 whereas in P_4, two TC clones showed polymorphism. In TCC P_1–1 the polymorphism was exhibited as two additional bands whereas in P_2, the variant TCC P_2–7 lacked 5 bands present in the rest of the TC clones. In P_4, both the variants TCC P_4–4 and TCC P_4–8 showed monomorphism between them but when compared with the rest of TC clones of the variety, they lacked four bands. In variety Subhakara, all TC clones were monomorphic.

But these variants were undetectable by morphological traits. Beer *et al.* (1993) also reported such instances where, they had specifically stated that, though the isozyme diversity helped in prediction of relative level of morphological diversity, the correlation between isozyme and morphological proximities was too low for accessions of *Avena steriliz*. There are several other reports, which also indicated that isozyme and

morphological variations were often not correlated (Giles, 1984; Jain *et al.*, 1980 and Perry *et al.*, 1991).

5.3.3.2 Inter clonal polymorphism

When the conventional clones of each variety were compared with the corresponding TC clones, in varieties P_1, P_2 and P_4 the two groups were monomorphic with respect to all the bands. It was observed that the characteristic bands for each variety were present in both CC group and TCC group. But unlike these varieties, in Subhakara, CC group differed from the TCC group by five additional bands present in the later (two slow moving bands of the zone PRX-I and three slow moving bands of PRX-II). The morphological markers also pointed out the difference between these two groups in the variety. Further, both markers indicated similarity of TC Subhakara with P_4 though not proved conclusively.

5.3.4 Isozymes of GOT for characterisation

The electrophoresis and staining for enzyme GOT was not as efficient as peroxidase due to the low intensity and poor resolution of the bands. Even then three major zones of activity and eight bands distributed in these three zones were identified. But only varietal discrimination was possible as the band resolution and intensity was very poor in electrophoresis of the conventional and TC clones of the different varieties. Even after several combinations of the various factors affecting the enzyme extraction, electrophoresis and staining the results were not promising.

To test the efficiency of protocol to detect GOT activity, electrophoresis was done with samples from related and unrelated genera such as *P. colubrinum*, *P. longum*, as well as *Myristica fragrance*, also a spice with high phenol content. The very high intensity of GOT obtained for *Myristica fragrance* proved the efficiency of the protocol. But for the

other two *Piper* spp. the intensity was similar to that of *P. nigrum*. One of the reasons for this low intensity of GOT in *Piper* may be genetic, where, the activity of the enzyme may be very low or controlled by limited number of loci. The limited number of loci for GOT was also reported in several crops such as date palm (Torres and Tisserat, 1980), pea (Weeden and Marx, 1984), walnut (Arulsekar *et al.*, 1985). Boyle *et al.* (1990) reported only two bands in pine and among them, the slower band was very indistinct and considered unsuitable for analysis.

Other possible reasons include physiological condition (Hart and Bhatia, 1967; Conklin and Smith, 1971 and Wendel and Weeden, 1989), developmental stage and season (Scandalios, 1969; Thorpe *et al.*, 1978; Endo, 1981; Everette *et al.*, 1985 and Tyson *et al.*, 1985) or interference of regulatory genes (Nishikawa and Nobuhara, 1971 and Smith and Conklin, 1975).

Conklin and Smith (1971) also stated that, in developmental studies, the changing pattern of the electrophoretic bands of isozymes could be interpreted as evidence of different gene activations at the various ontogenetic stages sampled. To identify the exact reason further studies are required.

5.3.5 Varietal polymorphism

Varietal characterisation was carried out using peroxidase. From three different experiments, each involving four varieties of black pepper, it was found that the varieties differed from each other with respect to peroxidase isozymes. In general, there were two major zones of activity in the green leaf sheathed varieties *viz*, P_1 and P_2 whereas one additional slow moving zone with a single isozyme was found in the red leaf sheathed varieties *viz*, P_4 and TC Subhakara.

There were 7 bands in the zone PRX-I, which was the zone with maximum anodal migration rate. Out of these, two isoperoxidases were monomorphic among the four varieties whereas five were polymorphic. PRX-I (5), the band corresponding to the standard band of *P.colubrinum* was the most prominent and intensely stained band in all varieties. But in P_1, this was comparatively of less intensity than in other varieties. This may be considered as a characteristic of P_1. Smith and Conklin (1975) reported that apart from the electrophoretic characters such as number and migration rate of the different isozymes, intensity was also a significant character for studying polymorphisms. In this zone, P_4 and Subhakara shared all four bands. Among the five polymorphic bands, PRX-I (2) and PRX-I (3) were characteristic of P_2, as the other three varieties were lacking these two. Apart from these two bands and PRX-I (6), P_2 resembled P_1.

In the second zone PRX-II there were eight isozymes of which, three were monomorphic. Of the five polymorphic bands, three were monomorphic when varieties P_1 and P_2 alone were considered. These two differed from each other with respect to two bands, whereas P_4 and Subhakara clearly differed with respect to four isozymes, three of which were present in Subhakara and one in P_4.

In the third zone, P_4 and TC Subhakara were identical with the presence of the single band of the zone whereas P_1 and P_2 were identical with respect to absence of this isozyme.

Among the four varieties P_2 had the largest number of isozymes; 13 out of the total 16 isoperoxidases in black pepper were present in this variety. There were two isozymes, which were characteristic of only this variety *viz.*, PRX-I (2) and PRX-I (3). P_4 was characterised by an additional slow migrating zone with a single isozyme. The comparison of zymograms of conventional clones of Subhakara, TC Subhakara and P_4 showed that TC subhakara was closer to P_4. Conventional clones of Subhakara lacked four isozymes, two fast moving (Rm = 0.96, 0.94) and three slow moving (Rm = 0.76, 0.72, 0.45) which were

present in TC subhakara. P_4 and TC plants of Subhakara showed more similar zymograms as they differed only with respect to PRX-II where, TC subhakara had six isozymes with Rm values 0.88, 0.84, 0.82, 0.80, 0.76 and 0.72 that were closely arranged. P_4 shared three out of these i.e. 0.84, 0.80 and 0.76 and had an additional slow moving band of Rm = 0.68. So in general, it appeared that to a certain extent, P_1 and P_2 resembled each other whereas P_4 and Subhakara resembled each other with respect to isoperoxidase banding pattern.

The genetic diversity analysis carried out supported this observation. It was found that, P_1 and P_2 were genetically closer (GD = 0.23) whereas P_4 was closer to Subhakara (GD = 0.33). The extent of similarity was found more in the former group.

Characterisation of the four varieties with GOT was also tried. For this enzyme, there were 8 isozymes in total distributed in the three major zones, of which, Subhakara had all the eight. P_1, P_2 and P_4 were monomorphic with respect to the zones GOT-I and GOT-II. The two fastest isozymes *viz.*, GOT-I (1) and GOT-I (2) as well as the slowest band *viz.*, GOT-III (1) were typical of variety Subhakara. Also, the zone GOT-II had higher intensity in this variety, compared to others.

5.4 RAPD ANALYSIS

The cultivated black pepper appears to be an ideal candidate for characterisation using molecular markers. There is a high level of intra specific genetic variation, while inter clonal variability would be expected to be low due to the vegetative propagation. In TC derived and conventionally propagated clones, the assessment of intra clonal variation, if any, using the present system of characterisation based on morphological descriptor alone is not sufficient. The reasons are, it is time consuming as both vegetative and reproductive characters have to be recorded, is influenced by environment and also, the existing morphological keys are not sufficient to discriminate between clones. The present study

revealed that, RAPD analysis can be effectively used in black pepper for monitoring inter clonal as well as inter varietal variation.

5.4.1 Standardisation of protocol

Newbury and Ford-Lloyd (1993) and Karp et al. (1997) have opined that for a given plant genotype the RAPD markers generated by a particular primer will not be clear and consistent unless a specific set of reaction conditions was standardised for the crop and rigidly followed henceforth.

5.4.4.1 Tissue sample

The fresh tender leaf sample (1g) was enough to provide good yield of good quality DNA. Most of the earlier reports mentioned the use of fresh leaf samples, the quantity varying from 0.01g to 2g (Vierling and Nguyen, 1992; Mori et al., 1993; Takemori et al., 1994; Bennet et al., 1995; Rani et al., 1995 and Damasco et al., 1996).

5.4.1.2 DNA isolation

The grinding in liquid nitrogen was found to improve the quality of DNA isolated. On grinding, the leaves of black pepper turned brown immediately due to phenolic oxidation. The quinones produced were known to be powerful oxidising agents, which damage DNA and proteins (Weising et al., 1995; Ram and Sreenath, 1999). The addition of antioxidants like β-mercaptoethanol and sodium metabisulfite in the extraction buffer or during grinding was found effective. Similar results were reported by Rogers and Bendich (1994) and also in coffee, which is a crop with high phenols (Ram and Sreenath, 1999).

The CTAB procedure (protocol II) was found superior in the present study. The advantageous effect of CTAB along with PVP on the quality of DNA was also reported by

Rogers and Bendich (1994), Gallego and Martinez (1996) and Ram and Sreenath (1999). It effectively disrupts the cell membrane and together with NaCl separates the polysaccharides. The EDTA in the extraction buffer protects the DNA from endonucleases by chelating the Mg^{2+} ions of DNA. Double treatment with chloroform: isoamyl alcohol mixture and centrifugation effectively removes the pigments and proteins. The addition of chilled isopropanol precipitates the DNA and washing the pellet with 70 percent alcohol followed by absolute alcohol removes the traces of CTAB, salt and other contaminants in the pellet. The high salt TE buffer rehydrates the DNA and dissolves it (Rogers and Bendich, 1994; Wettasinghf and Peffley, 1998 and Babu, 2000).

5.4.1.3 Purification and Quantification of DNA

The quality of the DNA was tested by subjecting it to agarose gel electrophoresis as well as by spectrophotometric method. In the former the DNA was visualized on 1.2 percent agarose gel under UV light by ethidium bromide staining. The stain was added directly to the melted agarose before casting the gel in order to have a better resolution. Nanda and Jain (1994) also reported similar results. In the latter the ratio of optical density at 260 and 280 nm was worked out to test the quality.

A DNA sample was reported as high quality if it had a band of high molecular weight with little smearing and a low amount of RNA (Wettasinghf and Peffley, 1998). The DNA extracted showed high amount of RNA as a smear below it. To remove RNA, Rnase A was used. Use of Rnase A was also reported by Rival et al. (1998), Wettasinghf and Peffley (1998) and Gallego and Martinez (1996). In the present investigation, the RNase treated DNA sample on electrophoresis showed a high molecular weight DNA, which formed a single band just below the well. This indicted that the DNA under test was of good quality.

The absorbance ratio was calculated as OD at 260/OD at 280, for the various samples, using spectrophotometer. Those samples with ratio in between 1.8 and 2 were considered to be of high quality. Lim *et al.* (1999) and Ram and Sreenath (1999) also reported the assessment of quality of isolated DNA by observing the ratio of absorbance at wavelengths 260nm and 280nm as well as by subjecting it to agarose gel electrophoresis.

Quantification of the DNA on the basis of UV absorbance at 260nm indicated substantial yield of DNA from the tissue samples. Spectrophotometric determination of concentration of DNA was also reported by Gallego and Martinez (1996), Ram and Sreenath (1999) and Lim *et al.* (1999).

