Study of genetic diversity and standardization of genetic transformation in *Camellia sinensis* (L.) O. Kuntze

Thesis

Submitted in fulfillment of the Degree of

Doctor of Philosophy

By

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Supervisor

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STOCK TAKING - 2011

This work is dedicated to my beloved parents

Sardar Jaswant Singh Gill

&

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CERTIFICATE

I certify that the thesis entitled, "Study of genetic diversity and standardization of genetic transformation in *Camellia sinensis* (L.) O. Kuntze" submitted by Ms. Manprit Gill for the award of PhD degree of the University of North Bengal, embodies the record of the original investigation carried by her under my supervision. She has been duly registered and the thesis presented is worthy of being considered for the award of Doctor of Philosophy (Science) degree in Botany. The work has not been submitted for any degree of this or any other university and is in accordance with the rules and regulations of the University of North Bengal.

Arnab Sen

(Arnab Sen)
Sr. Lecturer

Date: 21/11/06.

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Manpreet Gill

[Manprit Gill]

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The Way of Tea states

"One cup does all disorders cure
With two, your troubles will be fewer
Thrice, to the bone more vigour give
With four, forever you will live
as young as on your day of birth
A true immemorial on the earth"
Old Chinese Poem

Many of us would agree with the ancient Chinese saying: *"Better to be deprived of food for three days, than of tea for one"* (Ody 1993).Tea, *Camellia sinensis* (L.) O. Kuntze belongs to Thea section of the *Camellia* genus in Theaceae. Chemical composition of *Camellia sinensis (*Ukers 1935) is given in Table 1.1. It is the oldest caffeine containing beverage which has been used for two to three hundred years in Southeast China and continues to be the most popular and widely consumed beverage in India and across the world.

Tea plant is a woody perennial shrub or tree (9 -15 meters in height) under natural conditions and 1.5 meters under cultivated conditions (Fig.1.1). Leaves are alternate, elliptical on short stalks, leathery and with toothed margins. The flowers are white in colour and born singly or pairs at the axils. The fruits are green in colour with 2–3 seeds and start bearing within 5–6 years after planting. The cultivated varieties separate into two main groups on the basis of foliar and growth characteristics. China teas, *Camellia sinensis* var.*sinensis*, are slow growing, dwarf trees, with small, erect, comparatively narrow, dark green leaves and are resistant to cold. In contrast, Assam tea, *C. sinensis* var. *assamica,* is quick- growing taller tree with large, drooping leaves and resistant to cold, while natural triploid (2n=45) and tetraploid (2n=60) varieties adapted to this environment have also been discovered. Various hybrids between China and Assam types are planted according to easy intercrosses. Hybrids are characterized by the intermediate charac-

Table 1.1 Chemical Composition of tea (Ukers 1935)

Constituent	Percent (%)
Water	5.00-8.0
Caffeine	2.50-5.0
Nitrogen	4.75-5.5
Soluble matter	38.0-45.0
Tannin	7.00-14.0
Mineral elements	5.00-5.75

teristics of leaves and growth of trees when compared between the two types.

1.1. Distribution of tea (worldwide and in India)

Tea is cultivated in 31 countries, scattered from 45°N to 33°S of the equator. From the main centers of its primary origin in South-East Asia, tea has spread far and wide into tropical and subtropical areas and adopted broad characteristics corresponding to regions of tropical rainforest, tropical Savannah and summer rain areas (Eden 1976; Greenway 1945); Kingdon ward 1950; Kulasegaram 1980). The earliest knowledge of the tea- the oldest caffeine-containing beverage has been derived from China for about 3000 years back. It is believed to have originated somewhere in South-East Asia (Kingdon-Ward, 1950) but the current distribution patterns of tea varieties suggest that the centre of origin of tea is probably near Irrawady (Burma) region from where it has been dispersed to South-Eastern China, Indonesia and Assam.

In India tea seeds from china were brought and sown at Botanical Garden, Calcutta in 1780 (Bezbaruah, 1999). Tea (china type) was introduced in north east India in 1836, although in 1823, Major Robert Bruce discovered tea plants growing wild in some hills near Ragpur (now Sibsagar) the then capital of Assam (Ukers 1935). In South India one Dr. Christy has experimented on growing tea in Nilgiris in 1832 (Muralidharan 1991). But the tea did not get under way until 1893 when the planting of tea increased rapidly (Harler 1963). On the basis of its distribution tea is classified into two main varieties: var. sinensis which originated from mainland China recognized by its small leaf, slower growing bush withstanding colder climates (Wight 1962; Banerjee 1992) and var. assamica discovered in the nineteenth century in the Assam region in India and later in other regions of south east Asia as well (Kaundan & Park 2002) which can be easily identified by its large leaf, tall and quick growing characters which is well suited to very warm tropical climates (Wight 1962; Banerjee 1992). Besides these two basic varieties there exists *Camellia assamica* ssp. lasiocalyx (Planchon ex Watt) with its intermediate leaf size also called cambod variety.

1.2. Agro-Climatic conditions required for tea cultivation (Tea board of India)

A suitable climate for cultivation has a minimum annual rainfall of 45-50 inches (114.3-

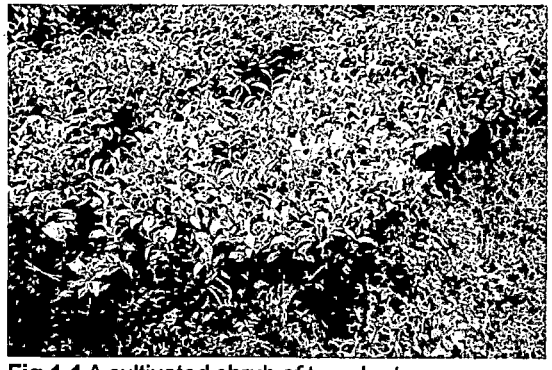


Fig 1.1 A cultivated shrub of tea plant.

127 centimeters). Tea soils must be acidic since tea plants will not grow in alkaline soils. A desirable pH value is 5.4-5.8 or less. Tea can be cultivated up to 7,218.2 feet (2,200 meters) above sea level and can grow between the equator and the forty-fifth latitude. The plants are reproduced through tile-laying or through seeds from trees that have grown freely.

1.2.1. Air Temperature

Tea is grown under a regime of air temperature that varies between 8° and 35°C. In Darjeeling, the extension growth stops at monthly mean maximum and minimum temperatures of 19.4°C and 12.4°C respectively in November and it start flushing during end of March when mean maximum and minimum temperatures exceed 21°C and 14°C respectively. The extension growth of the tea plant in general ceases below a minimum temperature of 13°C. In Darjeeling, highest yield can be achieved in June when mean maximum and minimum temperatures remains 23.5°C and 18.3°C respectively. The rapid decline in yield in Darjeeling during October and then stops during November until end of March indicates that low temperature is one of the major climatic variables, limiting yield. It has been reported that the higher yield in Darjeeling can be achieved during the period from June to September when the differences between maximum and minimum temperatures remains least in comparison with the rest of the year (Fig. 1.2).

1.2.2. Soil temperature

In many instances soil temperature is of greater importance to plant life than air temperature and soil temperature influences to growth and yields of tea. Soil temperature is an important variable, with a lower limit of

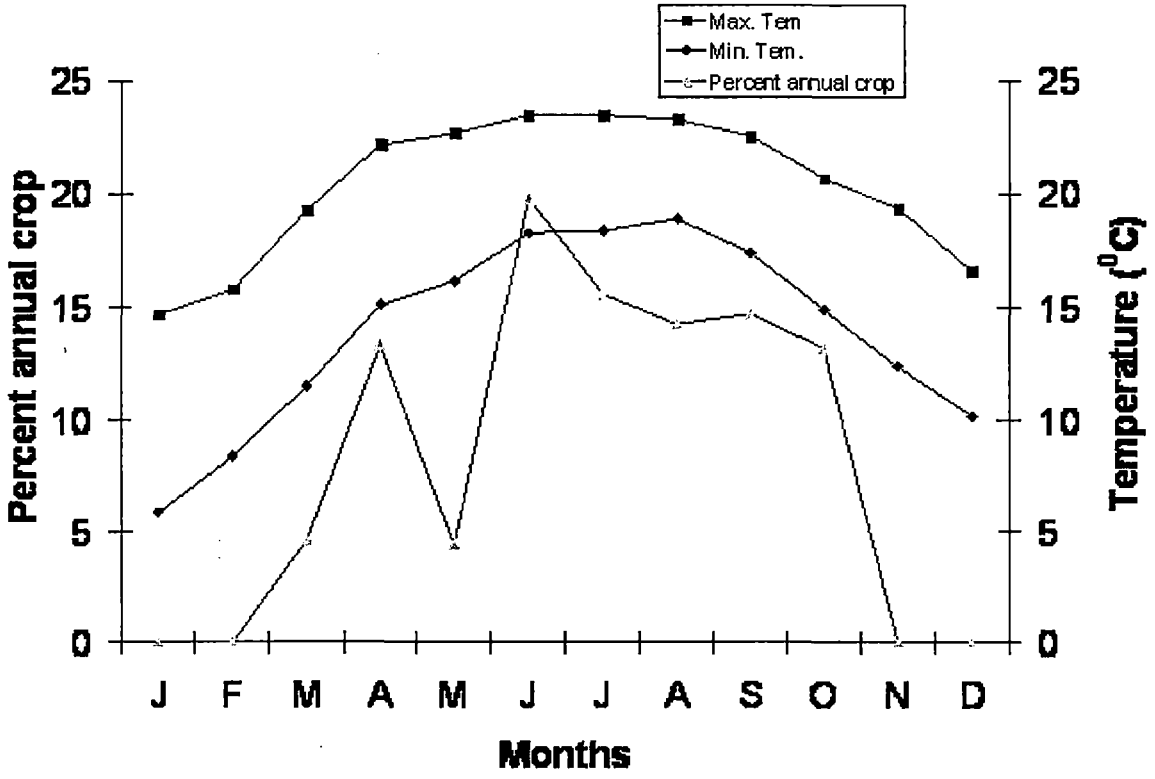


Fig. 1.2 The relationship between monthly yield and air temperature at Darjeeling. Source: Darjeeling Tea Research and Development Centre, Tea Board of India.

about 20°C, at 0.3 m under short grass surface (or 16°C beneath a canopy tea), below which shoot extension rates will be reduced. The corresponding upper levels are 29°C and 25°C respectively.

1.2.3. Rainfall

Tea is basically a rain-fed crop. It is grown well in areas where annual rainfall varies from 1150 to 6000 mm. Tea should not normally be grown in areas where the rainfall is below 1150 mm, unless irrigation is available. The effect of precipitation is perhaps more manifested by its influence on moisture status of the soil and in inducing vegetative growth. Therefore, distribution of rainfall over the year is as vital as the total annual rainfall. Annual rainfall varies from 2274 (Kalimpong sub-division) to 4082 mm (Kurseong sub-division) in Darjeeling where in May, the southerly winds reaches the hills and cause increased precipitation which is at times very high (Fig. 1.3). There is some residual effect of monsoon in November. But, there is almost no rain in November and December and the light showers which fall in January and February occur when shallow depressions are passing eastward over the plains. In October, northerly winds begins, cloud is much less than in previous months and rainfall occurs, mainly owing to cyclonic storms which generally recurve towards North Bengal at the end of the season

1.2.4. Humidity

It has influence in determining the loss of moisture by evapo-transpiration. The invisible water content of the air is expressed as relative humidity (RH), saturation vapour pressure deficit or dew point. RH of 80 - 90 % is favourable during the growth period of tea plants, below 50 %, shoot growth is inhibited

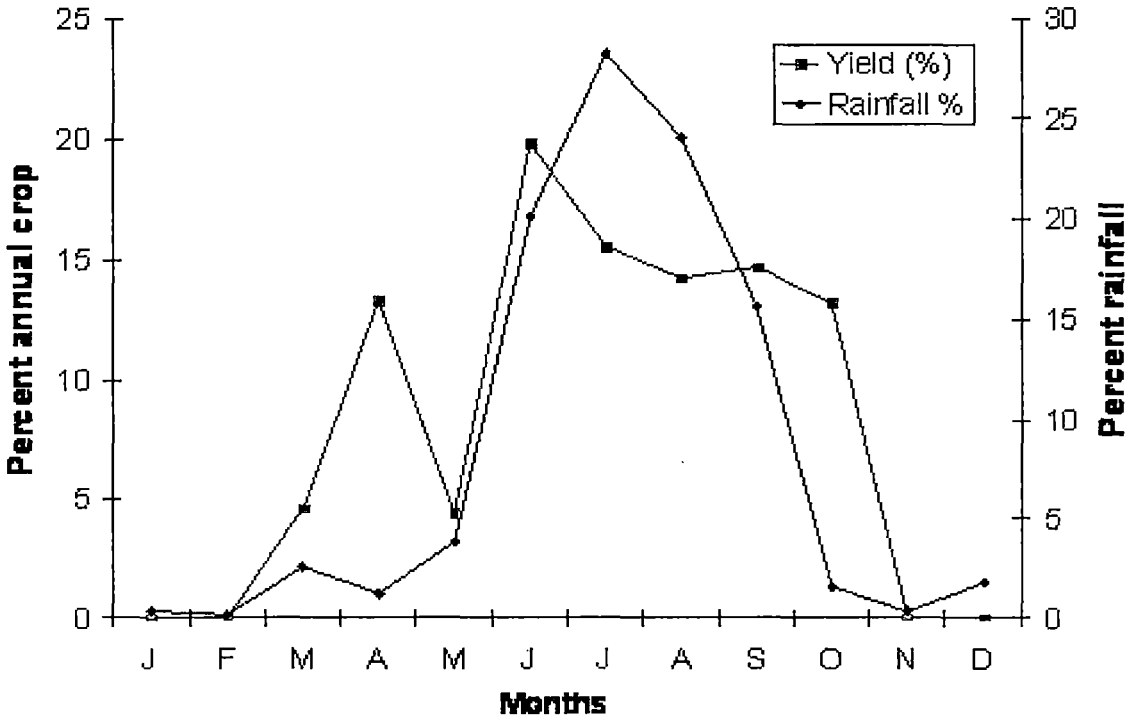


Fig. 1.3 The relationship between monthly yield of processed tea and rainfall at Darjeeling gardens. Source: Darjeeling Tea Research and Development Centre, Tea Board of India.

and below 40 % growth is adversely affected. In Darjeeling, even during the driest part of the year in March and April, mean RH never drops below 60 % in this region.