5.4.1.4 DNA amplification conditions

Since the reproducibility of banding pattern was reported to be influenced by type of the thermalcycler used (Penner *et al.*, 1993), it is essential to mention the model. The amplification conditions standardized in the present study were suited to PTC-200 model thermalcycler from MJ Research, USA.

a) Reaction mixture

The amplification pattern produced by the different combinations of the ingredients of the reaction mix indicated that the most important factors affecting the specificity and yield of amplification were the concentration of $MgCl_2$ in the buffer as well as concentration and type of DNA polymerase enzyme (Devos and Gale, 1992; Schierwater and Ender, 1993; Nanda and Jain 1994; Cipriani *et al.*, 1996; Gallego and Martinez, 1996 and Damasco *et al.*, 1996). In the present investigation the enzyme used was Taq DNA polymerase supplied by Genei, Bangalore. The molarities of primer as well as dNTPs were also found to affect the

intensity and number of amplifications. Based on the observations, the optimum set of reagents selected was as follows.

All reactions were performed in 25 μl final volume which contained 1x Taq buffer 10A with 1.5mM $MgCl_2$, 150μM each of dNTPs, 0.5units of Taq DNA polymerase (all from Genei, Bangalore), 4pmoles of primer (Operon Tech, USA) and 50ng template DNA. Nanda and Jain (1994) and Damasco et al. (1996) also reported that best results were obtained if Mg^{2+} ion concentration was between 1.5 to 4mM. The range of concentration of template DNA was reported to vary with different crops (Takemori et al 1994; Gallego and Martinez, 1996; Damasco et al., 1996 and Kumar et al., 1998).

b) Thermal profile

Two thermal profiles which differed from each other only in time of denaturation and extension steps, selected from the different profiles tried were found to give almost same number of amplification, but Thermal Profile II was found better with respect to intensity of the bands. Hence the second profile was selected as the most ideal one for pepper DNA. In previous reports annealing temperature was identified as the most critical with respect to number of amplified fragments and reproducibility of results. Cipriani et al. (1996) and Kumar et al. (1998) suggested 37^0C as the best, and hence only time was varied for this step.

c) Primer screening

From the 60 random decamer primers, 13 primers selected on the basis of a three level screening test considering the number, intensity and consistency of bands, were found useful for screening the black pepper vines. The RAPD markers generated using the selected primers were visualised by electrophoresis, in a 1.5 percent agarose gel stained

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with ethidium bromide. Though polyacrylamide gels with silver staining was suggested by a few, majority of earlier reports indicated the use of agarose gel due to ease of casting and handling of gel (Varghese *et al.*, 1997; Teparkum and Veilleux, 1998; Goswami and Ranade, 1999; Lim *et al.*, 1999).

5.4.2.Characterisation using RAPD

Based on the clarity (intensity), consistency and number of polymorphic bands, four primers were selected *viz.*, OPP 1, OPP 8, OPP 14 and OPF 13 for characterisation of the 20 conventional clones and 38 TC derived plants belonging to the four varieties. Neto *et al.*, (1995) reported using only three primers for efficient finger printing of four cashew clones.

5.4.2.1 Intra group characterisation

a) Within conventional clones

RAPD markers also proved the true to type nature of the conventional and TC clones which confirms the results of morphology, cytology and isozyme markers. Even though there were slight vine-to-vine variations exhibited for the various morphological traits, the DNA in all the clones within a variety seemed to be the same. Kongkiatngam *et al.* (1995) in red clover and Hoey *et al.* (1996) in pea also reported that the results of the three above mentioned marker system lead to the same conclusion, though efficiency was more for isozyme and RAPD.

b) Within TC clones

Unlike the conventional clones, certain vines in all the four varieties of the TC clones exhibited polymorphism with respect to the presence or absence of certain PCR products.

The result of the RAPD analysis of variety P_1 with the three selected primers (OPP 1, OPP 8 and OPP 14) showed amplification products that were monomorphic across all the conventionally and micro propagated plants except TCC P_1-1 and TCC P_1-2. These two were lacking two bands each produced by OPP 1 and OPP 8 and one band produced by OPP 14. But the amplification pattern of the primer OPF 13 showed that TCC P_1-8 was different from the rest of the TC plants with respect to three bands whereas TCC P_1-1 and TCC P_1-2, which were found different according to the other three primers, were similar to the rest of the TC plants. This primer was unable to discriminate the two variants detected by the other three primers. Among these, TCC P_1-1 was found different in zymogram pattern also. TCC P_1-1 also started flowering and had produced 45 spikes.

In variety P_2 the conventionally propagated and TC derived vines were identical except in the case of two TC plants *viz.*, TCC P_2-7 and TCC P_2-10. These two vines were different from the rest of the vines when tested with all the four primers. With respect to isozyme pattern also TCC P_1-7 was found different. But morphological traits failed to identify this variant.

Even though the PCR with OPP1 and OPP8 indicated that TCC P_4-2 and TCC P_4-9 might be different from the rest of the conventional as well as TC derived plants, further testing with OPP14, OPP13 and OPP12 clearly indicated that only TCC P_4-9 was different from the rest. All the other clones as well as TC plants were homogeneous. But the results of isozyme studies indicated TCC P_4-4 and TCC P_4-8 as variants, which were found monomorphic with others by RAPD.

Among TC Subhakara, there were two variants TCC Su_9 and TCC $Su_{10,}$ which showed polymorphism when compared with the rest of the vines, detected by all the four primers, OPP 1, OPP 8, OPP 14 and OPP 13. These variants were polymorphic when compared with the conventional clones also.

Identical work in which, detection of variants in micropropagated clones of *Populus deltoids* produced by enhanced axillary branching cultures were reported by Rani *et al.*, (1995). They found that out of the 23 plants 17 were uniform and six were different from these. But among the six plants, all showed complete homology within the group confirming the fact that a single mutation separated the 23 plants into two groups. But these 6 plants were morphologically indistinguishable from the remaining 17 clones.

Similarly in micropropagated cavendish banana, the frequency of production of off-types was reported to range from three to twenty five per cent (Hwang and Ko, 1987; Damasco *et al.*, 1996).

5.4.2.2 Inter clonal polymorphism

In varieties P_1, P_2 and P_4 The TC clones were monomorphic with the conventional clones, when tested with the different primers. But in variety Subhakara the two groups were found to differ.

Testing with the four primers, OPP 1, OPP 8, OPP 14 and OPP 13 clearly indicated that, the conventional clones of Subhakara were different from the TC derived plants under the experiment. To confirm this, PCR with OPP 14 was carried out taking DNA samples from Karimunda in field, TC derived Karimunda established in field at College of Horticulture, Vellanikkara, the currently used mother clone maintained in the green house for taking explants for tissue culture and the experimental vines CC Su_1, TCC Su_1, TCC Su_2 and TCC P_4-8. The result indicated that the banding pattern of TC derived Subhakara vines were exactly same as that of P_4 and it clearly differed from that of the other different Karimunda forms. Subhakara (KS27) is actually a selection from the local Karimunda germplasm collection.

All the three marker systems i.e., morphology, isozyme and RAPD results proved the difference of TC clones from conventional clones and similarity of these TC vines to those of P_4. So in the present investigation, the vines labeled as TC Subhakara were found to be TC clones of P_4. Analogous findings were reported in vetiver accessions (Kresovich et al., 1994) and also in cranberry (Novy and Vorsa, 1995) due to cultivar misclassification, resulting in the representation of a genotype by several names, which were detected by RAPD.

5.4.3 Intervarietal polymorphism

Using 13 primers, all the four varieties were subjected to PCR. The agarose gel electrophoresis showed a total of 89 bands from these 13 primers. These bands were scored for polymorphism to separate the monomorphic and polymorphic bands between the varieties. The average number of bands generated per primer was 6.85. Lower values such as 2.6 in rubber (Varghese et al., 1997) and 5.4 in oil palm (Rival et al., 1998); similar values like 6.6 in Stylosanthes (Kazan et al., 1993a, 1993b) and higher values such as 12.3 in Saskatoon (Weir and Pierre, 1997) and 14 in wild mustard (Moodie et al., 1997) were reported previously. Rafalski et al., (1991) observed that the number of bands in RAPD profiles was independent of genome complexity. Weir et al., (1997) suggested that these differences in number of bands generated per primer in various reports might be due to differences in primer length, primer sequence and primer-template DNA interaction.

Based upon the presence (+) or the absence (-) of polymorphic bands, the varieties were scored as suggested by Halward et al., (1992), Mori et al., (1993), Rani et al., (1995) and Demeke et al., (1996). Pair wise comparison of the four varieties was done with respect to the number of bands by which they differ. The number of RAPD products was highest in P_1 (73) followed by P_4 (69), Subhakara (67) and P_2 (66). It was found that P_1 was closer to

P_2 (difference in only seven out of 73 bands) whereas P_4 was closer to Subhakara (difference in only 16 out of 69 bands). P_2 differed from P_4 and Subhakara by 27 and 31 bands respectively. In the genetic distance analysis, which was based upon the coefficient of genetic distance (Demeke *et al.*, 1996) also, P_1 and P_2 came under the same group, whereas P_4 and Subhakara belonged to another group. The results revealed that RAPD analysis was an efficient tool for detecting inter varietal polymorphism in black pepper.

5.5 COMPARISON OF THE DIFFERENT MARKER SYSTEMS

In the present investigation, the use of four different marker systems viz., cytology (chromosome no.), morphology, isozyme (PRX), and RAPD for evaluation and characterisation of the different vines have endowed with an infallible identity for each of the experimental vines. Beer *et al.* (1993) also stressed the importance of using several marker systems together rather than the exclusive use of any one single system. A close scrutiny of these different markers revealed the following merits and demerits.

The determination of the somatic chromosome number is essential during the *in vitro* condition as the occurrence of callus and the resulting ploidy change is a common phenomenon. But this is inadequate to detect any finer variation produced during culturing such as those due to single gene mutation, transpositional changes etc.

Morphological markers are simple, easy to analyse and interpret and provide an overall picture of the individual vine. These were more suited to detect inter varietal variability. Here also the fine variation goes undetected and hence, not of much use to discriminate the clones within a variety. In other words, it may be assumed to have more of horizontal efficiency rather than vertical efficiency in discerning the polymorphism. The influence of environmental factors may further decrease the efficiency.

The isozyme markers were able to recognize finer changes in the genotype and were suitable to test intra group, inter clonal and inter varietal variability. They were influenced by the environmental changes to a limited extent. But in certain cases the zymogram may be complicated and difficult to interpret. Also different enzymes differ in their efficiency depending upon the genotype and may not produce bands that could be scored. The technique needs more skill, costly chemicals and equipments.

The RAPD banding pattern provided a very clear fingerprint of each individual which was easy to interpret and efficient to detect polymorphism at the genetic level. There are large numbers of scorable markers that are not influenced by the environment. Here both the horizontal and vertical efficiency was high and hence was useful to screen the intra group, inter clonal and inter varietal polymorphism. But the cost and technical skill needed is very high and the reproducibility of bands will be affected by a slight change in the reaction conditions.

Many earlier workers also discussed the merits and demerits of these marker systems, separately as well as in a comparative manner (Chandy and Pillay, 1979; Rahiman and Nair, 1983; Gordon *et al.*, 1988; Mori *et al.*, 1993; Newbury and Ford-Lloyd, 1993; Kongkiatngam *et al.*, 1995; Halden *et al.*, 1996; Baudracco-Arnas and Pitrat, 1996, Gallego and Martinez, 1996; Goswamy and Ranade, 1999 and Lakshmikant and Gulati, 2001).

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| SUMMARY... |

6. SUMMARY

The present investigation was carried out with the objective of characterisation of 40 tissue culture derived and 20 conventionally propagated black pepper vines of four different varieties *viz.*, Panniyur 1, Panniyur 2, Panniyur 4 and Subhakara established in the field during 1995. The micropropagated materials were produced at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara and were supplied to the Pepper Research Station, Panniyur for planting in the field. The conventional clones were produced as rooted cuttings from the existing mother plants at the research station itself and planted in the field along with the micropropagated plants. All the vines were receiving uniform agronomic and plant protection treatments.

The genetic identity of these TC and conventionally propagated vines were tested using a combination of conventional and molecular markers such as morphology, cytology, isozyme and RAPD analysis. Both qualitative and biometric observations were recorded for morphological characterisation and cytological investigations were conducted to determine the chromosome number. Isozyme analysis was done with respect to two enzyme systems. DNA finger printing and detection of polymorphism, if any, were carried out using RAPD analysis. All these four markers were used in combination with the aim of getting a comprehensive and perfect genetic identity of each vine, the different marker systems complementing each other, giving an infallible result. Additional information on the varietal response to the four marker systems were also obtained which helped to estimate the genetic distance between them as well as to compare the efficiency of the different marker systems.

The salient results obtained from the investigations are summarised below in relation with the objectives aimed at.

6.1 Genetic identity of TC and conventional clones

Based on the detailed descriptor involving 22 vegetative characters, 19 reproductive characters and 39 qualitative characters, each of the 58 vines were characterised. The salient differences or similarities were also discussed. The cytological studies confirmed the chromosome number in all the experimental vines as $2n = 52$ which is characteristic of the species. The somatic chromosome number was found constant in all the vines. Under the microscope, the chromosomes appeared as darkly stained dot like structures even at a magnification of 1000 times as the chromosomes were of comparatively smaller size in the species. Apparently, the micropropagation techniques did not affect the chromosome number of the plantlets and therefore were true-to-true type with the mother clones. Even though the chromosome number is high, the smaller size of the chromosomes is the reason for the low absolute chromosomal length of 62 to 74 µm as reported earlier. The molecular markers gave a more specific identity for each of the 58 vines. The zymogram and RAPD banding pattern has given a very clear fingerprint of the individual vines.

6.2 Detection of intra group, inter clonal and inter varietal variablity

The biometric observations and visual assessment of the vines based on qualitative traits brought out the intra group (within conventional clones or within TC clones), inter-clonal (CC vs. TC clones) and inter varietal variation of the different characters. Out of the of 22 biometric observations, 17 were found to be homogenous with respect to the extent of variability among conventional clones and also among the TC clones irrespective of varieties. However, five of the biometric observations were found to be heterogeneous for which, variety wise analysis was done to asses intra group variation. Most of the qualitative observations also showed uniformity in the conventional as well as TC clones.

The estimation of inter clonal variability proved the uniformity and better vigour of TC plants compared to the conventional clones. The biometric analysis of traits gave significantly higher values for 11 out of 17 characters, clearly indicating more vigorous growth and early bearing habits of the TC plants, irrespective of the variety. Visual ratings of the qualitative characters also supported the above finding. Slight, but negligible variation was observed in the vegetative characters of conventional clones as well as TC plants. This is to be expected, as vine-to-vine variation within a variety is an accepted rule in black pepper (Ratnambal *et al.*, 1985). Even this variability was found to be lesser in TC plants compared to conventional clones indicating the more uniform nature of TC plants. Another interesting observation was the great variability in qualitative and quantitative traits between TC and conventional clones of Subhakara.

Qualitative characters such as anthocyanin pigmentation, leaf shape and size, inter nodal length and thickness, branching nature, spike length, berry weight and volume etc. were found to be efficient for varietal discrimination and could be used as morphological markers for inter varietal polymorphism.

The molecular markers brought out the variability in the different types more specifically. In both isozyme and RAPD analysis, the conventional clones under each variety were found to be monomorphic whereas certain variants could be detected within TC plants. For example, the vines TCC P_1-1 and TCC P_2-7 of the varieties P_1 and P_2 were separated out as variants by both isozyme and RAPD markers. Other TC vines, which showed polymorphism, were P_1-2, P_2-10, P_4-4, P_4-8, P_4-9, Su-9 and Su-10. These vines should be subjected to further analysis using more number of RAPD primers and advanced computer programmes to assess their significance. If the variation was found significant these vines have to be studied separately after the stabilisation of yield. Based on this,

tagging the genes and marker assisted selection for yield as well as other economic traits such as disease resistance may also be practiced in the crop.

The assessment of inter clonal variability using molecular markers clearly pointed out the distinctly different traits of TC clones and conventional clones OF Subhakara. Morphological observations too supported this finding. In RAPD banding patterns, the vines labelled as TC Subhakara showed the typical traits of TC P_4. It is suspected that a mix up might have occurred during labelling, transportation and handling of the micropropagated plants from Vellanikkara to Panniyur.

The inter varietal polymorphism brought out by the molecular markers was useful in discriminating the four black pepper varieties and to group them according to genetic distance. Both the marker systems, separately and jointly, suggested that P_1 and P_2 belonged to one group whereas P_4 and Subhakara fell into a second and different group. Several morphological traits such as colour of leaf sheath, stem thickness, time taken for the maturity of berries etc. also pointed out a closer relationship between P_1 and P_2 than with the other two. The information is very much valuable in breeding as the genetic distance of the varieties is an important criterion in the selection of parents for hybridisation.

6.3 Standardisation of various techniques

The cytological, isozyme and RAPD analysis in black pepper faced several problems like very small sized chromosomes, high phenol content which denature the enzymes and DNA, critical influence of pH and molarity of different chemicals used etc. So, to standardise these techniques and to fine-tune them to suite the crop, a lot of repeated efforts were necessary. Guided by the experience of previous workers and by trial and error method, all the techniques could be standardised for the black pepper genotype.

6.4 Comparative efficiency of the techniques

The combined use of four different marker systems in a single experiment helped to assess the relative efficiency of each of the method. All the four techniques had definite advantages as well as drawbacks, the difference being determined by their relative percentage.

The morphological markers were easy to use, required lesser skill and had cost effectiveness. Since these were influenced by environment, the specificity was lesser. But an overall assessment of each vine and its growth habits could be obtained only by this system. The cytological studies on chromosome number provided the general character of the species with respect to ploidy level and helped to check out the possibility of variation in chromosome number caused by *in vitro* culturing but here again, it could not mark the individual variations. Among the four techniques tried, the molecular markers, in general, were found superior. Direct analysis of the gene itself using the RAPD technique gave highest specificity in characterisation. Isozymes being the gene products, were influenced by developmental and ontogenic factors to a certain extent. Also, different enzyme systems behaved differently in the genotype.

REFERENCES...

8. REFERENCES

Adkins, S.W., Kunanuvatchaidach, R., and Godwin, I.D. 1995. Somaclonal variation in rice- drought tolerance and other agronomic characters. *Austr. J. Bot.* **43**: 201-209

Alarmelu, S., Balasubramaniam, T. and Padmanabhan, C. 1999. Isozyme changes during morphogenesis in the *in vitro* cultures of *Panicum maximum. Indian J. Genet.* **59**(1): 47-52

Anand, A. 1997. Morphological investigations in *Piper* spp. M.Sc. (Hort.) thesis, Kerala Agricultural University, Thrissur, pp. 52-61

Andrews, A. 1981. *Electrophoresis: Theory, Techniques and Biochemical and Clinical Applications.* Clarendeon Press, Oxford, pp. 82-126

Angel, F., Barney, V.E., Tohme, J. and Roca, W.M. 1996. Stability of cassava plants at the DNA level after retrieval from 10 years of *in vitro* storage. *Euphytica* **90**: 307-313

Armstrong, K. C. 1995. Staining procedures for chromosome analysis. *Pl. Cell, Tissue Org. Cult.* (Eds. Gamborg, O.L. and Philips, G.C.), Narosa Publishing House, New Delhi, pp. 251-266

Arulsekar, S., Parfitt, D.E. and McGranahan, G.H. 1985. Isozyme gene markers in *Juglans* species. *J. Hered.* **76**: 103-106

Arunachalam,V., Prabhu, K.V. and Sujatha, V. 1996. Isozyme parameters in genetic evaluation in *Brassica* –A tribute to Haldane's vision on the synergy between biochemistry and genetics. *Curr. Sci.* **70**: 1062-1064

Babu, H.T.P. 2000. RAPD analysis to assess the genetic stability in TC derived black pepper (*Piper nigrum* L.) M.Sc. (Hort.) thesis, Kerala Agricultural University, Thrissur, pp. 57-83

Babu, N.K. and Ravindran, P.N. 1992. Improved varieties of black pepper. *Problems and Prospects in Black Pepper and Cardamom.* Indian Institute of Spices Research, Calicut, pp. 61-64

Babu, N.K., Lukose, R. and Ravindran, P.N. 1993. Tissue culture of tropical spices. *Proceedings of International Symposium on Genetic Engineering and Tissue Culture for Crop Pest and Disease Management* (Ed. Vidhyasekharan), Daya publishing House, New Delhi, pp. 257-267

Barber, C.A. 1906. The varieties of cultivated pepper. *Dept. Agri. Madras Bull.* **3**(26): 126-132

Bartlett, M.S. 1937. Some examples of statistical methods of research in agriculture and applied biology. *Suppl. J. R. Stat. Soc.* **4**: 137-170

Baudracco-Arnas, S. and Pitrat, M. 1996. A genetic map of melon (*Cucumis melo* L.) with RFLP, RAPD, isozyme, disease resistance and morphological markers. *Theor. Appl. Genet.* **93**: 57-64

Bebeli, P.J., Karp, A. and Kaltsikes, P.J. 1990. Somaclonal variation from cultured immature embryos of sister lines of rye differing in heterochromatin content. *Genome* **33**: 177-183

Beer, S.C., Goffreda, J., Philips, T.D., Murphy, J.P. and Sorrelle, M.E. 1993. Assessment of genetic variation in *Avena sterilis* using morphological traits, isozymes and RAPDs. *Crop Sci.* **33**: 1386-1393

Bennet, H., Guries, R.P., Boury, S. and Smalley, E.B. 1995. Identification of RAPD markers related to a black leaf spot resistance gene in Chinese elm. *Theor. Appl. Genet.* **90**: 1068-1073

Bergmeyer, H.V., Bernt, E., Gawehn, K. and Michael, G. 1974. Handling of biochemical reagents and samples. In: *Methods of enzymatic analysis Vol. 1* (Ed. Bergmeyer, H.V.), Academic Press Inc., New York, pp. 168-170

Bhat, K.V., Bhat, S.R. and Chandel, K.P.S. 1992a. Survey of isozyme polymorphism for clonal identification in *Musa*- I. Esterase, acid phosphatase and catalase. *J. Hort. Sci.* **67** (4): 501-507

Bhat, K.V., Bhat, S.R. and Chandel, K.P.S. 1992b. Survey of isozyme polymorphism for clonal identification in *Musa*- II. Peroxidase, superoxide dismutase and malate dehydrogenase. *J. Hort. Sci.* **67** (6): 737-743

Bhat, K. and Lakhanpaul, S. 1995. Study of polymorphism. In: *Plant Germplasm Conservation: Biochemical Approaches* (Ed. Rana) NBPGR, New Delhi, pp. 243-250

Bhatia, C.R. and Nilson, T.P. 1969. Isoenzyme changes accompanying germination of wheat seeds. *Biochem. Genet.* **3**: 207-214

Bhojwani, S.S., Dhawan, V. and Cocking, E.C. 1986. *Plant Tissue Culture – A Classified Bibliography.* Elsevier, Amsterdam, pp. 399-432