1.2.5. Solar radiation

It is the source of energy which sustains organic life on the earth surface. Crop production is in fact an exploitation of solar radiation. The intensity and duration of sunshine has also an important influence on the growth of tea plant. The hours of bright sunshine are fewer in the rainy season of Darjeeling hills due to the overcast clouds.

1.2.6. Day length

There are remarkable variations in day length between 9 and 15 h prevailing in Darjeeling. In Darjeeling, highest yield was recorded during June when average day length was 13 h 49 m. In fact, 50 % annual crop is produced in Darjeeling during June to August when day length obtained in between 12-13.5 h is a factor contributing to high productivity during this period.

1.2.7. Altitude

The China variety in particular and the hybrids in general produce very fine, flavoury tea when they are grown above an altitude of about 1100 m as in the case of Darjeeling. China hybrid bushes from Darjeeling fails to reproduce the full hill muscatel flavour if grown in the plains of Assam and Dooars. It may thus be stated that the Darjeeling teas owe their unique flavour partly to the type of bush and partly to the climate.

1.2.8. Hail

It is unpredictable and of a localized nature. Hail in Darjeeling is almost a regular phenomenon every year during spring and cause severe losses. Hailstorms occur in one valley or other during March to May, but end of March to early April is the period when it occurs most. The severity of damage ranges from shattering of leaves to peeling of bark in trunks, young stems breaks and bushes are defoliated. It can be particularly harmful when the bushes are recovering from the effect of a severe drought and are re-foliating after pruning. In Darjeeling hills, loss of higher priced quality crop during first and second flush due to hail damage is actually the real damage in terms of income. Despite destruction of current crop it damage stems which lead to disease and pest problems (Fig. 1.4)

1.3. Methods of propagation

Tea is traditionally propagated through either seeds or stem cuttings, with a life span of more than 100 years. The plants are obtained from seeds because the flower has high cross pollinating ability. Biclonal seeds i.e., trees planted in two clones in the seed garden are used for propagation. Cross fertilized seed bushes are selected for yield and quality or other characteristics. Seeds are grown in special seed gardens and seedlings show high heterogeneity. Moreover, since seeds become recalcitrant and unable to retain their viability through long term storage, seeds need to be sown immediately. Vegetative propagation by using cuttings has become one of the most frequently used

Fig. 1.4 Hail damaged tea plant in Darjeeling

methods. Usually single internode cuttings, taken immediately above a leaf and axillary bud, are planted into the rooting bed. New growing shoots with five to six leaves are also used for cutting materials. The cuttings from selected trees are planted in shaded propagating beds and given moderate amounts of water. Rooting ability varies among cultivars. Methods of propagation with seeds or cuttings require a longer period of time and space for production of nurslings. Shaping and pruning are done to maintain a convenient height for plucking, to induce vigorous vegetative growth and ensure a continuous supply of flushes. China types, being dwarf and slow growing, require relatively little pruning, where as Assam types and hybrids have to be kept within bounds. Pruning should be done during a dormant period (Purseglove 1974). Newly grown terminal buds with two or three leaves are plucked and brought to the factory.

1.4. Processing and marketing of Tea

The young leaves are processed into different types of tea, such as black, green, and oolong. The *assamica* variety contains large amounts of tannin and Catechin and is particularly used for black tea, where as *sinensis* tea accounts for most of the green tea production (Kaundan & Matsumoto 2003). Health benefits attributed to tea consumption are well proven. The processing of black tea comprises four main steps: withering or drying, rolling, fermentation and firing. During firing, the fermentation is stopped by destroying the enzyme with heat. This is done either by steaming or roasting. The process takes about 40 minutes at 71°C and 15 minutes at 99°C (Kihlman 1977). The fermented tea leaf enters the heating room, where it immediately undergoes heating at a high temperature of 90 to 95°C. The firing process is performed in special dryers. The hot air from the heater reduces tea moisture content to 3% to 4%. The firing stage is followed by a sorting process to make the half finished product marketable (Bokuchava and Skobeleva 1980).

Tea (*Camellia sinensis* L.; family Theaceae) is the oldest non-alcoholic caffeine-containing beverage crop in the world, and India is currently the foremost producer, consumer, and exporter of commercial tea. India with 5.11 lakh hectares of tea under cultivation produces an average of 850 million Kg of tea per year. It accounts for a fifth of the global tea area and nearly a third of the total world production. At present tea is grown in 16 states. Major tea growing states are Assam (50.7%), West Bengal (22%), Tamil Nadu (15.9%) and Kerala (8.3%) (Boriah, 2004).

Darjeeling produces the world's finest quality tea in the steep hill slopes of Eastern Himalayas. It is regarded as the most important beverage consumed in India and worldwide after water. This minor crop contributes significantly to the economy of many asian countries including India (Kaundan & Park 2002). Despite India's historical success with the tea industry, in recent years, Indian teas have faced serious competition in the international market. The UK now imports tea cheaper from Kenya and Malawi. To combat some of these challenges advertisers have made efforts toward building marketing cam-

paigns in order to boost the tea market. Some efforts have even been made to promote tea like Coca Cola. Yet despite these efforts, tea producers in India continue to face serious challenges: how to keep quality, production, and exports up without driving prices down.

1.5. Diseases of tea

Diseases of tea plants differ within the types of plant and planting areas. In areas where Assam teas (var. *assamica*) are grown, as in India, Srilanka and many other countries, blister blight caused by *Exobasidium vexans Massee* remains the most dangerous disease. Air borne basidiospores of *Exobasidium vexans* spread and form white blisters on young leaves and stems. Buds can be attacked and whole shoots may die. Anthracnose caused by *Gloeosporium theae-sinensis* Miyake is the most serious disease in Japan and China. It spreads over the area where the China tea (var. *sinensis*) is produced. It has been found very recently that fungus invades the plant only through the tricomes of three young leaves from the top of the growing shoots (Hamaya 1981, 1982). The fungus, however, does not attack the stem. Variety *assamica* and its hybrids are highly resistant to this disease. Grey blight caused by *Pestalottia theae* Sawada, *Pestalotia longiseta* Spegazzini and brown blight or copper blight by *Glomerella cingulata* (Stomen) Spalauding et Schrenck, white scab by *Elsinoe leuospila* Bitancourt et Jenkins (*Sphaceloma theae* Kurosawa) infect the leaves or growing shoots. Root disease, red root disease caused by *Poria hypolateritia* Berk. is very serious in Srilanka, India and Indonesia, but not observed in Japan. In its mode of development it resembles *Armillaria*, by causing the sudden death of the tree. *Armillaria mella* (Vahl: Fr.) Kummer is common in Africa but remains rare in Srilanka, India and Japan. Charcoal stump rot caused by *Ustilina deusta* (Fr.) Petrak and black root disease by *Rosellinia arculata* Petch are serious diseases in India, Srilanka and Indonesia, while charcoal stump rot is sporadically found in Africa. In Japanese *Pythium* cuttings, root rot by *Pythium spp.* is becoming a prevalent disease with the extention of vegetative propagation. The affected roots become watery, whitish brown then turn a brown colour and often flatten without inner substance. White root rot caused by *Rosellia necatrix* (Hartig) Berlese infests the plant and is also a very common disease in ornamental fruit trees. Stem disease, brunch canker caused by *Phomopsis theae* Hara, *Nacrophoma theicola* Petch, Nectria spp. and *Poria hypobrunnea* Petch are common in Srilanka and India. It is considered that a viral disease causes necrosis of phloem which can be present in root, stem or leaf bud. However, the vector has not been discovered and the diagnostic symptoms are spreading in tea producing areas of Srilanka and India. According to variety, tea plants differ in tolerance to some diseases such as anthracnose. It is suggested that there is scope for breeding of resistant varieties. Since some diseases are common in one area or country but not in others, the prevention of epidemics in international trade still represents a very important goal.

1.6. Uses of Tea (*Camellia sinensis*)

Tea has been consumed socially and habitu-

Table 1.2 Nutritive value of tea/tea infusion

Nutrients	Amount from 1 cup of tea	Actual requirement
Riboflavin	1mg	1.5mg per day
Nicotinic Acid	7.5mg	16mg per day
Pantothenic	2.5mg	6-10mg per day

ally by people for so long (since ±3000 BC), that aside from the astringent taste and boost it provides its medicinal properties are often over-looked. However, traditional healers have long believed that drinking tea is a means of prolonging life (Chopra 2000). It helps cure digestive complaints, infections and pain relief. Tea is one of the most popular beverages and is consumed by nearly half of the world's population. Though it is not a favoured drink for children, it is consumed by almost all adult population. Nutritive value of tea/tea infusion is given in (Table 1.2). However, a cup of tea with one teaspoon of sugar and two tablespoon of milk gives 42Kcals (1 teaspoon sugar=20 Kcalorie, 2 tablespoon milk=22 Kcalorie).

1.7. Tea Genetic Resources, conservation and its yield worldwide

1.7.1. Tea Genetic resources and conservation

A total of 2532 accessions have been collected and preserved in the field gene bank at Tocklai Experimental Station (Singh 1999). This collection has undoubtedly helped India as well as other tea growing countries in evolving superior plant materials (Bezbaruah, 1974; Singh, 1979). In South India, a majority of the selections available in the germplasm at present have been selected from some of the commercial tea estates of that region (Satyanarayana and Sharma, 1986). East Africa's tea germplasm is predominantly of the Assam type (*C.sinensis* var. *assamica*), and although appreciably diverse, the variability is not sufficiently broad since many of the clones are genealogically related (Wachira *et al.*, 1995). The preservation of tea germplasm has great importance primarily because the seed-grown sections of tea are being massively uprooted, the seed sources of which no longer exist and are lost forever. The wide variability in the commercial tea populations offers scope for the selection of elite mother bushes with desirable attributes in a practical plant improvement programme (Richards 1960; Bezbaruah 1975). Therefore, it needs to be preserved and utilized judiciously.

1.7.2. Tea Yield

Tea yield not only varies seasonally (Ghosh Hajra and Kumar 1999) but also with genetype, altitudes, climatic and edaphic factors (Carrr 1972) The exact contribution of each factor to total variability of yield is not yet known but the effect of environment seems to be quite profound. Since in selection and breeding, genetype environment interactions assume greater relevance (Wickramaratne 1981), therefore, a plant selected solely on the basis of yield in a particular environment should invariably be evaluated under different agroclimatic conditions. Harvest index is one of the determinants of yield in any crop. Tea is the one commercial crop with very low har-

vest index (HI) of 16 percent observed for clone UPASI 3 in South India (Murty and Sharma 1986), 12-13% in Assam tea in North East India (Hadfield 1974), 24 percent for clone S15/10 for the period two to four years after field planting in the Mufindi district of Southern Tanzania (Burgess and Carr 1996) and 8 percent reported for clone 6/8 at high altitude in Kenya (Magambo and Cannell 1981). In tea, yield potential realization is limited by inefficient conversion of intercepted radiation to dry matter and low HI (Singh 1999). Therefore, breeding more efficient plant type and to increase the partitioning of more photosynthates towards the harvestable dry matter (so that Hi can be raised to 20-25%) is the solution (Jain and Tamang 1988).

1.8. Problem areas in Tea

It is a well known fact that tea is highly outcrossing and clinal introgressants arising from these two extremes are frequently observed. Thus, a large variation in several important and desirable characters occurs from bush to bush in the existing tea populations (Ghosh 2001). Selection and breeding for the production of desirable traits in this perennial crop have resulted in severe erosion of its genetic base over the time. Lack of proper conservation programmes have caused major reduction in its gene pool, as most of the breeding and conservation programs are still based on conventional morphological and agronomical descriptors, which are dependent on environmental and developmental factors thus reflecting the base of the gene pool (Green 1971; Wilkremaratne 1981) with no true genetic relatedness. The breeding of experimental planting material of a perennial, heterozygous crop like tea is difficult. However, like any other crop, the main objective of tea breeding is to improve the quantity and quality of the end product. The methods of introduction, selection, and hybridization have been used with success for tea improvement. The different varieties have been developed to suit the requirements of the various agroclimatic regions. However, tea genetics is still poorly understood. Proper selection criteria have not yet been established. This apart, the prediction of the performance of mature tea based on their evaluation in the early years has not been perfected. Application of modern techniques, as has been done in other crops, is a greater challenge to tea breeders and tea biotechnologists.