Birecka, H., Shih, L.M. and Galston, A.W. 1972. Isoperoxidase patterns in tobacco pith and their alteration following tissue excision. *Jap. J. Bot.* **23**: 655-666

Blackshear, P.J. 1984. Systems for polyacrylamide gel electrophoresis. *Methods in Enzymol.* **104**: 237-255

Bonner, J.W., Warner, R.M. and Brewbaker, J.L. 1974. A chemo systematic study of *Musa* cultivars. *Hort. Sci.* **9**: 325-328

Boyle, T.J.B., Liengsiri, C. and Piewluang, C. 1990. Genetic studies in a tropical pine-*Pinus kesiya* I. Inheritance of some isoenzymes and linkage among allozyme loci. *J. Trop. For. Sci.* **3** (1): 35-43

Bradshaw, H.D., Villar, M., Watson, B.D., Otto, K.G., Stewart, S. and Stettler, R.F. 1994. Molecular genetics of growth and development in *Populus*. III. A genetic linkage map of a hybrid poplar composed of RFLP, STS and RAPD markers. *Theor. Appl. Genet.* **89**: 167-178

Bregitzer, P. and Poulson, M. 1995. Agronomic performance of barley lines derived from tissue culture. *Crop Sci.* **35**: 1144-1148

Bretting, P., Godman, M. and Stuber, C. 1987. Karyological and isozyme variation in West Indian and allied American mainland races of maize. *Amer. J. Bot.* **74**: 1601-1613

Bretting, P.K. and Widrlechner, M.D. 1995. Genetic markers and horticultural germplasm management. *Hort. Sci.* **30**: 1349-1356

Brewbaker, J.L. and Hasegawa, Y. 1975. Polymorphisms of the twelve major peroxidases of maize. *III. Isozymes: Developmental Biology.* (Ed. Markert, E. L.), Academic Press, New York, pp. 659-673

Brewbaker, J.L., Upadhya, M.D., Makinen, Y. and MacDonald, T. 1968. Isoenzyme polymorphism in flowering plants. III - Gel electrophoretic methods and applications. *Physiol. Plant.* **21**: 930-940

Brown, A.H.D. and Clegg, M.T. 1983. Isozyme assessment of plant genetic resources. *Isozymes: Current Topics in Biological and Medical Research. Vol. II.* (Eds. Rattazzi, M.D., Scandalios, J.G. and Whitt, G.S.), Alan R. Liss, New York, pp. 285-295

Caetano-Anolles, G., Bassam, B. J. and Gresshoff, P. M. 1991. DNA amplification fingerprinting; a strategy for genome analysis. *Plant Molec. Biol. Rep.* **9**: 294-307

Campion, B., Bohancec, B. and Javorrik, B. 1995. Gynogenic lines of onion (*Allium cepa*): evidence of their homozygosity. *Theor. Appl. Genet.* **91**: 598-602

Castiglione, S.G., Wang, G., Damiani, G., Bandi, C., Bisoffi, S. and Sala, S. 1993. RAPD fingerprinting for identification and for taxonomic studies of elite poplar (*Populus* spp.) clones. *Theor. Appl. Genet.* **87**: 54-59

Chandola, R.D. and Jain, S.N. 1970. Karyomorphological studies in *Pennisetum typhoides*. *Cytologia* **35**: 181-196

Chandy, K.C. and Pillai, V. S. 1979. Functional differentiation in the shoot system of pepper vine (*Piper nigrum* L.). *Indian Spices* **14**(3): 8-11

Chandy, K.C., Pillai, V.S. and Nambiar, P.K.V. 1979. Occurrence of abortive spikes in pepper. *Agric. Res. J. Kerala* **17**(1): 148-150

Chu, Y.E. 1967. Variations in peroxidase isozymes of *Oryza perennis* and *O. sativa*. *Jap. J. Genet.* **42**: 233-244

Cipriani, G., Bella, R.D. and Testolin, R. 1996. Screening RAPD primers for molecular taxonomy and cultivar fingerprinting in the genus *Actinidia*. *Euphytica* **90**: 169-174

Conklin, M.E. and Smith, H.H. 1971. Peroxidase isozymes: A measurement of molecular variation in ten herbaceous species of *Datura*. *Amer. J. Bot.* **58** (7): 688-696

*Curn, V. 1993. Variation in chromosome number of *in vitro* cloned and regenerated plants of *Trifolium alexandrianum*. *Fytotechnija Rada* **10**: 137-145

Damasco, O.P., Graham, G.C., Henry, R.J., Adkins, S.W., Smith, M.K. and Godwin, I.D. 1996. Random amplified polymorphic DNA (RAPD) detection of dwarf off-types in micropropagated cavendish (*Musa* spp. AAA) bananas. *Pl. Cell Rep.* **16**: 118-123

Das, S., Rajagopal, J., Bhatia, S., Srivasthava, P.S. and Lakshmikumaran, M. 1999. Assessment of genetic variation within *Brassia campestris* cultivars using amplified fragment length polymorphism and random amplification of polymorphic DNA markers. *J. Biosci.* **24**: 433-440

Dasgupta, A. and Datta, P.C. 1976. Cytotaxonomy of *Piperacea. Cytologia* **41**: 697-706

Davis, B.J. 1964. Disc electrophoresis II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121**: 404-427

Dellaporta, S. I., Wood, J. and Hicks, J. B. 1983. A plant DNA minipreparation; version II. *Pl. Molec. Biol. Rep.* **1**: 19-21

Demeke, T., Adams, R.P. and Chibbar, R. 1992. Potential taxonomic use of random amplified polymorphic DNA (RAPD): a case study in *Brassica. Theor. Appl. Genet.* **84**: 990-994

Demeke, T., Lynch, D.R., Kawchuk, L.M., Kozub, G.C. and Armstrong, J.D. 1996. Genetic diversity of potato determined by random amplified polymorphic DNA analysis. *Pl. Cell Rep.* **15**: 662-667

Desai, S.A. and Singh, R.D. 2001. Combining ability studies for some morphological and biochemical traits related to drought tolerance in maize (*Zea mays* L.). *Indian J. Genet.* **61** (1): 34-36

Devos, K.M. and Gale, M.D. 1992. The use of random amplified polymorphic DNA markers in wheat. *Theor. Appl. Genet.* **84**: 567-572

De Waard, P.W.F. and Zeven, A.C. 1969. Pepper (*Piper nigrum* L.). *Outlines of Perennial Crop Breeding in the Tropics* (Eds. Ferwerda, F. D. and Wit, F.). H. Weeman and Zomen N.V., Wageningen, Netherlands, pp. 409-425

DeWald, M.G., Moore, G.A. and Sherman, W. B. 1988. Identification of pineapple cultivars by isozyme genotypes. *J. Amer. Soc. Hort. Sci.* **113** (6): 935-938

Doyle, J.J. and Doyle J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **19**: 11-15

Doyle, J.J. and Doyle, J.L. 1990. Isolation of plant DNA from fresh tissue. *Focus* **12**: 13-15

Ellsworth, D.L., Rittenhouse, K.D. and Honeycutt, R.L. 1993. Artifactual variation in randomly amplified polymorphic DNA banding patterns. *Biotechniques* **14**: 214-217

Endo, T. 1967. Comparision of the effects of gamma rays and maleic hydrazide on enzyme systems of maize seed. *Radiation Bot.* **7**: 35-40

Endo, T. 1981. Developmental modification and hybridisation of allelic acid phosphatase isozymes in homo- and heterozygotes for the Acp-1 locus in rice. *Biochem. Genet.* **19**: 373-384

Evans, J.J. and Alldridge, N.A. 1965. The distribution of peroxidases in extreme dwarf and normal tomato (*Lycopersicon esculentum*). *Phytochemistry* **4**: 499

Evans, D.A. and Reed, S.M. 1981. Cytogenetic techniques. *Plant Tissue Culture – Methods and Applications in Agriculture*. Academic press, New York, pp. 213-240

Everett, N.P., Wach, M.J. and Ashworth, D.J. 1985. Biochemical markers of embryogenesis in tissue cultures of the maize inbred B.73. *Pl. Sci.* **41**: 133-140

Gallego, F.J. and Martinez, I. 1996. Molecular typing of rose cultivars using RAPDs. *J. Hort. Sci.* **71**: 901-908

Gaur, P.M. and Gour, V.K. 2001. A gene inhibiting flower colour in chickpea (*Cicer arietinum*). *Indian J. Genet.* **61**: 41-44

Garvin, D.F., Roose, M.L., Waines, J.G. 1989. Isozyme genetics and linkage in tepary bean, *Phaseolus acutifolius* A. Gray. *J. Hered.* **80**: 373-376

Gasper, T., Penel, C. and Greppin, H. 1975. Peroxidase and isoperoxidases in relation to root and flower formation. *Pl. Biochem. J.* **2**: 33-47

George, M. and Mercy, S.T. 1978. Origin and botany of pepper (*Piper nigrum* L.). *Silver Jubilee Souvenir*, Pepper Research Station, Panniyur, pp. 11-12

Geetha, C.K. and Aravindakshan, M. 1993. A comparison of growth and dry matter production in bush pepper and vine pepper as influenced by applied NPK. *Indian Cocoa, Arecanut Spices J.* **15**(4): 95-98

Giles, B.E. 1984. A comparison between quantitative and biochemical variation in the wild barley *Hordeum murinum. Evolution* **38**: 34-41

Gordon, M., Huang, X., Pentoney, S. and Zare, R. 1988. Capillary electrophoresis. *Science* **242**: 224-228

Ghosh, S.P., Pal, R.N., Peter, K.V. and Ravindran, P.N. 1999. Four decades of spices research and development- an overview. *Indian Spices* **36** (4): 11-18

Goswami, M. and Ranade, S.A. 1999. Analysis of variations in RAPD profiles among accessions of *Prosopis. J. Genet.* **78**: 141-147

Gottlieb, L.D. 1973. Genetic control of glutamate oxaloacetate transaminase in the diploid plant *Staphanomeria exigua* and its allotetraploid derivative. *Biochem. Genet.* **9**: 97-107

Gottlieb, L.D. 1977. Electrophoretic evidence and plant systematics. *Ann. Missouri Bot. Gard.* **64**: 161-180

Halden, C., Hansen, M., Nilsson, N.O., Hjerdin, A. and Sall, T. 1996. Competition as a source of errors in RAPD analysis. *Theor. Appl. Genet.* **93**: 1185-1192

Halward, T., Stalker, T., Larue, E. and Kochert, G. 1992. Use of single-primer DNA amplifications in genetic studies of peanut (*Arachis hypogaea* L.). *Pl. Molec. Biol.* **18**: 315-325

Hart, G.E. 1975. Glutamate oxaloacetate transaminase isozymes of *Triticum:* evidence for multiple systems of triplicate structural genes in hexaploid wheat. *Isozyme III* (Ed. Markert, C.L.) Academic Press, New York, pp. 637-650

Hart, G. E. and Bhatia, C. R. 1967. Acrylamide gel electrophoresis of soluble leaf proteins from *Nicotiana* sp. *Can. J. Genet. Cytol.* **9:** 367-374

Heinze, B. and Schmidt, J. 1995. Monitoring genetic fidelity vs. somaclonal variation in Norway spruce (*Picea abies*) somatic embryogenesis by RAPD analysis. *Euphytica* **85**: 341-345

Hoey, B.K., Crowe, K.R., Jones, V.M. and Polans, N.O. 1996. A phylogenetic analysis of *Pisum* based on hybrid seed production orchard. *J. Trop. For. Sci.* **2**(2): 157-164

Hooker, J.D. 1885. *Flora of British India (Vol.5)* L.Reeve and Co., London, pp. 78-79

Horwitz, W. (Ed). 1980. *Official Methods of Analysis.* Association of Official Analytical Chemists, Washington D. C., p. 497

Hu, J. and Quiros, C.F. 1991. Identification of broccoli and cauliflower cultivars with RAPD markers. *Pl. Cell Rep.* **10**: 505-511

Hunter, R.L. and Markert, C.L. 1957. Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. *Science* **125**: 1294-1295

Hwang, S.C. and Ko, W.H. 1987. Banana and plantain breeding strategies. *ACIAR Proceedings No. 21.* (Eds. Persley, G.J. and De Langhe, E.A.), pp. 151-156

Ibrahim, K.K., Pillai, V.S. and Sasikumaran, S. 1984. Discriminant functions in distinguishing between Travancore and Malabar cultivars of black pepper (*Piper nigrum* L.). *Indian Spices* **22**(1): 3-9

Ibrahim, K.K., Pillai, V.S. and Sasikumaran, S. 1985a. Methods for the estimation of leaf area in black pepper (*Piper nigrum* L.) and nature of association between various traits relating to leaf lamina. *South Indian Hort.* **33**: 316-322

Ibrahim, K.K., Pillai, V.S. and Sasikumaran, S. 1985b. Genotypic and phenotypic correlations among yield and its components in black pepper (*Piper nigrum* L.). *Agric. Res .J. Kerala* **23** (2): 150-153

Ibrahim, K.K., Pillai, V.S. and Sasikumaran, S. 1986a. Comparative genetic variability within the open pollinated seedlings of certain varieties of black pepper (*Piper nigrum* L.). *Agric. Res. J. Kerala* **24** (1): 74-76

Ibrahim, K.K., Pillai, V.S. and Sasikumaran, S. 1986b. Inheritance of anthocyanin pigmentation on stipules in black pepper. *Indian Cocoa, Arecanut Spices J.* **9**(1): 12-13

Isabel, N.L., Tremblay, M., Mchaud, F.M. and Bosquet, J. 1993. RAPDs as an aid to evaluate the genetic integrity of somatic embryogenesis derived populations of *Picea mariana* (Mill) B.S.P. *Theor. Appl. Genet.* **86**: 81-87

*Jaccard, P. 1908 *Nouvelles recherches sur la distribution florate. Bull. Soc. Vaud Sci. Nat.* **44**: 223-270

Jain, S.K., Wu, L. and Vaidya, K.R. 1980. Levels of morphological and allozyme variation in Indian amaranthus: a striking contrast. *J. Hered.* **71**: 283-285

Jarret, R.L. and Litz, R.E. 1986. Isozymes as genetic markers in bananas and plantains. *Euphytica* **35**: 539-549

Jermyn, M.A. and Thomas, R. 1954. Multiple components in horseradish peroxidase. *Biochem. J.* **56**: 631-639

Jose, J. 1981. Karyomorphological studies on *Piper attenuatum* HAM, a new record. *Curr. Sci.* **50**: 646-647

Jose, J. and Sharma, A.K. 1983. Chromosome composition in relation to chemical constitution in varieties of *Piper nigrum* L. *Nucleus* **26** (2): 78-86

Joseph, L. Nazeem, P.A., Thampi, M.S., Philip, S. and Balachandran, M. 1996. *In vitro* techniques for mass multiplication for black pepper and *ex vitro* performance of the plantlets. *J. Plant. Crops* **24** (Supplement): 511-516

Joseph, R. 1999. Isozyme variation in *Curcuma* with special reference to *Curcuma longa* L. M.Sc. (Hort.) thesis, Kerala Agricultural University, Thrissur, p. 98

Kaemmer, D., Afsa, R., Weising, K., Kahl, G. and Novak, F.J. 1992. Oligonucleotide and amplification fingerprinting of wild species and cultivars of banana (*Musa* spp.). *Bio/Technology* **10**: 1030-1035

Kanakamany, M.T. 1982. Formulation of a key for identification of the different types of pepper (*Piper nigrum.* L.). M.Sc. (Ag.) thesis, Kerala Agricultural University, Thrissur, pp. 78-92

Kannan, K. 1985. Panniyur 1 comes up well in Wynad district in Kerala. *Indian Cocoa Arecanut Spices J.* **9**(1): 5

Kao, K.N. 1975. A chromosomal staining method for cultured cells. *Plant Tissue Culture Methods* (Eds. Gamborg, O. L. and Wetter, L.R.). National Research Council, Prairie Regional Lab., Canada, pp. 63-64

Karp, A. 1995. Somaclonal variation as a tool for crop improvement. *Euphytica* **85**: 1-3

Karp, A., and Maddock, S.E. 1984. Chromosome variation in wheat plants regenerated from cultured immature embryos. *Theor. Appl. Genet.* **67**: 249-255

Karp, A., Jones, M.G.K., Foulger, D., Fish, N. and Bright, S.W.J. 1989a. Variability in potato tissue culture. *Am. Potato J.* **66**: 669-684

Karp, A., Rissiot, R., Jones, M.G.K. and Bright, S.W.J. 1989b. Chromosome doubling in monohaploid and dihaploid potatoes by regeneration from cultured leaf explants. *Pl. Cell Tissue Org. Cult.* **3**: 363-373

Karp, A., Kresovich, S., Bhat, K.V., Agad, W.G. and Hodgkin, T. 1997. Molecular tools in plant genetic resources conservation - a guide to the technologies. *International Plant Genetic Resource Institute Technical Bulletin* **2**: 5-9

KAU. 1996. *Package of Practices Recommendations- Crops.* Kerala Agricultural University, Thrissur, pp.120-126

Kawatta, M., Harada, S., Antonio, B. and Dono, K. 1992. Protoclonal variation of plant regeneration in rice. *Pl. Cell Tissue Org. Cult.* **28**(1): 1-10

Kazan, K., Manners, J.M. and Cameron, D.F. 1993a. Inheritance of random amplified polymorphic DNA markers in an interspecific cross in the genus *Stylosanthes*. *Genome* **36**: 50-55

Kazan, K., Manners, J.M. and Cameron, D.F. 1993b. Genetic variation in agronomically important species of *Stylosanthes* determined using random amplified polymorphic DNA markers. *Theor. Appl. Genet.* **85**: 882-888

Kelley, W.A. and Adams, R.P. 1977. Preparation of extracts from juniper leaves for electrophoresis. *Phytochemistry* **16**: 513-516

Kephart, S.R. 1990. Starch gel electrophoresis of plant isozymes: A comparative analysis of techniques. *Am. J. Bot.* **77**(5): 693-712

Kertadikara, A.K.W.S and Pratt, D. 1995. Isozyme variation among teak (*Tectona grandis* L. F.) provenances. *Theor. Appl. Genet* **90**: 803-810

Khandka, D.K., Nejidat, A. and Goldhirsh, G.A. 1996. Polymorphism and DNA markers for asparagus cultivars identified by random amplified polymorphic DNA. *Euphytica* **87**: 39-44

Klaas, M. 1998. Applications and impact of molecular markers on evolutionary and diversity studies in the genus *Allium*. *Pl. Breed.* **117**: 297-308

Kochhar, V. K., Kochhar, S., Ravat, K.S. and Balasubramanyan, V. R. 1984. Peroxidase isozymes in some cultivars of betel vine and their taxonomic significance. *Improvement of Betel Vine Cultivation in India*. National Botanical Research Institute, Lucknow, pp. 165-169

Kongkiatngam, P., Waterway, M.J., Fortin, M.J. and Coulman, B.E. 1995. Genetic variation within and between two cultivars of red clover (*Trifolium pratense* L.): comparisons of morphological, isozyme and RAPD markers. *Euphytica* **84**: 237-246

Kresovich, S., Lamboy, W.F., Rugang, L., Ren, J., Szewe-McFadden, A.K. and Bliek, S.M. 1994. Application of molecular methods and statistical analysis for discrimination of accessions and clones of vetiver grass. *Crop. Sci.* **34**: 805-809

Krishnamoorthy, A. 1969. *The wealth of India: Raw materials* Vol 8, New Delhi, p.71

Kumar, P.P., Janice, C.K. and Goh, C.J. 1998. Genetic analysis of *Heliconia* sp. and cultivars with randomly amplified polymorphic DNA (RAPD) markers. *J. Amer. Soc. Hort. Sci.* **123** (1): 91-97

Kumar, R.S. and Navaneethan, N. 1981. Chromosome number reports 72. *Taxon* **30**: 696

Lakshmikant and Gulati, S.C. 2001. Genetic analysis for yield, its components and oil content in Indian mustard (*Brassica juncea*). *Indian J. Genet.* **61**: 37-40

Lagrimini, L.M., Burkhart, W., Moyer, M. and Rothstein, S. 1987. Molecular cloning of complementary DNA encoding the lignin forming peroxidase from tobacco: molecular analysis and tissue specific expression. *Proc. nat. Acad. Sci.* USA **84**: 7542-7546

Larkin, P.J. and Scowcroft, W.R. 1981. Somaclonal variation- a novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet.* **60**: 197- 214

Lavee, S. and Galston, A.W. 1968. Structural, physiological and biochemical gradients in tobacco pith tissue. *Pl. Physiol.* **43**: 1760-1768

Lefebvre, V., Caranta, C., Pflieger, S., Moury, B., Daubeze, A.M., Blattes, A., Ferriere, C., Phaly, T., Nemouchi, G., Ruffinatto, A. and Palloix, A. 1997. Updated intraspecific maps of pepper. *Capsicum and Eggplant Newsletter* **16**: 35-41

Lim, S., Teng, P.C., Lee, Y. and Goh, C. 1999. RAPD analysis of some species in the genus *Vanda* (Orchidaceae). *Ann. Bot.* **83**: 193-196

Liu, E.H. 1975. Substrate specificities of plant peroxidase isozymes. *Isozymes II-Physiological Function* (Ed. Markert, C.L.). Academic Press, New York, pp. 837-849

Liu, Z. and Furnier, G.R. 1993. Comparison of isozyme, RFLP and RAPD markers for revealing genetic variation within and between trembling aspen and bigtooth aspen. *Theor. Appl. Genet.* **87**: 97-105

Lyman, J. and Ellstrand, N. 1984. Clonal diversity in *Taraxacum officinale* (Compositae): an apomict. *Heredity* **53**: 1-10

Lyslova, E.M., Serevrenikova, T.P. and Verzhbinskaya, N.A. 1975. Isozymes of aspartate aminotransferase and glycogen phosphorelase in muscles of lower vertebrates. *Isozymes II- Physiological Function* (Ed. Markert, C.L.), Academic Press, New York, pp. 547-566

MaaB, H.I. and Klass, M. 1995. Intraspecific differentiation of garlic (*Allium sativum*), by isozyme and RAPD markers. *Theor. Appl. Genet.* **91**: 89-97

Madan, M.S. 2000. The Indian black pepper: economics and marketing. *Centennial Conference on Spices and Aromatic Plants,* September 20-23. Indian Institute of Spices Research, Calicut, Kerala, pp. 225-232

Markert, C.L. 1975. *Biology of isozymes.* Academic Press, New York, p. 547-566

Markert, C.L and Moeller, F. 1959. Multiple forms of enzymes: tissue, ontogenetic and species specific patterns. *Proc. nat. Acad. Sci.* USA **45**: 753-763

Martin, F.W. and Gregory, L.E. 1962. Mode of pollination and factors affecting fruit set in *Piper nigrum* L. *Crop Sci.* **2**: 295-299