Since the chemical composition of calli in the tea plant was analyzed (Ogutuga and Northcote 1970a, b), callus induction and organogenesis have been reported in several papers from the year 1980 to 2001. In tea research the previous era held major emphasis on standardizing parameters of the *in vitro* protocol, such as using a suitable explant, overcoming microbial contamination, and optimizing media composition combined with growth regulation for better proliferation (Kato1989). Following this era the efforts turned towards hardening micro-shoots to achieve a higher survival percentage. Accordingly, several non-conventional approaches, such as a CO_2-enriched hardening chamber, biological hardening, and micrografting, were developed till 2001 (Mondal *et al.*, 2001). Presently, attention is increasingly focused on evaluating field performance of the transformed

in vitro grown whole plantlets. Although there is no stable technique developed so far to produce transformed invitro grown whole plantlets of tea except the one made by Mondal *et al.*, in 2001 by micrografting seedling grown roots on the *in vitro* grown shoots of tea.

Besides this the other major concern for loss in productivity of an important crop like tea in India is the damage caused by various diseases e.g., foliar fungal disease as it grows mainly under prescribed moisture conditions. Some of the most important foliar fungal diseases of tea such as blister blight, black rot, brown blight and grey blight caused by *Exobasidium vexans*, *Corticium invisum*, *Glomerella cingulata* and *Pestalotiopsis theae* respectively are responsible for considerable economic losses. Drought is another major constraint over the years on production of tea in India. In order to stop further reduction in its gene pool and to breed for new tea types with more productiveness; less prone to natural calamities, diseases, as well as new flavors, a thorough knowledge of the existing genetic diversity, *in vitro* culture studies and improvement of the existing varieties through various Molecular biology, tissue culture and biotechnological techniques is a pre-requisite in tea research.

Therefore the research needs

- The choice of disparate parents is critical for the realization of genetic advance, and to avoid inbreeding and narrowing of the genetic base in advanced generations. Although the prospect of production of high yielding tea plants during last two decades have improved, it did not help in widening the genetic variability of tea, as attempts were mostly restricted to selecting elite mother bushes or progenitors from within the natural hybrid populations of tea (Banerjee 1992). The danger of narrowing the genetic base in most tea growing countries due to popularity of a few clones used for infilling/replanting and extension. In particular it has recently been established through the use of DNA markers that close genetic relatedness exists within certain clones developed by the African Highlands Produce and Brooke Bond companies (Wachira 1997). A narrow genetic base can pose risk to natural hazards like pests, diseases and drought. Appropriate strategies should be designed to broaden and maintain a sufficiently large genetic base.
- The danger of loosing many valuable tea germplasm is increasing due to fast uprooting of older sections of tea estates and clearing of jungles practically all over the world. Reexamination of the wealth of this diverse material should be continued.
- For a better yield, breeding for more efficient plant type with efficient interception, absorption and photosynthetic utilization of light energy is required.
- Development of polyploid plants by gene cloning which can provide avenue through which genetic base can be broadened and vigor and greater variability can be introduced into the genetic pool of the tea germplasm (Wachira 1994).
- Search for new biochemical markers associated with quality parameters and genes. Once such genes are identified using such markers and their segregation

pattern would be possible to breed specifically for quality (Wachira 1990).

- Standardization of a protocol for production of completely *in vitro* grown plantlets from cotyledon cultured embryos.
- Thus, standardization of the gene transfer in tea plants using various methods like *Agrobacterium* and Biolistics is a prerequisite.

Objectives

1. DNA finger printing study of tea

- Collection and maintenance of various tea cultivars available in different regions of North Bengal.
- Genomic DNA isolation from fresh & tender leaf samples of various cultivars.
- Detection of genetic variability and the phylogenetic relationship among these tea cultivars by different PCR based fingerprinting methods like Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP) and Microsatellite markers.
- Sequencing of the tea varieties exhibiting a diverse genetic base.

2. *In vitro* culture studies in tea

- Induction of embryogenic lines from cotyledonary explant.
- Maintenance and multiplication of embryogenic lines.
- Maturation of somatic embryos and conversion into viable plantlets.
- Histological study of the early developmental stages and structural organization of the resulting embryos from cotyledons.

3. Genetic transformation study of tea

- Trial of different gene transfer methods to evaluate the suitability of their use in tea.
- Induction and multiplication of callus tissue from the genetically transformed explants on the antibiotic selective medium.
- Differentiation of genetically transformed tissue by subjecting it to various hormone combination & concentrations.
- Confirmation for the integration of transgene into the tea nuclear genome using *GUS* assay and PCR analysis using *npt*-II specific primers.

Chapter 2
Review of Literature

2.1 History of tea (*Camellia sinensis*)

2.1.1. History of tea world wide

Tea is the oldest caffeine containing beverage, and has been used for two or three thousand years in south-east China (Eden 1958). It is also the most widely consumed hot beverage as being the cheapest. To date, the habit of tea drinking has become well established for more than half of the world's population. India is also the largest consumer of tea and accounts for 22% of global tea consumption. Based on FAO records (1984), 2.7 million hectares of land are under tea cultivation and 2.2 million tons are produced every year in the world. Over 80% of the world's tea exports come from India and Srilanka. Exports from China rank next to these two countries. The United Kingdom is the largest importer of the world, importing over 20% of the production. The largest importer of the world is the United States, but there only about half a pound per person is drunk (Kato, 1989) India with 5.11 lakh hectares under tea with an average annual production of 850 million Kg is the largest producer of tea in the world. It accounts for a fifth of the global tea area and nearly a third of the total world production. At present tea is grown in 16 states in India. Major tea growing states are Assam (50.7%), West-Bengal (22.1%), Tamil-Nadu (15.9%) and Kerala (8.3%) (Boriah, 2004).

The centre of origin of the tea plant is considered to be near the source of the Irrawady River and further North. It is variously stated that tea is found wild in Assam and Upper Burma and South Yunnan in upper Indo-China (Eden 1958; Purseglove 1974), but its real origin is still the subject of various theories. In China, cultivation of tea has long been established, while in Japan tea was introduced dur-

ing the 12th century from China.

From the main centers of its primary origin in South-East Asia, tea has spread far and wide into tropical and subtropical areas and adopted broad characteristics corresponding to regions of tropical rain forest, tropical Savannah and summer rain areas (Eden, 1976; Greenway, 1945; Kingdon-Ward, 1950; Kulasegaram; 1980).

In India, tea seeds from China were brought and sown at Botanical Garden, Calcutta in 1780 (Bezbaruah, 1999). Tea (China type) was introduced in North East India in 1836, although in 1823, Major Robert Bruce discovered tea plants growing wild in some hills near Ragpur (now Sibsagar), the then capital of Assam (Ukers, 1935). In South India, one Dr. Christy has experiments on growing tea in Nilgiris in 1832 (Muraleedharan, 1991). But the tea did not get under way until 1893 when the planting of tea increased rapidly (Harler, 1963).

In the early part of the eighth century, tea was introduced into Japan from China and in Srilanka; seeds were imported from Assam in 1839 and planted in Royal botanic Gardens at Peradeniya near Kandy (Ghosh Hajra, 2001). The commercial planting in Srilanka was taken up from 1867 (Kulasegaram, 1978). In Taiwan, tea growing on a considerable scale started at the conclusion of the Sino-Japanese war of 1895 (Njuguna, 1984). In Indonesia, tea seeds were imported from Japan in 1826 and planted in the Bogor (Buitenzorg) Botanical Gardens though commercial planting started only in 1878 using Assam seeds collected from Srilanka (Arfin and Semangun, 1999). Tea was introduced in Bangladesh in 1839 (Ghosh Hajra, 2001), and in Vietnam it took commercial shape in 1918 (Tien, 1993).

However, in other countries tea cultivation was introduced as a commercial production since the discovery of the black tea variety *assamica* by R.Bruce in 1823.

In Africa, tea was first grown South of Limpopo river in the Durban Botanic gardens, Natal, in 1850 (Paterson, 1995). The commercial production in Natal, South Africa, dates from 1878 (Greenway, 1945). Tea seeds obtained from Botanic Garden in Edenburgh were introduced into Malawi in 1878 but there were no survivors. Another batch of seeds was brought in 1886 from Edinburgh and Kew Gardens in 1888 (Whittle, 1999). In Kenya, tea was introduced at Limuru in 1903 but commercial tea growing, however, did not start until 1920s. In Uganda and Tanzania, experimental planting of tea was made in 1900 and 1904 and commercial production did not begin until 1931 and 1926 respectively (Ghosh Hajra, 2001; Carr et al., 1988).

In Turkey, tea was first planted in 1888 but it was unsuccessful and later cultivation started in 1939-1940 dominated by China hybrid seeds obtained from Georgia in the former Soviet Union (Vanli, 1991). Cultivation of tea started in Russia in 1885 in Chakva village near Batumi city with the seeds transported from China (Kravotsov *et al.*, 1999).

In addition to the above countries to the above countries, tea is also grown in Iran, Malaysia, Mayanmar, Nepal, Thailand, Burundi, Mauritius, Mozambique, Etiopia, Cameroon, Republic of Congo, Rwanda, Zimbabwe, Argentina, Brazil, Ecuador, Peru, Papua New-

Fig. 2.1 Darjeeling tea garden

Guinea and Australia. The latitude of these countries ranged from 41°N to 33°S.

2.1.2. History of Tea in India

The credit for creating India's vast tea empire goes to the British, who discovered tea in India and cultivated and consumed it in enormous quantities between the early 1800's and India's independence from Great Britain in 1947. The Scottish adventurer, Robert Bruce, discovered tea plants growing in Assam in the 1820's. At this time, no one thought that tea existed in India; however Major Bruce discovered the plants growing wild in the jungles controlled by the tribal chiefs. The British East India Company's monopoly in China ended in 1832 and it became necessary to find other sources to supply the English consumers of tea. In 1834, a tea committee was appointed to investigate the possibility of cultivating tea in India. After a thorough investigation and study of the crop, the first commercial batch of tea ever produced outside of China came from Assam in 1839. Two of India's major teas are the Darjeeling tea and the Assam tea. India's famed Darjeeling tea is named after the summer capital of the Government of Bengal, where tea is cultivated at altitudes of 4,000-10,000 feet in the Darjeeling hills (Fig.2.1). India's other major tea, Assam tea is named for the district in which it is grown, which lies in northeast India along the border between India and Burma. This region produced more black tea than any other area in the world, with the exception of some parts of China.

2.2. Taxonomy of tea

Tea has been addressed under various botanical names viz. *Camellia thea* Dyer, *Camellia sinensis* (L.) O. kuntze (Wight and Barua, 1957), *Thea sinensis*, *T. bohea* or *T. viridis* (Eden, 1976).It belongs to Thea

section of the *Camellia* genus under Theaceae family. However, today tea is botanically referred to as *Camellia sinensis*, irrespective of species specific differences. But it has been found that tea has various distinct species, or forms and subspecies. Different authors have recognized various intra-specific categories of *Camellia sinensis* (Wight and Barua, 1957; Wight, 1959; Barua, 1963) but the only ones which have met general acceptance were described by Kitamura, quoted by Wight and barua (1957) as *C. sinensis* var. sinensis and *C. sinensis* var. assamica (Masters). For simplicity, these two species are generally reffered to as *C.sinensis* and *C. assamica* (Barua, 1963; Eden, 1976; Kulasegaram, 1978; Sharma and Venkataramani, 1974).

A third form which resembles Planchon's Thea *lasiocalyx* (Barua, 1963), was proposed by Wight (1962) as subspecies of *C. assamica* and named *C. assamica* subsp. l*asiocalyx* (Planchon ex Watt). A fourth group called hybrid variety with mixed taxonomic characters of cultivated China, Assam and Cambod types and to a lesser extent some species hybrids from other species of *Camellia* (Barua,1963; Kulasegaram,1978; Sharma and Venkataramani, 1974), occupies the largest tea area under cultivation through seed propagation (Wight, 1958).

2.3. Genome diversity

The genus *Camellia* had 82 species in 1958 (Sealy, 1958) and accounts for more than 325 species in 2000 (Mondal, 2002a) that indicates genetical instability and high outbreeding nature of the genus. Presently, worldwide over 600 cultivated varieties are available, of which many have unique traits such as high caffeine content, blister blight disease tolerant etc. Owing to extensive internal hybridization between different *Camellia* taxa, several intergrades, introgressants and putative hybrids have been formed. These can be arranged in a gradient based on morphological characters that extend from China types through intermediates to those of Assam types. Indeed, because of the extreme homogenization, existence of the pure archetypes of tea is doubtful (Visser, 1969). Till date, numerous hybrids currently available are still referred to as China, Assam or Cambod tea depending on morphological proximity to the main taxon (Banerjee, 1992). Tea breeds well with wild relatives and thus taxonomists have always been interested to identify such hybrids due to suspected involvement in tea genetic pool. Two particularly interesting taxa are *C. irrawadiensis* and *C. taliensis* whose morphological distributions overlap with that of tea (Banerjee, 1992). It has also been postulated that some desirable traits such as anthocyanin pigmentation or special quality characters of Darjeeling tea might have introduced from wild species (Wood and Barua, 1958).