Martin, G.B., Williams, J.G.K. and Tanksley, S.D. 1991. Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near-isogeneic lines. *Proc. nat. Acad. Sci.* USA **88**: 2336-2340

Mathai, C.K., Kumaran, P.M. and Chandy, K.C. 1981. Evaluation of commercially important chemical constituents in wild black pepper types. *Qual. Plant Food Hum. Nutr.* **30**: 199-202

Mathew, P. M. 1958. Studies on *Piperacea. J. Indian bot. Soc.* **37**: 155-171

Mathew, P.M. 1973. Karyomorphological studies on *Piper nigrum* L. *J. Plant. Crops* **1** (Supplement): 15-18

Mathew, P.M and Mathew, D.J. 1982. Cytology of the hybrid pepper variety Panniyur 1. *Curr. Sci.* **51**: 530-531

McCune, D.C. 1967. Multiple peroxidases in corn. *Ann. N.Y. Acad. Sci.* **94**: 723-730

Menon, R. and Nair, P.C.S. 1989. Growth and flowering in pepper variety Panniyur 1. *Indian Cocoa Arecanut Spices J.* **12**(3): 82-83

Moodie, M., Finch, R.P. and Marshall, G. 1997. Analysis of genetic variation in wild mustard (*Sinapis arvensis*) using molecular markers. *Weed Sci.* **45**: 102-107 Mori, M., Hosaka, K. and Umemura, Y. 1993. Rapid identification of Japanese potato cultivars by RAPDs. *Jap. J. Genet.* **68**: 167-174

Mullis, K.B. and Faloona, F. 1987. Specific synthesis of DNA *in vitro* via the polymerase chain reaction. *Methods Enzymol.* **155**: 335-341

Munthali, M.T., Newbury, H.J. and Ford-Lloyd, B.V. 1996. The detection of somaclonal variants of beet using RAPD. *Pl. Cell Rep.* **15**: 474-478

Nair, R.R., Sasikumar, B. and Ravindran, P.N. 1991. Polyploidy in cultivar of black pepper (*Piper nigrum* L.) and its open pollinated progenies. *Cytologia* **58**: 27-31

Nambiar, P.K.V., Pillai, V.S., Sasikumaran, S. and Chandy, K.C. 1978. Pepper research at Panniyur – a resume. *J. Plant. Crops* **6**(1): 4-11

Nanda, S.K. and Jain, S.K. 1994. *In vitro* nucleic acid amplification systems. *Curr. Sci.* **66** (6): 421-430

Neto, S.P.S., Maruta, I., Takaiwa, F., Oono, K. and Matsumoto, K. 1995. Identification of cashew (*Anacardium occidentale*) seedlings with RAPD markers. *Acta Hort.* **370**: 21-26

Newbury, H.J and Ford-Lloyd, B.V. 1993. The use of RAPD for assessing variation in plants. *Pl. Growth Reg.* **12**: 43-51

Nisselbaum, J.S. and Kopelovich, L. 1975. The synthesis and regulation of aspartate aminotransferase isozymes. *Isozymes III, Developmental Biology* (Ed. Markert, C.L.), Academic Press, New York, pp. 159-175

Nishikawa, K. and Nobuhara, M. 1971. Genetic studies of α-amylase isozymes in wheat. I. Location of genes and variation in tetra – and hexaploid wheat. *Jap. J. Genet.* **46**: 345-353

Novy, R.G. and Vorsa, N. 1995. Identification of intra cultivar genetic heterogeneity in cranberry using silver-stained RAPDs. *Hort. Sci.* **30**: 600-604

Oliver, J.L. and Martinez-Zapater, J.M. 1985. A genetic classification of potato cultivars based on allozyme patterns. *Theor. Appl. Genet.* **69**: 305-311

Ornstein, L. 1964. Disc electrophoresis I. Background and theory. *Ann. N. Y. Acad. Sci.* **141**: 13-23

Pandey, R.M. and Singh, S.P. 1989. Field performance of *in vitro* raised plants. *Indian Hort.* **33**: 1-7

Papa, R., Attene, G., Barcaccia, G., Ohgata, A. and Konishi, T. 1998. Genetic diversity in landrace populations of *Hordeum vulgare* L. from Sardinia, Italy, as revealed by RAPDs, isozymes and morphophenological traits. *Pl. Breed.* **117**: 523-530

Parani, M., Anand, A. and Parider, A. 1997. Application of RAPD fingerprinting of micropropagated plants of *Piper longum* for conservation. *Curr. Sci.* **73**(1): 81-84

Pasteur, N., Pasteur, G., Bonhomme, F., Catalan, J. and Britton-Daidian, J. 1988. *Practical Isozyme Genetics*. Halsted Press, New York, pp. 125-134

Penner, G.A., Bush, A., Wise, R., Kim, W., Domier, L., Kasha, K., Laroche, A., Scoles, G., Molnar, S.J. and Fedak, G. 1993. Reproducibility of random amplified polymorphic DNA (RAPD) analysis among laboratories. *PCR Methods Appl.* **2**: 341-345

Perbal, B. 1988. *A Practical Guide to Molecular Cloning*. John Wiley and Sons, NewYork, pp. 11-35

Perry, M.C., McIntosh, M.S. and Stoner, A.K. 1991. Geographical patterns of variation in the USDA soybean germplasm collection: II Allozyme frequencies. *Crop Sci.* **31**: 1356-1360

Pillai, V.S. 1997. Production technology of black pepper (*Piper nigrum* L.) in India. *Proc. int. Seminar Black Pepper Cupuacu (Cocoa)*. Japan International Co-operation Agency, Belem, Brazil, pp. 27-44

Pillai, V.S., Cheeran, A. and Sasikumaran, S. 1985. '*Kurumulaku*'. Kerala Agricultural University, Trissur, pp. 1-47

Pillai, V.S., Ibrahim, K.K. and Sasikumaran, S. 1987. Heterosis in Panniyur 1 variety of black pepper (*Piper nigrum* L.). *Agric. Res .J. Kerala* **25** (1): 116-118

Pillai, V.S., Renjith, A.M. and Sasikumaran, S. 1989. Pepper research station, Panniyur -a resume. *Annual Report 1989-1990*, Pepper Research Station, Panniyur, p.1-13

Prabakaran, K.A.A.J., Vaidyanathan, P. and Rangasamy, S.R. 1991. Karyo-morphological variations in *Pennisetum americanum* (L.)Leeke. *Cytologia* **56**: 233-238

Price, S.C., Shumaker, K.M., Kahler, A.L., Allard, R.W. and Hill, J.E. 1984. Estimates of population differentiation obtained from enzyme polymorphisms and quantitative characters. *J. Hered.* **75**: 141-142

Purseglove, J.W. 1969. *Tropical Crops Dicotyledons.* Longman, London, pp. 441-450

Racusen, D. and Foote, M. 1966. Peroxidase isozymes in bean leaves by preparative disc electrophoresis. *Canad. J. Bot.* **44**: 1633-1638

Rafalski, J.A., Tingey, S.V. and Williams, J.G.K. 1991. RAPD markers- a new technology for genetic mapping and plant breeding. *Agr. Biotech. News Info.* **3**: 645-648

Rahiman, B.A. and Nair, M.K. 1983. Morphology, cytology and chemical constituents of some *Piper* species from India. *J. Plant. Crops* **11** (2): 72-90

Rahiman, B.A. and Nair, M.K. 1986. Cytology of *Piper* species from the Western Ghats. *J. Plant. Crops* **14** (1): 52-56

Raja, K., Ravindran, P.N. and Nair, M.K. 1983. Quality evaluation of black pepper cultivars. *Indian Spices* **20**: 3-5

Raje, R.S. and Rao, S.K. 2001. Genetic diversity in a germplasm collection of mung bean (*Vigna radiata*). *Indian J. Genet.* **61** (1): 50-52

Ram, A.S. and Sreenath, H.L. 1999. A method for isolation and amplification of coffee DNA using random octamer and decamer primers. *J. Plant. Crops* **27**(2): 125-130

Ramankutty, N.N. 1977. A note on pepper in Wynad. *Agric. Res. J. Kerala* **15**(1): 89-90

Rani, V., Parida, A. and Raina, S.N. 1995. Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus deltoids* Marsh. *Pl. Cell Rep.* **14**: 459-462

Ratnambal, M.J., Ravindran, P.N. and Nair, M.K. 1985. Variability in black pepper cultivar Karimunda *J. Plant. Crops* **13**: 154-158

Reiter, R.S., Williams, J.G.K., Feldmann, K.A., Rafalski, J.A., Tingey, S.V. and Scolnik, P.A. 1992. Global and local genome mapping in *Arabidopsis thaliana* by using recombinant inbred lines and random amplified polymorphic DNAs. *Proc. nat. Acad. Sci. USA* **89**: 1477-1481

Reyes, L.M.C., Martinez, O.W. and Beltran, M. 1998. Chemical variability in the genus *Musa*: Genetic characterisation using nine enzyme systems. *Infomusa* 7: 6-8

Rick, C.M. and Fobes, J.F. 1975. Allozyme variation in the cultivated tomato and closely related species. *Bull. Torbey bot. Club* **102**(6): 376-384

Rick, C.M., Zobel, R.W. and Fobes, J.F. 1974. Four peroxidase loci in red fruited tomato species: genetic and geographical distribution. *DNAs* **71**: 835-839

Rieseberg, L.H. 1996. Homology among RAPD fragments in interspecific comparisons. *Mol. Ecol.* **5**: 99-105

Rival, A., Bertrand, L., Beule, T., Combes, M.C., Trouslot, P. and Lashermes, P. 1998. Suitability of RAPD analysis for the detection of somaclonal variants in oil palm *(Elaeis guineensis* Jacq.). *Pl. Breed.* **117**: 73-76

Rogers, S.O. and Bendich, A.J. 1994. Extraction of total cellular DNA from plants, algae and fungi. *Pl. Mol. Biol. Manual* **501**: 1-8

Rolfs, A., Schuller, M. I., Finckh, V. and Weber-Rolfs, I. 1992. *PCR: Clinical Diagnostics and Research.* Springer-Verlag, New York, p. 86

Sadasivam, S. and Manickam, A. 1996. *Biochemical Methods.* New Age International (P) Ltd. and TNAU, Coimbatore, pp. 143-189

Sambrooke, J., Fritsch, E.F. and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Course Manual.* Cold Spring Harber Laboratory Press, New York, pp. 70-93

Samuel, M.R.A. and Bavappa, K.V.A. 1981. Chromosome numbers in the genus *Piper.* *Curr. Sci.* **50**(4): 197-198

Samuel, R. 1986. Chromosome numbers in *Piper.* *Kew Bull.* **42**: 465-469

Satovic, Z., Torres, A.M. and Cubero, J.I. 1996. Genetic mapping of new morphological isozyme and RAPD marker in *Vicia faba* (L.) using trisomics. *Theor. Appl. Genet.* **93**: 1130-1138

Saunders, B.C., Holmes-Siedle, A.G. and Stark, B.P. 1964. *Peroxidase.* Butter-Worths, London, pp. 134-140

Scandalios, J.G. 1964. Tissue specific isozyme variation in maize. *J. Hered.* **55**: 281-285

Scandalios, J.G. 1969. Genetic controls of multiple molecular forms of enzymes in plants; a review. *Biochem. Genet.* **3**: 37-79

Schierwater, B. and Ender, A. 1993. Different thermostable DNA polymerases may amplify different RAPD products. *Nucleic Acid Res.* **21**: 4674-4678

Sebastian, A. 1995. Isozyme variation in *Piper* spp. M.Sc. (Hort.) thesis, Kerala Agricultural University, Thrissur, pp. 55-87

Shah, F.H. and Parveez, G.K.A. 1992. Construction of recombinant plasmids harbouring single copy genomic DNA of oil palm in *E. coli. Elaeis* **4**: 11-18

Sharma, A. 1991. *Chromosomes.* Oxford and IBH Publishing Company, New Delhi, p. 121

Sharma, A.K. and Bhattacharya, N.K. 1959. Chromosome studies on two genera of the family *Piperaceae. Genetica* **29**: 256-289

Shaw, C.R. 1970. How many genes evolve? *Biochem. Genet.* **4**: 275-283

Shaw, C.R. and Koen, P.L. 1968. Starch gel zone electrophoresis of enzymes. *Chromatogaphic and Electrophoretic Techniques Vol.2* (Ed. Smith, I.), John Wiley, New York, pp. 325-359

Siegel, B. and Galston, A. 1967. The isoperoxidases of *Pisum sativum. Pl. Physiol.* **42**: 221-226

Sikka, R.K., Lakshmanachar, M.S. and George, C.K. 1984. Some studies on the commercial quality of important varieties of pepper. *Indian Cocoa Arecanut Spices J.* **7**(3): 85-86

Simmonds, N.W. (Ed.) 1976. *Evolution of crop plants.* Longmans, London, pp. 86-90

Smith, J.B. 1966. Chromosome numbers in *Peperomia* Ruiz and Pav (Piperaceae) and note on chromosome number of *Piper magnificum. Kew Bull.* **20**: 521-526

Smith, H.H. and Conklin, M.E. 1975. Effects of gene dosage on peroxidase isozymes in *Datura stramonium* trisomics. *III. Isozymes: Developmental Biology* (Ed. Markert, C.L.), Academic Press, New York, pp. 337-341

Smith, J.S.C. and Williams, J.G.K. 1994. Arbitrary primer mediated fingerprinting in plants: case studies in plant breeding, taxonomy and phylogeny. *Molecular Ecology and Evolution: Approaches and Applications* (Eds. Sibierwater, B., Street, B., Wagner, G.P and de Salle, R.) Birkhauser Verlag, Basel, Switzerland, pp. 5-15

Son, S.H., Moon, H.K. and Hall, R.B. 1993. Somaclonal variation in plants regenerated from callus cultures of hybrid aspen (*Populus alba* x *P. grandidentata*). *Plant Sci.* **90**: 89-94

Sreekumari, M.T. and Mathew, P.M. 1991. Karyomorphology of five morphotypes of Taro (*Colocasia esculenta* (L) SCHOTT). *Cytologia* **56**: 215-218

StellaBai, G.V. and Subramanian, D. 1985. Cytotaxonomical studies of South Indian *Piperaceae . Cytologia* **50**: 583-592

Stuber, C. and Goodman, M. 1983. Inheritance, intracellular localization and genetic variation of phosphoglucomutase isozymes in maize (*Zea mays* L.). *Biochem. Genet.* **21**: 667-689

Sujatha, R. 1991. Variability in inter varietal F_1 hybrids and open pollinated seed progenies of black pepper (*Piper nigrum* L.). M.Sc. (Ag.) thesis, Kerala Agricultural University, Thrissur, p. 103

Sujatha, R. and Namboodiri, K.M.N. 1995. Influence of plant characters on yield in black pepper (*Piper nigrum* L.). *J. Tropic. Agric.* **33**: 11-15

Swartz, H. J., Galleta, G. J. and Zimmerman, R. H. 1983. Field performance and phenotypic stability of tissue culture propagated thorn less blackberries. *J. Amer. Soc. Hort. Sci.* **108**: 285-290

Takemori, N., Shinoda, K. and Kadotani, N. 1994. RAPD markers for confirmation of somatic hybrids in the dihaploid breeding of potato (*Solanum tuberosum* L.). *Pl. Cell Rep.* **13**: 367-371

Tanksley, S.D. and Orton, T.J. 1983. *Isozymes in Plant Genetics and Breeding- Part A.* Elsevier, Amsterdam. p. 516

Tatineni, V., Cantrell, R.G. and Davis, D.D. 1996. Genetic diversity in elite cotton germplasm determined by morphological characteristics and RAPDs. *Crop Sci.* **36**: 186-192

Taylor, P.W.J., Geijskes, J.R., Ko, H.L., Fraiser, T.A., Henry, R.J. and Birch, R.G. 1995. Sensitivity of random amplified polymorphic DNA analysis to detect genetic change in sugarcane during tissue culture. *Theor. Appl. Genet.* **90**: 1169-1173

Teparkum, S. and Veilleux, R.E. 1998. Indifference of potato anther culture to colchicine and genetic similarity among anther-derived monoploid regenerants determined by RAPD analysis. *Pl. Cell Tissue Org. Cult.* **53**: 49-58

Thorpe, T.A., Van, M.T.T. and Gasper, T. 1978. Isoperoxidases in epidermal layers of tobacco and changes during organ formation *in vitro*. *Physiol. Plant.* **44**: 388-394

Thomas H. and Stoddart, H. 1980. Heat senescence. *An. Rev. Pl. Physiol.* **31**: 83-11

Tingey, S.V. and del Tufo, J.P. 1993. Genetic analysis with random amplified polymorphic DNA markers. *Pl. Physiol.* **101**: 349-352

Todorovska, E., Trifonova, A., Petrova, M., Vitanova, Z., Marinova, E., Gramatikova, M., Valcheva, D., Zaprianova, S., Merkinkov, N. and Atanassov, A. 1997. Agronomic performance and molecular assessment of tissue culture derived barley lines. *Pl. Breed.* **116**: 511-517

Torres, A.M., Diedenhofen, V., Bergh, B.O. and Knight, R.J. 1978a. Enzyme polymorphisms as genetic markers in the avocado. *Amer J. Bot.* **65**: 134-139

Torres, A.M., Soost, R.K. and Diedenhofen, V. 1978b. Leaf isozymes as genetic markers in citrus. *Amer. J. Bot.* **65**: 869-881

Torres, A.M. and Tisserat, B. 1980. Leaf isozyme markers in date palms. *Amer. J. Bot.* **67**: 162-167

Torres, A. M., Weeden, N.F. and Martin, A. 1993. Linkage among isozyme, RFLP and RAPD markers in *Vicia faba*. *Theor. Appl. Genet.* **85**: 937-945

Tyson, H., Fields, M.A., Cheung, C. and Starubin, J. 1985. Isozyme relative mobility (Rm) changes related to leaf position: apparently smooth Rm trends and some implications. *Biochem. Genet.* **23**: 641-654

Upadhyaya, M.D. and Yee, J. 1968. Isoenzyme polymorphism in flowering plants. VII. Isoenzyme variation in tissues of barley seedlings. *Phytochemistry* **7**: 937-943

Varghese, Y.A., Knaak, C., Sethuraj, M.R. and Ecke, W. 1997. Evaluation of random amplified polymorphic DNA (RAPD) markers in *Hevea brasiliensis*. *Pl. Breed.* **116**: 47-52

Van Sumere, C.F., Albrecht, J., Deonder, A., de Pooter, H. and Pe, I. 1975. Plant proteins and phenolics. *The chemistry and biochemistry of plant proteins* (Eds. Harborne, J.B and Van Sumere, C.F.), Academic press, London, pp. 226-231

Versha, I. and Ravi 1995. Differential chromosome staining in *Oryza sativa* L. cultivars. *Crop Improv.* **22**(2): 154-159

Vierling, R.A. and Nguyen, H.T. 1992. Use of RAPD markers to determine the genetic diversity of diploid wheat genotypes. *Theor. Appl. Genet.* **84**: 835-838

Virmani, S.S. and Jill, B.S. 1972. Somatic chromosomes of *Pennisetum typhoides*. *Cytologia* **37**: 257-260

Visedo, G., Fernendez-Piqueras, J and Garcia, A. 1990. Isozyme profiles associated with the hypersensitive response of *Chenopodium foetidum* to plumpox virus infection. *Physiol. Plant.* **78**: 218-224

Wallner, E., Weising, K., Rompf, R., Kahl, G. and Kopp, B. 1996. Oligonudeotide fingerprinting and RAPD analysis of *Achillea* species: characterisation and long term monitoring of micropropagated clones. *Pl. Cell Rep.* **15**: 647-652

Walters, K.J., Hosfield, G.L. Uebersax, M.A. and Kelly, J.D. 1997. Navy bean canning quality: correlations, heritability estimates and randomly amplified polymorphic DNA markers associated with component traits. *J. Amer. Soc. Hort. Sci.* **122**: 338-343

Wang, H. and Holl, F.B. 1988. *In vitro* culture and incidence of somaclonal variation in regenerated plants of *Trifolium pratense* L. *Pl. Sci.* **55**: 159-167

Weeden, N.F. and Marx, G.A. 1984. Chromosomal location of twelve isozyme loci in *Pisum sativum*. *J. Hered.* **75**: 365-370

Weeden, N.F. and Marx, G.A. 1987. Further genetic analysis and linkage relationships of isozyme loci in the pea. *J. Hered.* **78**: 153-159

Weeden, N.F., Reisch, B.I and Martens, M.H.E. 1988. Genetic analysis of isozyme polymorphism in grape. *J. Amer. Soc. Hort. Sci.* **113**(5): 765-769

Weeden, N.F., Hemmat, M., Lawson, D.M., Lodhi, M., Bell, R. L., Mangabaris, A.G., Resch, B.I., Brown, S.K. and Ye, G.N. 1994. Development and application of molecular marker linkage maps in woody fruit crops. *Euphytica* **77**: 71-75

*Weiland, T. and Pfleiderer, G. 1957. *Nachweis der heterogenitat von milchsaure-dehydrogenasen verschiedenen ursprungs durch tragerelektrophorese. Biochem. Z.* **329**: 112-116

Wieme, R.J. 1974. Measurement of enzyme activities after electrophoresis. *Methods of Enzymatic Analysis, Vol. I* (Ed. Bergmeyer, H.V.). Academic Press. Inc., New York, pp. 261-282

Weining, S. and Langridge, P. 1991. Identification and mapping of polymorphisms in cereals based on the polymerase chain reaction. *Theor. Appl. Genet.* **82**: 209-216

Weir, B.J. and Pierre, S. 1997. RAPD marker polymorphism among Saskatoon cultivars, clones and seedlings. *Hort. Sci.* **32**: 1109-1113

Weising, K., Nybom, H., Wolf, K. and Meyer, W. 1995. *DNA Fingerprinting in Plants and Fungi*, CRC press, BocaRaton, pp. 21-34

Welsh, J. and Mc Clelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18**: 7213-7218

Wendel, J.F. and Weeden, N.F. 1989. Visualization and interpretation of plant isozymes. *Isozymes in Plant Biology*. (Eds. Soltis, D.E. and Soltis, P.S.) Dioscorides Press, Portland, pp. 5-45

Wettasinghf, R. and Peffley, E.B. 1998. A rapid and efficient extraction method for onion DNA. *Pl. Breed.* **117**: 588-589

Wickneswari, R. 1990. Use of isozyme analysis in proposed *Acacia mangium* x *Acacia auriculiformis* hybrid seed production orchard. *J. Trop.For. Sci.* **2**(2): 157-164

Wilde, J., Waugh, R. and Powell, W. 1992. Genetic fingerprinting of *Theobroma* clones using randomly amplified polymorphic DNA markers. *Theor. Appl. Genet.* **83**: 871-877

Wilkinson, J.H. 1970. *Isoenzymes*. Science Paperbacks, Chapman and Hall, London, p. 369

Williams, J.G.K., Kubelik, A.R., Liva, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**: 6531-6535

Zaman, A., Islam, R. and Joarder, O.I. 1997. Field performance and biochemical evaluation of micropropagated mulberry plants. *Pl. Cell Tissue Org. Cult.* **51**: 61-64

Zimmerman, R.H. 1986. Propagation of fruit, nut and vegetable crops. *Tissue Culture as a Plant Propagation System for Horticultural Crops* (Eds. Zimmerman, R.H., Grieisback, R.J., Hammerschlag, F.A. and R.H. Lowson), Martinus Nijhoff Publishers, Dordrecht, pp. 183-200

*Original not seen

APPENDICES...