Other *Camellia* species, which are suspected to have contributed to the tea genetic pool by hybridization, include *C. flava* (Pifard) Sealy, *C. petelotii* (Merrill) Sealy (Wight, 1962) and possibly *C. lutescens* Dyer (Sharma and Venkataramani, 1974). The role of *C.taliensis* is however, not clear because the species itself is considered to be a hybrid between *C. sinensis* and *C. irrawadiensis* (Wood and Barua, 1958; Visser, 1969). Therefore, it is generally agreed that at least three taxa i.e. *C. assamica*; *C. sinensis*; *C. assamica* sub

lasiocalyx and to an extent *C. irrawadiensis* have mainly contributed to the genetic pool of tea. The term 'tea' should therefore, cover progenies of these taxa and the hybrids thereof or between them.

2.4. Economic importance and health benefits

The economic importance of the genus *Camellia* is primarily due to the tea. Tea was initially used as a medicine and subsequently as beverage and now has proven well to be a future potential as an important raw material for the pharmaceutical industry. Tea is mainly consumed in the form of 'fermented tea' or 'black tea'. However, 'non-fermented' or 'green tea' and semi-fermented or 'oolong tea' are also popular in some countries e.g. Japan and China. Apart from being used as beverage, green leaves are also used as vegetables such as 'leppet tea' in Burma and 'meing tea' in Thailand. Though the tea seed oil is used as lubricant, yet extraction from seed is not economical (Wealth of India, 1950). Additionally tea seed cakes contain saponins but has got poor value as fertilizer as well as unfit for animal feed due to low nitrogen, phosphorus and potassium content but can be used successfully in the manufacture of nematocide (Wealth of India, 1950). Tea leaves have more than 700 chemical constituents, among which flavanoides, amino acids, vitamins (C, E, K), caffeine and polysaccharides are important to human health. Importantly, the vitamin C content in leaves is comparable to that of lemon. 'Tea drinking' is now being associated with cell-mediated immune responses of the human body and reported to improve the growth of beneficial micro flora in the intestinal. Tea also imparts immunity against intestinal disorders, protects the cell membranes from oxidative damages, prevents dental caries due to presence of fluorine, normalize blood pressure, prevents coronary heart diseases due to lipid depressing activity, reduces the blood-glucose activity and normalizes diabetes (Chen, 1999). Tea also possesses germicidal and germistatic activities against various gram-positive and gram negative human pathogenic bacteria such as *Vibrio cholera, Salmonella, Clostridium* etc. (Chen, 1999). Both green and black tea infusions contain a number of antioxidants like catechins and have anti-carcinogenic, anti-mutagenic and anti-tumorous properties. Among the different catechins, epigallo catechin-gallate is the most active component. Several epidemiological studies have also proved that tea consumption plays a protective role against human cancer.

2.5. Conventional propagation and breeding

Tea is propagated either through seeds or cuttings. Usually seeds are collected from orchard, stratified in sand and then sown in polythene sleeves in the nursery where it takes 12–18 months before transferring to the field. Nevertheless, seed-grown plants show a high degree of variability. Therefore, the alternative choice is through vegetative propagation of the elite variety wherein single leaf internode cuttings, with an axillary bud are planted in polythene sleeves under shade for 12–18 months followed by the transfer of these rooted plants to the field. Recently, grafting as an alternative propagation technique has gained considerable popularity. In this technique, fresh

single leaf internode cutting of both root-stock and scion are generally taken. Scion, commonly a quality cultivar, is grafted on root-stock, which is either drought tolerant or high yielding cultivar. Upon grafting, the scion and stock influence each other for the characters and thus composite plants combine both yield and quality characters resulting 100% increase of yield with better quality than either of the non-grafted cultivar. Further, a modified improved 'second generation' grafting has been developed where tender shoots were grafted on the young seedlings of tea which have an additional advantage over conventional grafting due to presence of tap root system (Prakash *et al.*, 1999). Tea breeding consists of hybridization as well as selection. Hybridization can be either natural or hand pollination. In natural hybridization, based on better performance of yield, quality or diseases resistance capability, two parents are planted side by side in an isolated place and allow them to bear fruits. Subsequently seeds (F1) are harvested, raised and planted. If average performance of these plants is found to be better than either parent, then seeds (F1) are released as hybrid seed or Bi-clonal seed. However, some of the outstanding performers among the progenies are marked and verified for multi-locational trial and still if found suitable released as clone. These clones are geographically specific and most of the tea research institutes in the world have generated clones for their own region. Sometimes more than two parents are used and known as poly-clonal seeds. The idea is to introduce more variability among the F1 seeds. Since it is difficult to know about the pedigree of the cultivars (as pollen may come from any male), hence the chance of reproducibility is low and least preferred presently. Alternatively, pollination or control cross, despite being an important approach has made a limited success in tea breeding. However, recently, few clones have been released in Kenya and Malawi using this technique. Selection is the most popular, age-old practice in tea breeding. Since commercial tea gardens earlier were established with seeds, hence lot of variability exit among them. Many instances the elite plant has been identified the existing bushes and released as clones. Majority of the tea clones have been developed through selection. However, pedigrees of the clone remain unknown. Though breeding work is limited up to F1 progenies presently yet F2 population holds greater promise for varietal improvement of tea. The advantage of this approach is better segregation of characters and with the help of molecular biology this can be exploited for marker assisted selections for a particular trait and construction of linkage map which is till not available for tea. Although, conventional tea breeding is well established and contributed much for tea improvement over the past several decades, but the process is slow due to some bottlenecks. Specifically in tea, they are:

- perennial nature,
- long gestation periods,
- high inbreeding depression,
- self-incompatibility,
- unavailability of distinct mutant of different biotic and abiotic stress,
- lack of distinct selection criteria,
- low success rate of hand pollination,
- short flowering time (2–3 months),

- long duration for seed maturation (12–18 months),
- clonal difference of flowering time and fruit bearing capability of some clones.

Similarly, vegetative propagation is an effective method of tea propagation, yet it is limited by several factors such as:

- slower rates of propagation,
- unavailability of suitable planting material due to winter dormancy, drought in some tea growing area etc.,
- poor survival rate at nursery due to poor root formation of some clones and
- seasonal dependent rooting ability of the cuttings.

Therefore, micropropagation technique appears to be an ideal choice for circumvention of the problems related to conventional propagation. Additionally, transgenic technology has the potential for varietal improvement of tea through means other than conventional breeding. However, central to any successful transgenic technology is an efficient *in vitro* regeneration protocol. While an efficient regeneration protocol is essential for introduction of the foreign gene into plant tissues, micropropagation is important for the transfer of large number of genetically modified plants to the field within a short span of time. *Camellia sinensis* being a polymorphic species offers a lot of practical difficulties in the field of breeding and evolution of pure line races from them. Thus, there occurs a large variation in several important and desirable characters from bush to bush in the existing tea populations. These variations offers a ready means to exploit such plants and develop improved planting material in this perennial crop by careful selection for high yield, excellence of cup characters of made tea, fair resistance to drought and some important pests and diseases and adaptations for different environment. Since commercialization of tea, improved planting materials have been developed primarily by selection of the desired plant type. Conventional tea breeding is well established, though time-consuming and labor intensive due to its perennial nature and long gestation period (4—5 years). Vegetative propagation is standard, yet limited by slow multiplication rate, poor survivability of some clones, and need for copious initial planting material. Seed-borne plants are heterogeneous due to their highly allogamous nature; consequently, it is difficult to maintain their superior character. Additionally, tea breeding has been slowed by lack of reliable selection criteria. Although few morpho-chemical markers are available for identification of superior cultivars, these markers are greatly influenced by environmental factors and show a continuous variation with a high degree of plasticity. To overcome these problems, a limited number of isozyme markers have been used, resulting in less polymorphism. With the advancements of molecular biology, however, efforts have shifted to using various DNA markers. Understanding genetic diversity at the molecular level of tea germplasm will help to: (1) preserve the intellectual property rights of the tea breeder; (2) identify individual tea cultivars through use of a molecular passport; (3) prevent duplicate entry of different genotypes into the tea gene pool; (4) increase efficient selection of varieties for hybridization, composite plant production, etc.; (5) classify

tea genotypes taxonomically using molecular markers; and (6) improve tea varieties for agronomically important characteristics through marker assisted selection. Consequently, biotechnological tools appear to be the ideal choice to circumvent problems of conventional tea breeding.

2.6. DNA finger printing study of tea

Morphological markers such as leaf pose, dry matter production, partitioning, flesh evenness, etc. and biochemical markers such as total catechin/polyphenol content, caffeine, etc. are used to identify the superior tea plant. However, tea breeders are often unable to use markers effectively because they are greatly influenced by environmental factors and show a continuous variation with a high degree of plasticity. Hence, to overcome these problems, research has shifted to using more sensitive DNA marker technology.

2.6.1. Morphological markers

Tea has been classified into different taxa by morphological characters. Barua (1963) provided morphoanatomical descriptions, which later was elaborated by Bezbaruah (1971).Morphological parameters such as leaf architect, growth habits and floral biology are important criteria used by tea taxonomists (Banerjee, 1992). While bush vigour, pruning weight, period of recovery from pruning time, plant height, root mass, root-shoot ratio, plucking point density, dry matter production and partitioning are considered as yield indicator of tea (Banerjee, 1992), caffeine, volatile compounds (Seurei, 1996), green leaf pigmentation (Banerjee, 1992), leaf pubescence (Wight and Barua, 1954), total catechine content and total tannin content etc. (Takeda, 1994) have been used as potential determinants for tea quality. Despite the several disadvantages, these are the most adopted markers used by tea breeder globally.

2.6.2. Cytological markers

Cytological markers of the genus *Camellia* were elaborately studied in the early 1970s with many interesting features. Chromosome number has been established for the most available taxa of *Camellia* including tea (Bezbarua, 1971), which was reviewed by Kondo (1977). Generally tea chromosomes are small in size and tend to clump together due to 'stickness'. Tea is diploid (2n=30; basic chromosome number, X=15) and karyotype ranges from 1.28 μ to 3.44 μ (Bezbaruah, 1971). The *r* value (ratio of long arm to short arm) for all the 15 pairs of chromosomes range from 1.00 to 1.91. This consistency in diploid chromosomes number suggests a monophyletic origin of all *Camellia* species. However few higher ploidy level such as triploids e.g., TV-29, HS-10 A, UPASI-3, UPASI-20 (2n=45), tetraploids (2n=60), pentaploids (2n = 75) and aneuploids (2n +1 to 29) have also been identified (Singh, 1980; Zhan et al., 1987). Karyotypic data had also been accumulated in past for the other species of this genus (Fukushima *et al.*, 1966; Kato and Siura, 1971; Ackerman, 1971; Kondo, 1975, 1978a, b, 1979). In karyotype analysis, unfortunately, grouping by chromosome size was difficult in the *Camellia* taxa, since the chromosome grade imperceptibly from the largest to the smallest. Furthermore, even in the best preparation, homologous chromosome pairs could not be appeared identical in *Camellia* (Kondo, 1975, 1978a, b). Relatively little in-

traspecific karyotypic variation had been observed in the cultivated species of *Camellia* studied (Kondo, 1975, 1979). Sat-chromosomes in karyotypes within mass accessions of certain *Camellia* species are morphologically and quantitatively variable. Thus karyotypes including characteristics of sat-chromosomes are not of taxonomic significance for *Camellia* taxa. Among the diploid species of *Camellia* studied, *C. japonica* L. *sensu lato* showed the greatest karyotypic variation, many of the accessions studied indicated similar karyotypic patterns to each other (Kondo, 1975). For instance, *C. japonica* L. var. *Spontanea* (Makino), *C. japonica* L. var. *macrocarpa* Masamune, *C. japonica* L. subsp. *rusticana* (Honda) Kitamura and four cultivars namely 'Aka-Wabisuke', 'Fukurin-Wabisuke', Kuro-Wabisuke', and 'Wabisuke' carried same, most common standered acetoorcein-stained karyotype if presence of satellites is not considered; 16 metacentric, 8 submetacentric and 6 subtelocentric chromosomes. Actually, *C. japonica* L. var. *Macrocarpa* Masamune had satellites on four submetacentric chromosomes and the other accessions had satellites on two submetacentric chromosomes (Kondo and Parks, 1980). Later, it was shown by Kondo and Parks (1979) that the C-banding method can be applied to the somatic mid-metaphase chromosomes in *Camellia* taxa. These differentially stained bands in somatic midmetaphase chromosomes permit the identification of individual chromosomes and make it possible to match the homologous pairs of chromosomes more precisely and possibly even measure chromosome divergence between different clones within the same species with same or similar karyotypes. Karyotypic variability and divergence among the seven accessions of *C. japonica* L. *sensu lato* with same acetoorcein stained karyotype were revealed by C-banding method (Kondo and Parks, 1981). These way cytological markers were used to sort and classify the vast number of cultivars. However, due to the development of more sensitive biochemical techniques, attention was shifted towards the search of biochemical markers.

2.6.3. Biochemical markers

Biochemical markers were widely used for characterization of different plant germplasm (Das *et al.*, 2002). Presence of calcium oxalate crystals and it's quantity in paranchymatous tissue of leaf petioles nomenclatured as phloem index, have been suggested to be a suitable criterion for classifying tea hybrids (Wight, 1958). The variations in quantity and morphology of the sclereids in the leaf lamina were also utilized for differentiating tea taxa (Barua, 1958; Barua and Dutta, 1959). Takeo (1983) suggested a chemo-taxonomic method of classifying tea clones based on a ratio referred to as the Terpene Index (T.I), which expresses the ratio between linalools and linalools plus geraniols. However, with the advancement of high performance liquid chromatogarphy, considerable success has been achieved in the identification of tea quality indicators (Takeo, 1981; Owuor *et al.*, 1986a). These indicators have also found wider use in distinguishing the two main species of tea, namely *C. sinensis* and *C. assamica* and their respective clones (Owuor, 1989). Although not fully exploited,

the polyphenol oxidase activity, individual polyphenols, amino acids and chlorophyll content are considered to be potential parameters in tea taxonomy (Sanderson, 1964). The presence or absence of certain phenolic substances in tea shoots has also been used in establishing relationships among various taxa (Roberts *et al.*, 1958). Quantitative changes in chlorophyll-*a*, chlorophyll-*b* and four carotenoids (*β*-carotene, lutein, violaxanthine and neoxanthine) were used for characterization of Assam, China, and Cambod (Hazarika and Mahanta, 1984). Total catechin concentration and the ratio of dihydroxylated to trihydroxylated catechins of green leaf were used to establish genetic relationship among the 102 Kenyan tea accession (Magoma *et al.*, 2000). Upon multivariate analysis, accumulation of the various catechins separated the tea clones into 3 major and 5 minor groups, according to their phylogenetic origin. They found that Cambod tea had the highest ratio (7:10) followed by China tea (3:5) while Assam tea had the lowest ratio (1:4). This biochemical differentiation indicates that there is potential for broadening the genetic base of mainly Assam tea in Kenya with the putative China and Cambod tea. Though detection accuracy is higher, yet accumulation of such chemicals is subjected to post-transcriptional modification, which restricts the utility of biochemical markers (Staub *et al.*, 1982).

2.6.4. Isozymes markers

Genetic analysis of isozyme variation was used for cultivar identification in a wide range of plants (Ferguson and Grabe, 1986; Hirai and Kozaki, 1986). Similarly in tea also, isozymes have been analysed by several workers (Hairong *et al.*, 1987; Xu *et al.*, 1987; Anderson, 1994) for studying the genetic tendencies, cultivar identifications and implications in hybrid breeding. Among the isozymes, peroxidase and esterase are extensively studied in different tea cultivars (Ikeda *et al.*, 1991; Chengyin *et al.*, 1992; Singh and Ravindranath, 1994; Yang and Sun, 1994; Borthakur *et al.*, 1995; Chen, 1996). However, other isozymes such as tetrazolium oxidase, aspartate aminotransferase and alpha-amylase were also studied among 7 different tea cultivars along with 3 different species (Sen *et al.*, 2000). The electrophoretic analysis revealed both the qualitative and quantitative variation in the isozyme banding pattern among different species of tea and their clones. The tetrazolium oxidase enzyme system showed the highest variability among all the enzymes. Cluster analysis using isozyme banding pattern produced a dendrogram which clearly differentiated characteristics of both the clones and species studied. In general, isozyme studies in tea were limited to few enzymes with inadequate polymorphism (Wachira *et al.*, 1995). However, isozymes were extensively studied in *C. japonica*. Wendel and Parks (1982) reported the analysis of 17 isozymes in different cultivars of *C. japonica*. They found that 15 isozymes produced 2-9 polymorphic loci while 2 produced 1–3 monomorphic bands. Based on the segregation of 12 loci by 8 enzymes, they suggested that genes of aspartate amino-transferase with phospho-glucomutase and 6-phosphogluconate dehydrogenase with phosphoglucomutase are linked. Further, the same

group (Wendel and Parks, 1983) resolved isozyme variation at 15 loci from 12 enzymes in 205 cultivated genotypes of *C. japonica*. All loci were polymorphic and a total of 64 alleles were detected. The sensitivity of electrophoresis in distinguishing clones was high so much so that 95.4% of the clones uniquely characterized by their 15-locus genotypes. Isozyme analysis was also used to study the genetic linkage and segregation pattern of alcohol dehydrogenease genes (Wendel and Parks, 1984). They found that alcohol dehydrogenease isozymes in *C. japonica* are encoded by two genes Adh-1 and Adh-2. Both loci are expressed in seeds and their products are randomly associated with intragenic and intergenic dimmers. Electrophoresis of leaf extracts produces only the products of Adh-2. Formal genetic analysis indicated that the two Adh loci are tightly linked. Most segregations fit expected Mendelian ratios but in some families distorted segregation was also observed at Adh-1, Adh-2 or both. Starch gel electrophoresis was used to score allelic variation at 20 loci resolved from 13 enzyme systems in seeds of *C. japonica* collected from 60 populations distributed over throughout the Japan. The genetic diversity within the population was higher i.e. 66.2% of loci were polymorphic per population on an average within a mean number of 2.16 allele per locus; the mean observed and heterozygosities were 0.230 and 0.265, respectively. They also reported genotypic proportions at most of the loci in majority of the population fit the Hardy-Weinberg expectations (Wendel and Parks, 1985). The dispute of identification of two oldest *C. japonica* plants at New Zealand as cv. Middle Mist Red was concluded using 10 different isozymes (Spoth *et al.*, 1999). Among them 3 isozyme could not detected polymorphism. The analysis based on rest of the 7 enzymes confirmed that those plants were not actually belong to the Middle Mist Red cultivars. However, limited number of loci of isozyme showed the lesser polymorphism but with the advancement of molecular biology such efforts were shifted towards various DNA based markers.

Plant genetics and by association plant breeding, is based on the analysis of inheritance of characteristic and traits and elucidation of gene expression as related to the genotype and environmental interaction. Biochemistry and molecular biology have impacted these activities through the application of molecular markers. Initially molecular markers were based on isozymes (i.e., polymorphic mobility variants of enzymes detected usually in starch gels using activity staining), which are valuable but not numerous enough to cover the extensive regions of genome found in most plants. Recently, a large number of DNA markers are in use as tools for large scale genetic studies providing the best estimate of genetic diversity and are not influenced by the environmental factors. Various classes of the molecular markers are available but those based on PCR are considered to meet many applications in genetic studies (Powell *et al* 1995).By using the polymerase chain reaction, smaller amounts of DNA are needed, facilitating analysis of badly preserved samples (e.g., field material, museum specimen, forensic samples, plant parts). Most PCR based

marker technologies use arbitrary primers. One interesting alternative has been the use of telomere-associated sequence derived PCR primers (Kolchinsky and Gresshoff, 1994).

2.7. Molecular marker technology

In addition to the problems described earlier in the 'Conventional propagation and breeding' section, progress of tea breeding has also been slowed down due to the lack of reliable selection criteria (Kulasegaram, 1980). Though a number of morpho-biochemical markers has been reviewed in past (Wachira, 1990; Singh, 1999; Ghosh Hazra, 2001), yet they have only marginally improved the efficacy of selection for desired agronomic traits. This is mainly due to the fact that most of the morphological markers defined so far, are influenced greatly by the environmental factors and hence show a continuous variation with a high degree of plasticity. Therefore, these markers cannot be separated into discrete groups for identification (Wickremaratne, 1981). Recently, development of the molecular biology has resulted in alternative DNA-based markers for crop improvement of tea. These markers can assist the process of traditional breeding with several efficacies. The greatest advantages of molecular markers are:

- free from the environmental influence and
- detection of polymorphism at an early stage.

The different markers, which have been employed for varietal improvement of tea, are reviewed below.

Various types of molecular markers studied are as follows:

2.7.1. Restriction Fragment Length Polymorphism (RFLP)

The first DNA-based markers were RFLPs (restriction fragment length polymorphisms), which were detected using either random genomic or cDNA clones. RFLPs are still of great value today and effective co-mapping requires near complete map saturation as is achieved in only a few plant species such as Arabidopsis, maize and rice.

By determining the segregation of molecular polymorphism in segregating families, it is possible to determine their degree of linkage and recombination. This generates genetic maps in which distances are measured in units of recombination (called centimorgans) or physical distances (in Kilo or megabases; Funke *et al*, 1993). In plant genetics maps are usually based on F2 families or thereof derived recombinant inbred line populations (Keim *et al*, 1990; Lark *et al* 1993). The latter have the advantage of immortality, inexhaustibility of DNA, ability to be shared globally, as well as homozygosity at each locus, facilitating the application of molecular markers which detect dominant loci only (e.g., DAF, RAPD and AFLP markers; Ceatano-Anolles *et al*, 1991; Williams *et al*, 1990; Vos *et al*, 1995). Potential applications are frequently thwarted by the requirement for significant quantities of DNA in the case of RFLP analysis or by lack of relevant sequence information in the case of PCR based techniques. Recent criticisms are that DNA fingerprinting requires special molecular training, is labour-intensive and is relatively expensive (Weatherhead & Montgomerie 1991).

2.7.2. Random Amplified Polymorphic DNA (RAPD)

Williams *et al* (1990), described a novel type

of genetic marker based on DNA amplification, which does not require prior information of target DNA sequences. These markers called RAPD (Random Amplified Polymorphic DNA) markers are generated by the amplification of random DNA segments with single primers of arbitrary chosen primers. However not much work has been done on the studies of genetic diversity of tea worldwide, though Wachira *et al* (1995) and Chen & Yamaguchi (2005) have done some studies in a very limited way. Use of random amplified polymorphic DNA (RAPD) markers, detected by PCR amplification of small inverted repeats scattered throughout the genome, adds a new technology of DNA fingerprinting to the molecular analysis of relatedness between genotypes. The PCR based RAPD technique (Williams *et al* 1990) is an attractive complement to conventional DNA fingerprinting. RAPD analysis is conceptually simple. Nanogram amounts of total genomic DNA are subjected to PCR using short synthetic oligonucleotides of random sequence. The amplification protocol differs from the standard PCR conditions (Erlich 1989) in that only a single random oligonucleotide primer is employed and no prior knowledge of the genome subjected to analysis is required. When the primer is short (e.g. 10 mer), there is a high probability that the genome contains several priming sites close to one another that are in an inverted orientation. The technique essentially scans a genome for these small inverted repeats and amplifies intervening DNA segments of variable length. The profile of amplification products depends on the template-primer combination and is reproducible for any given combination. The amplification products are resolved on agarose gels and polymorphisms serve as dominant genetic markers, which are inherited in a Mendelian fashion (Williams *et al* 1990; Carlson *et al* 1991; Welsh, Peterson & McClelland 1991). Amplification of non-nuclear RAPD markers is negligible because of the relatively small non-nuclear genome sizes. Since discovered, random amplified polymorphic DNA (RAPD) assay (Williams *et al*., 1990) is being used for a number of areas in plant taxonomy. At the present it is the most preferred DNA markers due to greater speed, easy-to-perform and non-requirement of radioactive materials etc. In tea and other species of *Camellia*, a considerable amount of work has been carried out which are summarized below.

2.7.2.1. Germplasm characterization

Present day tea plantation is developed largely from the selected genotypes based on the performance of yield, quality, biotic and abiotic stress resistance amongst the previously existing planting materials. As a consequence, widespread cultivation of clonal tea can diminish the genetic diversity if care is not taken to use clones of disparate origin. Therefore germplasm characterization at molecular level of tea will help:

- varietal improvement of tea for agronomically important character.
- to preserve the intellectual property right of tea breeder.
- identification of individual tea cultivar by making a molecular passport.
- prevention of duplicate entry of different genotypes in tea gene pool.

- efficient selection of the varieties for hybridization program, graft compatibility in composite plant production etc and
- taxonomic classification of tea genotypes on the basis of molecular markers.

Wachira *et al.* (1995) were the first to characterize 38 different cultivars of Kenyan tea. A total of 23 primers which were used could generated 157 polymorphic bands. The maximum polymorphism of 20 bands were detected by primer SC10-56. The amplified fragments and similarity matrix ranged from 0.3 to 3 kb and 43% to 96%, respectively, among the clones. Based on average linkage cluster analysis, a dendrogram was constructed which clearly discriminate different varieties of Assam, Cambod and China tea. Further, to examine the evolutionary relationship the principal co-ordinate analysis (PCA) was undertaken which was able to classify into three varietal types of tea in a manner consistent with both the present taxonomy of tea and with the known pedigrees of some clones. In a preliminary study, Tanaka *et al.* (1995) used several 10-mer and 12-mer primers to detect variation among Korean, Japanese, Chinese, Indian and Vietnamese tea. Among all primers, OPF- 2 was found to be the most polymorphic. Their study concluded that after introduction from China, Korean tea undergone little genetic diversification. On the contrary, Japanese tea showed a closer relationship with their Chinese and Indian counter part, which reveal the fact that tea in Japan might have brought from China as well as India. Twenty-five Indian tea cultivars and 2 ornamental species were characterized using RAPD markers (Mondal, 2000). In a separate study, the diversity of 27 accessions comprising Korean, Japanese and Taiwanese tea was examined with RAPD markers. The RAPD system has been used in linkage map construction (Grattapaglia and Sedroff 1994), insect resistance gene localization (Dweikat *et al* 1997), hybrid origin identification (Friesen *et al* 1997), and breeding utilization (Durham and Korban 1994; Baril *et al* 1997). RAPDs may also be useful for the design of collection strategies to maximize the sampling of genetic variation within the available gene pool (Dawson *et al* 1993; Huff *et al* 1993; Liu and Furnier 1993; Nesbitt *et al* 1995). Moreover RAPD markers are capable of detecting variation in non-coding regions of the genome.