APPENDIX - I

LIST OF IMPORTANT CHEMICALS USED

I. Cytological Analysis

Orcein dye	-SRL
Basic Fuchsine	-SRL
Carmine dye	-SRL

II. Isozyme analysis

TRIS buffer	-SRL
PVP	-SRL
PVPP	-Sigma, USA; E Merck, Germany
Triton x100	- SRL
Acrylamide	-SRL
Bis-Acrylamide	-SRL
TEMED	-SRL
Ammonium persulphate	-SRL
Glycine	-SRL
PMSF	-SRL
Benzidne	-Sigma
Fastviolet B salt	-Sigma

III. RAPD Analysis

TRIS buffer	- Sigma, USA
CTAB	- E Merck, Germany
Agarose	- Sigma, USA; Genei, Bangalore
Lithium Bromide	- Sigma, USA
RNAse	- Pancreatic RNase (RNase A)
Assay buffer (10x TRIS with $MgCl_2$)	- Genei, Bangalore
Taq DNA Polymerase	- Genei, Bangalore
DNTPs	- Genei, Bangalore
Primers	- Operon Technologies, USA
Mineral Oil	- Sigma, USA
Molecular marker (Hind III)	- Genei, Bangalore
Sodium dodicyl sulphate)	- Sigma, USA
β-mercapto ethanol	- E Merck, Germany
Bromophenol blue	- Sigma, USA
Liquid N_2	- Madras Oxyacetelene Company, Coimbatore
EDTA	-Sigma, USA

APPENDIX - II

LAB EQUIPMENTS

I.   Cytological Analysis

1. Trinocular Research microscope (Biomed)
2. Camera (Nickon)

II.   Isoenzyme Analysis

1. Gel electrophoretic system- Hoefer, USA; Genei, Bangalore
2. Micro centrifuge, Spinwin
3. Remi cooling centrifuge
4. Refrigerated highspeed centrifuge- Kubota 9000, Japan
5. Deep freezer- Sanyo, Japan

III.   RAPD

1. Refrigerated highspeed centrifuge - Kubota 9000, Japan
2. Spectrophotometer- Spectronic Genesys –5, Spectronic Instruments Inc., USA
3. Drybath incubator- Genei, Bangalore
4. Water purification system- Millipore, Germany
5. Autoclave
6. Laminarflow- Kirloskar, India
7. Thermal cycler- PTC-200 Peltier Thermal cycler, MJ Research, USA
8. Deepfreezer- Sanyo, Japan
9. Electronic Balance- Sarturius
10. Mycrocentrifuge- Spinwin
11. Electrophoresis system- Hoefer, USA, Biotech, Madras; Genei, Bangalore
12. Transilluminator- Herolab, Germany
13. Documentation system- Alpha Infotech,USA
14. Ice flaking machine- Icematics
15. Glasswares - Borosil India Ltd.
16. Plastic ware (Polypropylene)- Merck India, Tarson's India Ltd.

APPENDIX -III

OPERON DECAMER PRIMERS USED FOR THE INVESTIGATION

Code	5' to 3'	M.W.	pmoles	µg/tube
OPE-01	CCCAAGGTCC	2964	5615	16.5
OPE-02	GGTGCGGGAA	3124	4771	15.0
OPE-03	CCAGATGCAC	2988	5192	15.5
OPE-04	GTGACATGCC	3019	5300	16.0
OPE-05	TCAGGGAGGT	3099	4892	15.0
OPE-06	AAGACCCCTC	2948	5413	16.0
OPE-07	AGATGCAGCC	3028	4988	15.0
OPE-08	TCACCADGGT	2979	5531	16.5
OPE-09	CTTCACCCGA	2939	5783	17.0
OPE-10	CACCAGGTGA	3028	4988	15.0
OPE-11	GAGTCTCAGG	3059	5088	15.5
OPE-12	TTATCGCCCC	2930	6207	18.0
OPE-13	CCCGATTCGG	2995	5742	17.0
OPE-14	TGCGGCTGAG	3075	5265	16.0
OPE-15	ACGCACAACC	2957	5088	15.0
OPE-16	GGTGACTGTG	3090	5192	16.0
OPE-17	CTACTGCCGT	2970	5918	17.5
OPE-18	GGACTGCAGA	3068	4799	14.5
OPE-19	ACGGCGTATG	3059	5088	15.5
OPE-20	AACGGTGACC	3028	4988	15.0

Code	5' to 3'	M.W.	pmoles	µg/tube
OPP-01	GTAGCACTCC	2979	5531	16.5
OPP-02	TCGGCACGCA	3004	5377	16.0
OPP-03	CTGATACGCC	2979	5531	16.5
OPP-04	GTGTCTCAGG	3050	5413	16.5
OPP-05	CCCCGGTAAC	2964	5615	16.5
OPP-06	GTGGGCTGAC	3075	5265	16.0
OPP-07	GTCCATGCCA	2979	5531	16.5
OPP-08	ACATCGCCCA	4948	5413	16.0
OPP-09	GTGGTCCGCA	3035	5493	16.5
OPP-10	TCCCGCCTAC	2915	6313	18.5
OPP-11	AACGCGTCGG	3044	5158	15.5
OPP-12	AAGGGCGAGT	3108	4625	14.5
OPP-13	GGAGTGCCTC	3035	5493	16.5
OPP-14	CCAGCCGAAC	2973	5265	15.5
OPP-15	GGAAGCCAAC	3037	4710	14.5
OPP-16	CCAAGCTGCC	2964	5615	16.5
OPP-17	TGACCCGCCT	2955	6014	18.0
OPP-18	GGCTTGGCCT	3026	5874	18.0
OPP-19	GGGAAGGACA	3117	4385	13.5
OPP-20	GACCCTAGTC	2979	5531	16.5

Code	5' to 3'	M.W.	pmoles	µg/tube
OPF-01	ACGGATCCTG	3019	5300	16.0
OPF-02	GAGGATCCCT	3019	5300	16.0
OPF-03	CCTGATCACC	2939	5783	17.0
OPF-04	GGTGATCAGG	3099	4892	15.0
OPF-05	CCGAATTCCC	2939	5783	17.0
OPF-06	GGGAATTCGG	3099	4892	15.0
OPF-07	CCGATATCCC	2939	5783	17.0
OPF-08	GGGATATCGG	3099	4892	15.0
OPF-09	CCAAGCTTCC	2939	5783	17.0
OPF-10	GGAAGCTTGG	3099	4892	15.0
OPF-11	TTGGTACCCC	2970	5918	17.5
OPF-12	ACGGTACCAG	3028	4988	15.0
OPF-13	GGCTGCAGAA	3068	4799	14.5
OPF-14	TGCTGCAGGT	3050	5413	16.5
OPF-15	CCAGTACTCC	2939	5783	17.0
OPF-16	GGAGTACTGG	3099	4892	15.0
OPF-17	AACCCGGGAA	3037	4715	14.5
OPF-18	TTCCCGGGTT	3001	6059	18.0
OPF-19	CCTCTAGACC	2939	5783	17.0
OPF-20	GGTCTAGAGG	3099	4892	15.0

CHARACTERISATION OF FIELD ESTABLISHED TISSUE CULTURE DERIVED BLACK PEPPER *(Piper nigrum L.)* PLANTS USING MORPHOLOGICAL, CYTOLOGICAL AND MOLECULAR MARKERS

By

R. SUJATHA

ABSTRACT OF THE THESIS

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COLLEGE OF HORTICULTURE

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ABSTRACT

Universally acclaimed as the ' King of Spices', black pepper (*Piper nigrum* L.) enjoys a unique position as a commercial crop of historical importance and assumes great economic importance to several nations of the world. Efforts to evolve high yielding types of black pepper through selection as well as hybridisation started as early as in 1953 (Nambiar *et al.*, 1978) and by now, about ten improved varieties were evolved and released. In order to meet the insatiable demand from farmers for the planting materials of high yielding pepper varieties, mass multiplication protocol through micro-propagation has been standardised (Joseph *et al.*, 1996). Babu (2000) tested the fidelity of TC plantlets of black pepper during the hardening stage using RAPD assay. A thorough evaluation and characterisation of these TC plants in relation to their performance in the field is a prerequisite to establish their heritage and usefulness. In this background, the present study was taken up with the main objectives of establishing the genetic identity of TC derived black pepper plants in field; testing the intra-group, inter-clonal and inter-varietal polymorphism and obtaining a thorough and fool proof finger print of the TC plants using a combination of molecular marker techniques and conventional markers.

Each of the experimental vines was characterised using morphological traits (biometric as well as qualitative) based on the descriptor formulated by NBPGR. The cytological studies ruled out any variation caused by a change in the chromosome number, which is possible in *in vitro* cultured plants. The somatic chromosome number was found uniform in all the vines with 2n = 52, which is characteristic of the species. The molecular markers provided a more specific identity for each of the experimental vines. The

zymogram based on peroxidase as well as RAPD banding pattern has given a very clear fingerprint characteristic of each vine.

The biometric observations and visual assessment of the vines based on qualitative traits brought out the intra group (within conventional clones or within TC clones), inter-clonal (CC vs. TC clones) and inter varietal variation of the different characters. Among the 22 biometric observations, 17 were found homogenous within clonal groups and also within the TC groups irrespective of varieties. The remaining five traits were discussed with respect to each variety. Most of the qualitative observations also showed uniformity in the clones as well as TC plants. The estimation of inter-clonal variablity proved the uniformity and better vigour of TC plants compared to the conventional clones. Another interesting observation was the great variablity in qualitative and quantitative traits between TC Subhakara and clonal Subhakara indicating a possible error in labelling.

The molecular markers brought out the variablity in the different vines more specifically. In both isozyme and RAPD analysis, the clones under each variety were found to be monomorphic whereas certain variants could be detected within TC plants with respect to a few bands. The vines TC P_2-7 and TC P_1-1 were separated out as variants by both isozyme and RAPD markers. Other TC vines, which showed polymorphism with respect to either of these markers were P_1-1, P_1-2, P_2-10, P_4-9, Su-9, Su-10, P_4-4 and P_4-8. The assessment of inter clonal variability using molecular markers clearly pointed out the distinctly different traits of TC clones and conventional clones Subhakara. Morphological observations too supported this finding. Using RAPD, it was conclusively proved that the vines labelled as TC Su were in fact TC P_4. This confirmed the possibility of an error in labelling while the TC plantlets were transported from Vellanikkara to Panniyur.

The inter-varietal polymorphism brought out by morphology and molecular markers was useful in discriminating the four black pepper varieties and to asses the genetic distance between them, which is an important criterion in the selection of parents for hybridisation. The results exposed the genetic proximity between P_1 and P_2 and between P_4 and Su. The standardisations of various protocols for cytological and molecular marker analysis in black pepper as well as the relative efficiency of the different marker systems were also discussed. The RAPD technique was found most effective in assessing the genetic constitution of the individual vine.