2.7.2.2. Detection of genetic fidelity among in vitro raised plants by RAPD analysis

The most important part of any *in vitro* propagation system is mass multiplication of plantlets which are phenotypically uniform and genetically akin to the mother plant, otherwise the advantage of desirable characters of elite supreme clones will not be achieved. Several approaches have been applied for identifying variants among micropropagated plants. These are phenotypic variation (Vuylsteke *et al.*, 1988), karyotypic analysis of metaphase chromosomes (Jha *et al.*, 1992) and biochemical analysis (Damaco *et al.*, 1996). Importantly, a major disadvantage of these techniques is the limited number of informative markers and the influence of environmental conditions or developmental process (Rani *et al.*, 1995). Besides these limitations, the above approaches are not fully suitable for detecting DNA sequence polymorphisms of *in vitro* raised plants. On the other hand, RAPD has

been used very advantageously for number of crop species to detect genetic diversity among micropropagated plants (Isabel *et al.*, 1993; Rani *et al.*, 1995; Damasco *et al.*, 1996).

2.7.2.3. Cultivar identification

RAPD markers were also employed for identification of the true-crossing progenies in tea breeding programme and to determine relationship between parents and their hybrids. For example, two Japanese tea cultivars, Yutakamidori and Meiryoku for which parentage identification for registration documents were identified using this marker (Tanaka and Yamaguchi, 1996). Wright *et al.* (1996) used the same technique to characterize 5 different South African tea cultivars namely SFS 150, SFS 204, PC1, PC81 and MFS87. Of the 20-arbitary primers tested, only one (ABI-17) yielded a unique set of fingerprints for each cultivar, which allowed cultivar discrimination. Singh *et al.* (1999) isolated DNA from 10 different processed dried commercial black and green tea samples. The isolated DNA was subjected for PCR amplification by random primers. Thus they demonstrated that this method has tremendous potential for testing the originality of commercial tea and for the identification of cultivars used by a tea manufacturer for a particular brand. Mondal et al. (2000a) described a simple method of DNA isolation from eight different polyphenol rich genus as well as from 20 commercially important tea cultivars. The method does not require liquid nitrogen or phenol purification step. The DNA was successfully used as a template in PCR amplification, which indicated the wide applicability of the marker. Liang *et al.* (2000) investigated the possibility of classification and identification of tea as well as closely related species. The results showed that the RAPD markers could specifically discriminate between species and varieties. While both Assam and China tea had a specific band, Japanese tea was closer to Chinese tea than others. Some of the tea varieties from Vietnam were the hybrids of Assam and China. Tanaka *et al.* (2001) used RAPDs to identify the pollen parent of popular Japanese green tea cultivar 'Sayamakaori'. They have screened the female parent 'Yabukita' along with 78 putative male tea plants, most of which were introduced from China and concluded that pollen parent of 'Sayamakaori' was not present amongst the putative population. However, due to dominant in nature and limited degree of polymorphism, attention was given for alternative advance markers.

2.7.3. DNA Amplification fingerprinting (DAF)

Two modifications of detecting RAPD markers have been described as DNA Amplification Fingerprinting (DAF) and Arbitrarily Primed Polymerase Chain Reaction (AP-PCR). An alternative technology, although not as widely used as RAPD is DNA Amplification Fingerprinting commonly called DAF (Caetano-Anolles *et al*, 1991; Caetano-Anolles and Gresshoff 1994a). This procedure uses single arbitrary primers to amplify multiple target regions using low template concentrations (20-100pg/μl) and high primer concentrations (usually about 3μM, but 30μM for mini-hairpin primers. With these major differences to RAPD, DAF is more optimized, can produce from 30-50 amplification prod-

ucts if separated on MiniProtean II gel rigs (BioRad Inc., uses primers of shorter length (5-mers can work, although 8-mers or mini-hairpin primers are commonly used), is possible with annealing temperatures of 55°C, and is resolved using thin 5 to 10% denaturing polyacrylamide gels commonly stained with silver (Bassam *et al* 1991; Caetano-Anolles and Gresshoff, 1994b). DAF markers are usually dominant, are mainly inherited as nuclear markers, have been mapped on the soybean map (Prabhu and Gresshoff 1994) can detect maternal inheritance and can be cloned from gels (Weaver *et al* 1994). DAF markers were shown to have the same resolution as RFLP markers, but at high efficiency caused by the larger number of assayed loci, and to confirm the genetic pedigree in a number of soybean lines. DAF was used to determine genetic distinctiveness in bacteria (Bassam *et al* 1992; Jayarao *et al* 1992), plants, humans, viruses and animals (Caetano-Anolles *et al* 1991). The ability to amplify populations of DNA molecules by arbitrary primers laid the basis for molecular expression studies using mRNA derived cDNA populations (such as Differential Display, Liang and Pardee 1992; cf. Caetano-Anolles *et al* 1991 refer to this possibility by coining the term cDAF for cDNA amplification fingerprinting). DAF uses short random primers of 5-8 bp and visualizes the relatively greater number of amplification products by polyacrylamide gel electrophoresis and silver staining (Caetano-Anolles, Bassam & Gresshof 1991). AP-PCR uses slightly longer primers (such as universal M13) and amplification products are radioactively labelled and also resolved by polyacrylamide gel electrophoresis (Welsh & McClelland 1990; Welsh *et al* 1991b).

Standard RAPD analysis is performed according to the original methods (Williams *et al* 1990) using short oligonucleotide primers of random sequence which are commercially available (Operon Technologies, Inc., Alameda, Calif.). Only high molecular weight i.e. non degraded DNA should be subjected to RAPD analyses. Amplification products can be resolved by gel electrophoresis on 1.4% agarose gel.

2.7.4. Amplification Fragment Length Polymorphism (AFLP)

A combination of RFLP, PCR and DAF is found in the recently developed AFLP procedure (Vos *et al*, 1995). The term AFLP was originally used by Caetano-Anolles *et al* (1991) to describe DAF polymorphisms. Independently KeyGene Inc. in Wageningen (The Netherlands) developed a related technique and called it AFLP. To avoid confusion, the term AFLP should be used in context of the KeyGene procedure. The technique is rather laborious and expensive. It combines elements of PCR, RFLP and DAF. Large amounts of genomic DNA are isolated and restricted with two restriction nucleases (usually a 6 base cutter (*EcoRI*) and a four base cutter (*MseI*). Restricted fragments are annealed to adapter molecules specific for the cohesive restriction site. PCR primer, specific for each adapter molecule, but extended by 2 or 3 nucleotides on the 3' end provide selectivity to amplify the interstitial DNA fragment (assuming its size does not exceed 2 to 3 kb). This produces about 100 bands per

amplification, which are resolved on a polyacrylamide sequencing gel and visualized by autoradiography. Alternatively the separation can occur in a modern DNA sequencer allowing automatic data acquisition. Most AFLP markers are dominant, cluster in chromosomal regions and represent predominantly repeated DNA. They thus are of great value for identity determination (DNA fingerprinting) but not for marker-assisted selection (MAS) or map-based cloning (positional cloning). Additionally AFLP's require large amounts of template DNA as well as high input costs (US$600-700 per kit) (Gresshoff *et al* 1997).

2.7.5. Microsatellites

Microsatellites arise in genomes by the repetition of single sequences (Akkaya *et al* 1992). The tandem repeats like microsatellites or simple sequence repeats (SSR) are densely interspersed in eukaryotic genomes (Hamada *et al* 1982; Tautz & Renz 1984; Weber & May 1989). These are usually 2-5 bp long, short DNA sequence motif that occur at multiple sites (Beckman & Weber 1992; Wang *et al* 1994) and reveal a high degree of allelic diversity which can be typed via polymerase chain reaction (Sclotterrer *et al* 1991). As PCR technology finds increased use in genetic analysis, additional novel variations of this technique are emerging. PCR analysis using anchored simple sequence repeats primers have gained attention recently as an alternative means of characterizing complex genomes. This approach employs oligonucleotides based on an SSR anchored at either the 5' or 3' end with two to four purine or pyrimidine residues, to initiate PCR amplification of genomic segments flanked by inversely oriented, closely spaced microsatellite repeats (Zietkiewicz *et al* 1994). The PCR products thus generated reveal multiple polymorphic products which can be resolved on agarose gel electrophoresis. The Inter SSR-PCR (ISSR-PCR) strategy is especially attractive because it avoids the need to carry out costly cloning and sequencing inherent in the original microsatellite- based approach. Moreover, locus specific probes and microsatellite containing sequences of interest can be developed by isolating and cloning or reamplifying individual bands (Wu *et al* 1994; Zietkiewiez *et al* 1994). ISSR-PCR has been profitably used for genetic characterization of various plant species (Kantley *et al* 1995; Charters *et al* 1996; Proven *et al* 1996; Tsumara *et al* 1996). Because of greater length of ISSR primers, they may show greater repeatability and stability of map position in the genome when comparing genotype of closely related individual (Zietkiewicz *et al* 1994). Simple sequence repeats (SSR) were derived from *C. japonica*, a closely related species of tea in Japan. Using these primer pairs, 53 *C. japonica* ecotypes were genotyped and population genetic parameters calculated. Later, the same group investigated the spatial genetic structure of *C. japonica* using four of these microsatellite primers. Spatial distribution of individuals was also assessed to obtain an insight into spatial relationships between individuals and allele. Use of molecular markers in the characterization and management of plant genetic resources including tea is well documented (Wachira *et al.* 1995; Lanaud 1999).

Thus, SSR's are PCR-based, which is

powerful because of ease of detection, large numbers of alleles per locus, and universal abundance. However, they require prior investment for their discovery and thus are of value only for elite crops such as corn, soybean, and tomato.

2.7.6. Organelle DNA analysis

Because of the relative resistance to evolutionary changes of organelle DNA than nuclear DNA, chloroplast (cp DNA) and mitochondrial DNA (mt DNA) sequences have been widely used to investigate interspecific relationships (Jorgensen and Cluster, 1989; Waugh *et al.*, 1990; Olmstead and Palmer, 1994). Chloroplasts DNA encodes for many agronomically important genes including large subunit of Rubp carboxylase oxygenase (Kung, 1977), the 32 kd thylakoid membrane protein (Bedrock and Kolodner, 1979) and some other coupling factors (Nelson *et al.*, 1980) etc. Non-coding regions display higher rates of evolution than coding regions; hence former is desirable target for phylogenic studies. The resolutions of many such non-coding regions have been amplified by the universal PCR primers (Taberlet *et al.*, 1991; Demesure *et al.*, 1995). However, the relatively high frequency of insertion/deletions may even, in some cases, make it possible to use the size of PCR product as a genetic marker. The choice of cp and mt DNA sequences that maximize phylogenic information however, depend upon the evolutionary time scale of the plant system. To date, systematic organelle DNA analysis for tea is not reported, though, we made a first attempt to study the cp DNA of tea (Borthakur *et al.*, 1998). Good quality of cp DNA using a discontinuous sucrose gradient was isolated from 10 different tea cultivars. To overcome the phenolic problem, 0.1% polyvinyl-pyrolidone along with 0.1% BSA was used in the homogenization buffer. The clear greenish yellow bands of intact chloroplasts were collected from the interface of 20–45% sucrose gradient from where cp DNA was isolated. Wachira *et al.* (1997) analysed species introgression into cultivated gene pool of tea using 5 different organelle-specific primers in 19 taxa as well as 9 tea cultivars. Out of the 5, 3 non-coding chloroplast regions as well as one mitochondrial region that amplified with universal primers did not reveal any polymorphism. Remaining one cp DNA specific PCR product revealed a single-strand conformation polymorphism (SSCP). This SSCP in the intergenic spacer between the *trnL* (UAA) 3_ exon and *trn* F (GAA) indicated that 4 species namely *C. furfuracea, C. assimilis, C. nokoensis* and *C. tsaii* shared a common haplotype. This may indicate a possible hybridization between species of the sections involved. Thakor (1997) re-examined taxonomic relationship among the genus *Camellia* with the help of 5 cp DNA sequences from 25 *Camellia* species covering 4 subgenera of tea family. Out of which, 4 showed a low degree of variability (less than 2%). Remaining one revealed a much higher degree of variability (3.8–20%). Interestingly, the phylogenic analysis using parsimony (PAUP) analysis of these sequence contradicted the sectional or subgeneric grouping of either Sealy's or Chang's monograph. Prince and Parks (1997) analyzed the evolutionary relationship in tea subfamily Theoideae based upon two cp DNA

regions, namely *rbc*L and *mat*K sequence data, for 4 species of subfamily Ternstroemioideae and 24 species from Theoideae. Later on, the same workers also examined the same cp DNA region (*rbc*L and *mat* K) to confirm the family Theacea, a natural group as well as to evaluate the validity of circumscription of tribes and genus of its subfamily Theoideae (Prince and Parks, 2000). The nucleotide sequences of *rbcL* gene in chloroplast DNA are determined on the native tea varieties of Japan, Korea, China, South East Asia, Sri Lanka and India. Direct sequencing of the amplified cp DNA products were carried out. The nucleotide sequences of the *rbcL* gene in cpDNA of China and Assam type were presented. Alignments were obtained by assuming two substitutions, at nucleotide position 40 (adenine in China tea) and 948 (guanine in China tea). The nucleotide sequences of the *rbcL* gene in China and Assam were 99.8% similar. On the other hand, the 1370 nucleotide sequences of *rbcL* gene among *C. irrawadiensis C. taliensis* and Assam tea were the same except a different base at position 627. At this position in *C. irrawadiensis* and *C. taliensis*, thymine and adenine were observed, respectively, as specific bases (Kato, 2001).

2.8. Various Molecular marker techniques applied to *Camellia* -Milestones

The genetic diversity of tea has been studied by restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) (Wachira *et al.*, 1995; Mondal *et al.*, 2000), amplified fragment length polymorphism (AFLP) (Paul *et al* 1997).

Randomly amplified polymorphic DNA (RAPD) (Williams *et al* 1990) has proven quite useful in plant discrimination studies (Hu and Quiros 1991, Yang and Quiros 1993, Khasa and Dancik 1996, Sedra *et al* 1998, Jia *et al* 2000, Conner and Wood 2001, Rajora and Rahman 2003). Recently RAPDs have also been used for the investigation of genetic relationship (Wachira *et al* 1995; 1997), identification of parentage (Tanaka *et al.* 2001). Genetic diversity (Kaundan *et al* 2000), genetic mapping (Hackett *et al* 2000) of tea plants (*C.sinensis*), as well as for the molecular systematics of the section Thea (Chen and Yamaguchi 2002).

In Japan, a wide range of markers has been used with various applications. The markers used for genetic characterization of different green tea cultivars are RAPD, AFLP, SSR, CAPS, and RFLP. Importantly, the RFLP technique was also applied in Japan to prevent adulteration of higher grade with lower grade tea. Several other minor tea-producing countries have used different molecular markers to characterize the tea gene pool of introduced tea cultivars available to that country. Such efforts were made using RAPD in Portugal, ISSR in Taiwan, and RAPD in South Africa. All work focused on the genetic characterization and molecular taxonomy of the introduced variety available in the respective countries. Similarly, South Korea and China tea cultivars were characterized through RAPD or AFLP, and RAPD, respectively.

Japanese researchers have isolated the cDNA chalcone synthase (CHS) gene as well as β-tubulin gene from the Japanese green tea cultivar 'Yabukita'. More recently, a few im-

portant genes such as phenyl ammonia lyase(PAL), caffeine synthetase, and primeverosidase have been isolated. The genetic diversity of tea *Camellia sinensis* (L.) O. Kuntze, including the two main cultivated *sinensis* and *assamica* varieties, was investigated based on PCR-RFLP analysis of PAL, CHS2 and DFR, three key genes involved in catechin and tannin synthesis and directly responsible for tea taste and quality. Polymorphisms were of two types: amplicon length polymorphism (ALP) due to the presence of indels in two introns of PAL and DFR, and point mutations detected after restriction of amplified fragments with appropriate enzymes. A progeny test showed that all markers segregated in a Mendelian fashion and that polymorphisms were exclusively co-dominant. CHS2, which belongs to a multi gene family, allowed for greater variation than the single copy PAL gene. Based on Nei's gene diversity index. Var. *sinensis* was revealed to be more variable than var. *assamica*, and that a higher proportion of overall diversity resisded within varieties as compared to between varieties. Even though no specific DNA profile was found for either tea varieties following any single PCR-RFLP analysis, a factorial correspondence analysis carried out on all genotypes and markers separated the tea samples into two distinct groups according to their varietal status. This reflects the large difference between var. *sinensis* and var. *assamica* in their polyphenolic profiles.

Researchers in different countries have made fingerprints of tea cultivars in their countries of origin. In India also RFLP technique was used to detect adulteration with cashew husk in 10 different tea samples. Some work has also been done on analysis of tea using the simple sequence repeat anchored polymerase chain reaction (SSR-anchored PCR) or Inter SSR-PCR (ISSR) (Mondal 2002).

Work is ongoing to develop a complete tea database with chemical as well as molecular data, which will assist with easy identification of the different cultivars. Simple sequence repeats (SSR) were derived from *C. japonica*, a closely related species of tea in Japan. Using these primer pairs, 53 *C. japonica* ecotypes were genotyped and population genetic parameters calculated (Ueno *et al* 1999). Later, the same group investigated the spatial genetic structure of C. japonica using four of these microsatellite primers. Spatial distribution of individuals was also assessed to obtain an insight into spatial relationships between individuals and alleles. Although techniques like RFLP and AFLP have been applied successfully and have provided considerable genetic information in a number of plant species (Zhang *et al*, 1992; Vos *et al*, 1995; Xu *et al* 2000) these techniques are comparatively slow and expensive requiring the nucleotide information and are not amenable for assessment of genetic studies. SSR markers require prior information of target DNA sequences and sophisticated electrophoretic systems with computer software for accurate band separation and scoring (Smulders *et al*, 1997; Sun *et al*, 1998). RAPD techniques overcome these limitations; a number of polymorphic markers can be obtained with ease from small amount of genomic DNA without the knowledge of

target sequences. There is only one drawback of this technique i.e., lack of reproducibility that may arise if the experimental conditions applied are not standardized properly (Prenner *et al* 1993). In spite of this fact RAPD methodology provides informative data consistent with other markers, especially at the intraspecific level (Dos Santos *et al* 1994; Lerceteau *et al* 1997). Moreover it is cost effective for large scale population genetic analysis. In preliminary studies the importance of molecular markers for the characterisation of tea genetic resources and estimation of genetic diversity were reported by various workers (Wachira *et al* 1995; Paul *et al* 1997; Kaundan & Park 2002). In the north east region of India some work was done on five varieties of tea by Bera and Saikia (1999) using sixteen RAPD markers. But the work mainly covered only the molecular characterization and genetic diversity of five tea varieties. Amplified fragment length polymorphism (AFLP) markers were also studied in depth to detect diversity and genetic differentiation of several important tea clones, including the famous 'Darjeeling tea', mainly to protect cultivars for intellectual property rights purposes.

2.9. *In vitro* culture studies in tea

The potential of tissue culture in various aspects of plant improvement has already been recognized and attracted the attention of scientists. Rapid multiplication of propagation materials through tissue culture, particularly in the initial selection, assumes importance in plant improvement programmes. Tissue culture offers opportunity to utilize genetic, physiological and biochemical procedures in developing ideotypes (Banerjee 1986). It also holds promise in the development of pure lines through haploid technology, production of triploids through endosperm culture, culture of embryos of incompatible crosses and isolation of somaclonal variants (Singh 1978). Banerjee (1992) suggested further possibilities of genetic manipulation for production of pure line of tea for inbreeding, breeding and propagation of interspecific hybrids derived from the various combination of crosses by cotyledon and embryo culture. Somatic cell hybridization using protoplast fusion has been tried to transfer the Darjeeling tea flavour of China clones to the Assam cultivars possessing strong and brisk liquors (Banerjee 1986). In Japan, the tissue culture is being used to produce new tea varieties (Wu 1976). Tissue culture studies in tea is also being carried out at a number of centres in Taiwan, Srilanka, Thailand and the former USSR (Kato 1986; Arulpragasam amd Latiff 1986; Koretskaya and Zaprometov 1975).

Since the chemical composition of calli in the tea plant was analyzed (Ogutuga and Northcote 1970a, b), callus induction and organogenesis have been reported in several papers from the year 1980 to 2001 (Table 2.1). Callus induction has been obtained from numerous organs in high frequency (Doi 1980; Wu *et al.* 1981; Kato 1982). Wu *et al.* (1981) have successfully obtained new clones derived from cotyledon callus. They reported from Taiwan that the callus-derived plantlets grew strongly and these plantlets were used as a female to cross with one of the highest-quality variety which appeared weak in growth. F_1 hybrids were expected to improve

Table 2.1 Summary of *in vitro* culture studies on *Camellia*

Plant species	Explant	Results	References
C.sinensis	Internode	Callus	Ogutuga and Northcote (1970)
C.sinensis	Stem	Callus and cell suspension	Bagratishvilli *et al* (1979)
C.japonica	Axillary buds	Shooting	Creze (1980)
C.sinensis	Anther	Callus and root	Doi (1981)
C.sinensis	Cotyledon	Callus, adventitious buds & plants	Wu et al (1981)
C.japonica	Cotyledon	Callus and plants	Bennet and Scheibert (1982)
C.sinensis&C.japonica	Cotyledon	Adventitious embryos and plants	Kato (1982)
C.sinensis	Anther	Haploid plants	Chen and Liao (1983)
C.japonica	Shoot tip	Shoot proliferation	Samartin *et al* (1984)
C.sinensis	Lateral buds	Buds and shoot proliferation	Tavartkiladze & Kutubidze (1984)
C.sinensis	Stem	Callus, adventitious buds&plants	Kato (1985)
C.chrysantha	Cotyledon	Adventitious embryos and plants	Zhuang and Liang (1985)
C.japonica	Shoot tip	Shoot micropropagation	Carlisi and Torres (1986)
C.sinensis&C.japonica	Cotyledon	Adventitious embryos and plants	Kato (1986a,b,c)
C.japonica	Shoot tip	Rooting	Samrtin *et al* (1986)
C.sinensis	Anther	Adventitious embryos	Shimokado *et al* (1986)

the yield and quality of oolong tea. Doi (1981) obtained roots from anther callus but not buds. The callus from the stem showed different capacities of differentiation, depending on the origin of the explant (Kato 1985). Although plantlets were regenerated from stem callus, in the callus of shoot tip, leaf and root bud formation has not been observed. The embryoids were formed directly from slices of cotyledon (Kato 1982, 1986a). Haploid plants derived from anther culture were established in China (Chen and Liao 1983). Chen and Liao (1983) cultured anthers which were stored at 5°C for 2 days. Shiny calli were formed on the N6 medium supplemented with 0.5mg/l 2, 4-D, 2mg/l Kin, 100mg/l serine and 800mg/l glutamine. Transfer of shiny calli to N6 medium containing 2mg/l Zeatin, 20mg/l adenine and 10mg/l Casein permitted shoot growth or continued proliferation of new shiny calli. Rooting was obtained on the 0.1mg/l IAA medium. The chromosome number of plantlets was observed with squashed root tips. Embryoids were obtained from anthers cultured on N6 medium supplemented with 0.05mg/l 2, 4-D and 0.2 mg/l Kin (Shimokado *et al* 1986). Haploid plants provide production of homozygous plants; they are of great importance in tea breeding, tea being heterogeneous.

Cell suspension culture has been investigated (Bagratishvilli *et al* 1979), but it gained small

aggregates with a few free cells. Since Creze (1980) reported that axillary buds of *C.japonica* were cultured and elongated, the propagation of tea and other plants in *Camellia* genus has been reported (Table 2.1). Numerous buds, as well as shoots, were induced from lateral buds of 3 month old tea seedlings (Tavatkiladze and Kutubidze 1984). Cultured shoot tip explants from 4 to 5 month old seedlings of *C. japonica* showed shoot proliferation followed by root initiation and later adaptation to soil (Samartin *et al* 1984, 1986). Also in *C. japonica* and *C. chrysantha*, embryoids formed directly or via callus of cotyledons (Bennet and Scheibert 1982; Kato 1982, 1986a; Zhuang and Liang 1985), but the regeneration from callus of organs other than the cotyledon scarcely has been reported to date in these plants (Kato 1989).

In tea research the previous era held major emphasis on standardizing parameters of the *in vitro* protocol, such as using a suitable explant, overcoming microbial contamination, and optimizing media composition combined with growth regulation for better proliferation.(Kato1989). Following this era the efforts turned towards hardening micro-shoots to achieve a higher survival percentage. Accordingly, several non- conventional approaches, such as a CO_2-enriched hardening chamber, biological hardening, and micrografting, were developed till 2001 (Mondal *et al.,* 2001). Presently, attention is increasingly focused on evaluating field performance of the transformed *in-vitro* grown whole plantlets. Although there is no stable technique developed so far to produce transformed *in-vitro* grown whole plantlets of tea except the one made by Mondal *et al.,* in 2001 by micrografting seedling grown roots on the *in-vitro* grown shoots of tea. Tissue culture studies on tea varieties cultivated in India is summarized in Table 2.2.

The development of micropropagation, a rapid *in vitro* multiplication method, of tea has passed through three phases. Until the 1980s, emphasis was on standardizing parameters of the in vitro protocol, such as using a suitable explant, overcoming microbial contamination, and optimizing media composition combined with growth regulation for better proliferation. It is now accepted that nodal segments (0.5-1 cm) cultured on MS medium with BAP (1-6 mg/l) are best for multiplication of shoots, along with either a high dose (500mg/l) pulse treatment or a low dose (1-2mg/l) long duration treatment of auxin such as IBA for in vitro rooting. Until the1990s, efforts turned toward hardening micro-shoots to achieve a higher survival percentage. Accordingly, several non-conventional approaches, such as a CO_2-enriched hardening chamber, biological hardening, and micro grafting, were developed. Presently, attention is increasingly focused on evaluating field performance of the micro propagated plant.One prerequisite for genetic transformation of tea is an efficient system of regenerating the complete plant from a single cell. Until today, somatic embryogenesis in tea was considered the most efficient regeneration system. Unlike micro propagation, tea somatic embryogenesis started in the late 1980s. Thus, emphasis was focused on standardizing parameters, such as genotypes, seed maturity, media formulation, growth regulator, physical condition, etc. A

Table 2.2 Tissue culture studies on tea in India

Explant Used	Reference
Callus	Complete plantlets (Das *et al* 1990;Jain *et al* 1990)
Two and a bud	Shoot formation (Palni *et al* 1991;Sood *et al* 1991)
Nodal segments	Direct and indirect shoot bud differentiation (Phukan and Mitra 1984); Rajasekaran and Mohan kumar 1992; Rajasekaran 1996; Rajkumar and Ayyapan 1992). Shoot multiplication and formation of rooted shoot (Banerjee and Agarwal 1990; Jha and Sen 1991; Palni *et al* 1991)
Stem segments	Leaf segments Callusing and direct rooting (Palni *et al* 1991) Callusing, shoot bud and embryo differentiation (Palni *et al*.1991)
Anther	Callusing and proembryoid formation (Raina and Iyer 1983; Palni *et al* 1991). Callusing (Sood *et al* 1991). Callusing and differentiation of shoot apices (Bhattcharya and Saha 1992)
Cotyledon segments	Non-organogenetic white friable callus (Bano *et al* 1991; Palni *et al* 1991) Organogenetic callus (Palni *et al*. Somatic embryogenesis (Bano *et al* 1991; Balasubramanian *et al* 2000) and formation of synthetic seeds (Palni *et al* 1991); Sood *et al* 1991). Rooting (Palni *et al* 1991).
Immature zygotic embryo	Complete plantlets (Palni *et al* 1991; Sood *et al* 1991)

bioreactor system for repetitive embryogenesis in tea has also been developed in Australia in which uniform sizes of globular somatic embryos were obtained for a bioreactor technology called the temporary immersion system (TIS). By controlling immersion cycles, synchronized multiplication (24 fold) and embryo development were achieved with greater consistency and with a high rate of plant recovery. Plantlets recovered through this method were hardy, with a well-formed taproot. Therefore, this technique was the first significant step for commercial application of bioreactor technology to produce large-scale tea somatic embryos.

The ultimate success of any *in vitro* protocol depends upon performance of plants in the field compared to vegetative counterparts. For the last several years, researchers at the Research and Development Department of Tata Tea Ltd, India, have transferred more than 45,000 plants of eight tea cultivars to the field, from which leaves are harvested regularly to manufacture black tea. A systematic study at 1.7, 4, and 8 year-old field-grown micro propagated and vegetatively propagated tea

plants demonstrated that overall yields and quality were comparable. Although different physiological parameters such as photosynthetic rate, chlorophyll content, etc. remained the same, two morphological variations were noticed. First, the number of lateral shoots produced after 'centering' were significantly greater in micropropagated-raised plants compared to vegetatively propagated plants. This is perhaps due to effects of various growth regulator treatments applied under *in vitro* conditions. Second, root volumes of tissue culture plants were also greater than in vegetatively-propagated plants. Micropropagated shoots were treated with IBA to induce rooting, which may be responsible for better root development in the field. Therefore, the conclusion was drawn that the micropropagation protocol should be used only when required to produce a large number of plantings from a limited source. Other techniques have been applied in tea with specific objectives, efforts to improve these techniques are ongoing at laboratories worldwide (Mondal 2004).

2.10. Histological study

Somatic embryogenesis involves control of 3 consecutive steps:

- Induction of embryogenic lines from sporophytic cells
- Maintenance and multiplication of embryogenic lines
- Maturation of somatic embryos and conversion into viable plantlets. (Williams *et al.*,1986)

Induction of embryogenic lines and their subsequent conversion into plantlets have been much studied by many workers (Sharp *et al.*, 1980; Tisserat *et al.*, 1979; Wann 1988) but the multiplication step has been comparatively less studied although it directly contributes to the ability of the resulting embryos to germinate and develop into growing plantlets (Redouane *et al.,* 2001).

Two main problems have been reported concerning the multiplication step. The first one is the difficulty in obtaining stable and subculture-suitable lines that will produce embryos for long periods of time (Tisserat *et al* 1979; Wann 1988). The second is the lack of synchrony in embryo development and the risk of morphological abnormalities such as pluricotyledony, multiple apex formation, fused cotyledons and/ or fasciation.

In angiosperm species, multiplication of embryogenic lines can be achieved either by regular subculturing of explants taken from compact or friable embryogenic calli (Tisserat *et al* 1979), or by the formation of new embryos from the previously developed somatic embryos themselves (Bornman 1991; Wann 1988; Williams and Maheswaran 1986). This second case is reffered to as secondary embryogenesis.

In *Quercus*, initiation of somatic embryogenesis has been described from a variety of sporophytic explants, namely stem segments, leaves and zygotic embryos. The multiplication of the embrogenic lines was first achieved from calli ageing on the same culture medium (Feraud-keller and Espagnac 1989; Gingas and Lineberger 1988) or via successive transfers onto fresh culture media with different growth regulator supplements (Feraud-keller and Espagnac 1989; Fernandez-Guijarro *et al* 1995). Embryogenic response from anthers and ovary tissues was also obtained using simi-

lar procedures (Jorgensen 1993).

Researchers have noted that

- Within one embryogenic line the somatic embryos could occur from different histological origin, as observed for example in *Theobromo cacao* (Adu-Ampomah *et al* 1988).
- The growth regulator composition of the culture medium influenced the histological origin of the somatic embryos (*Hevea brasiliensis* (Michaux-Ferriere *et al* 1992; Michaux-Ferriere *et al* 1993), *Elaeis guineesis* (Schwendiman *et al* 1988).
- Depending on their origin somatic embryos exhibited different potentials for germination and further growth (Michaux-Ferriere *et al* 1992; Williams and Maheswaran 1986)

In tea, initiation of somatic embryogenesis has been described from explants, like stem segments and cotyledons (Kato 1989; Mondal *et al.*, 2001). In all the reports of embryogenesis, emphasis has been given to manipulate the nutrient composition, growth regulators in culture medium, physical conditions of incubation and other stress treatments to induce somatic embryos. However, there is no report on the histological study of *in vitro* grown embryos of tea. None of these studies investigated the histological origin and structural organization of the somatic embryos.

2.11. Genetic transformation study of tea

Production of desired ecotypes of human choice through conventional breeding is an age-old practice. Transgenic technology offers several advantages such as:

- transferability of genes of any origin to plant,
- time effectiveness and
- self-expression of transgene i.e. free from any epistatic and dominant/recessive interaction etc.

Recent advances in tissue culture and recombinant DNA technology have opened new avenues in transformation of higher plants, which consequently produced many transgenic plants with new genetic properties. Transgenic plants broadly speaking refers to those plants in which functional foreign genes have been inserted in their genomes (Uchimiya *et al.*, 1989). For the improvement of crop species these developments in molecular biology and gene transfer technology have equipped scientists with powerful tools of biotechnology. Previously impossible techniques like gene identification, cloning and transfer have now enabled the workers to introduce specified alterations in plants. Today it is possible to transfer useful genes from sexually incompatible species or unrelated organisms to crop species of agricultural importance for developing transgenic crop plants. A number of methods are available for transfer of gene to plants and many transgenics have been generated with a number of useful traits in an array of crop species. The methods include a modified Ti plasmid system in *Agrobacterium tumefaciens* and the direct gene transfer including PEG- induced DNA uptake, microinjection of DNA into cultured cells and plant organs, electroporation, microprojectile bombardment and some other innovative means. The refinement in plant regeneration from cultured cells, efficient vector constructs and availability of defined selectable marker

genes has resulted in the production of transgenic plants. Though several techniques are available (Klee *et al.*, 1987; Kuhlemeiere *et al.*, 1987; Draper and Scott, 1991; Hooykaas and Schilperoort, 1992; Smith and Hood, 1995), yet few have been employed to produce the transgenic tea, detail accounts of which are elucided below.

2.11.1. Agrobacterium mediated genetic transformation

Plant transformation mediated by *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium, has become the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants (Table 2.3). *A. tumefaciens* naturally infects the wound sites in dicotyledonous plant causing the formation of the crown gall tumors. The first evidences indicating this bacterium as the causative agent of the crown gall goes back to more than ninety years (Smith and Townsend, 1907). Since that moment, for different reasons a large number of researches have focused on the study of this neoplastic disease and its causative pathogen. During the first and extensive period, scientific effort was devoted to disclose the mechanisms of crown gall tumor induction hoping to understand the mechanisms of oncogenesis in general, and to eventually apply this knowledge to develop drug treatments for cancer disease in animals and humans. When this hypothesis was discarded, the interest on crown gall disease largely decreased until it was evident that this tumor formation may be a result of the gene transfer from *A. tumefaciens* to infected plant cells. *A. tumefaciens* has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease (Nester *et al.*, 1984; Binns and Thomashaw, 1988). T-DNA contains two types of genes: the oncogenic genes, encoding for enzymes involved in the synthesis of auxins and cytokinins and responsible for tumor formation; and the genes encoding for the synthesis of opines. These compounds, produced by condensation between amino acids and sugars, are synthesized and excreted by the crown gall cells and consumed by *A. tumefaciens* as carbon and nitrogen sources. Outside the T-DNA, are located the genes for the opine catabolism, the genes involved in the process of T-DNA transfer from the bacterium to the plant cell and the genes involved in bacterium-bacterium plas-

Table 2.3 Transgenic plants obtained by *Agrobacterium* mediated gene transfer

Host plants	Foreign genes	References
Apium graveolens	*NOS, NPTII*	Catlin *et al* 1988
Arabidopsis thaliana	*NPTII*	An *et al* 1986
Brassica napus	*CAT, NPTII*	Charest *et al* 1988
Lycopersicon esculentum	*NPTII, PAT*	De Block *et al* 1987
Medicago varia	*NPTII*	Deak *et al* 1986
Nicotiana tabacum	*A.rhizogenes,*	Cardarelli *et al* 1987 Barton
	BT toxin, NPTII	*et al* 1987
	TMV CP	Abel *et al* 1986
	soybean hs6871	Baumann *et al* 1987
Solanum tuberosum	*NPTII, PAT*	De Block 1988

mid conjugative transfer. (Hooykaas and Schilperoort, 1992; Zupan and Zambrysky, 1995). Virulent strains of *A. tumefaciens* and *A. rhizogenes*, when interacting with susceptible dicotyledonous plant cells, induce diseases known as crow gall and hairy roots, respectively. These strains contain a large megaplasmid (more than 200 kb) which plays a key role in tumor induction and for this reason it was named Ti plasmid, or Ri in the case of *A. rhizogenes*. Ti plasmids are classified according to the opines, which are produced and excreted by the tumors they induce. During infection the T-DNA, a mobile segment of Ti or Ri plasmid, is transferred to the plant cell nucleus and integrated into the plant chromosome. The T-DNA fragment is flanked by 25-bp direct repeats, which act as a *cis* element signal for the transfer apparatus. The process of T-DNA transfer is mediated by the cooperative action of proteins encoded by genes determined in the Ti plasmid virulence region (*vir* genes) and in the bacterial chromosome. The Ti plasmid also contains the genes for opine catabolism produced by the crown gall cells, and regions for conjugative transfer and for its own integrity and stability. The 30 kb virulence (*vir*) region is a regulon organized in six operons that are essential for the T-DNA transfer (*virA, virB, virD,* and *virG*) or for the increasing of transfer efficiency (*virC* and *virE*) (Hooykaas and Schilperoort, 1992; Zupan and Zambryski, 1995, Jeon *et al.*, 1998). Different chromosomal-determined genetic elements have shown their functional role in the attachment of *A. tumefaciens* to the plant cell and bacterial colonization: the loci *chvA* and *chvB*, involved in the synthesis and excretion of the b -1,2 glucan (Cangelosi et al., 1989); the *chvE* required for the sugar enhancement of *vir* genes induction and bacterial chemotaxis (Ankenbauer et al., 1990, Cangelosi *et al.*, 1990, 1991); the *cel* locus, responsible for the synthesis of cellulose fibrils (Matthysse 1983); the *pscA* (*exoC*) locus, playing its role in the synthesis of both cyclic glucan and acid succinoglycan (Cangelosi *et* al., 1987, 1991); and the *att* locus, which is involved in the cell surface proteins (Matthysse, 1987). The initial results of the studies on T-DNA transfer process to plant cells demonstrate three important facts for the practical use of this process in plants transformation. Firstly, the tumor formation is a transformation process of plant cells resulted from transfer and integration of T-DNA and the subsequent expression of T-DNA genes. Secondly, the T-DNA genes are transcribed only in plant cells and do not play any role during the transfer process. Thirdly, any foreign DNA placed between the T-DNA borders can be transferred to plant cells, no matter where it comes from. These well-established facts, allowed the construction of the first vector and bacterial strain systems for plant transformation (Hooykaas and Schilperoort, 1992; Deblaere *et al.*, 1985; Hamilton, 1997; Torisky *et al.*, 1997).

Agrobacterium tumefaciences-mediated transformation has been the most widely used technique in woody plants. Despite the fact that several transgenic plants have been produced in a wide range of genera, production of the transgenic tea plant remains difficult till recently mainly due to low transformation efficiency as well as difficult regeneration

system. This may be perhaps due to the presence of high levels of polyphenols in tea explants, which has germicidal property (Biao *et al.*, 1998). Nevertheless, genetic transformation of tea through *Agrobacterium tumefaciens* has been attempted by several workers using different explants such as *in vitro* leaves, somatic embryos etc. (Matsumoto and Fukui, 1998, 1999; Biao *et al.*, 1998; Mondal *et al.*, 1999, 2001c; Luo and Liang, 2000). While only stable transformed callus could be achieved from leaf explants (Matsumoto and Fukui, 1989, 1999; Biao *et al.*, 1998; Mondal *et al.*, 1999; Siswanto and Chaidamsari, 1999), the first healthy transgenic plants were produced by Mondal *et al.* (2001c), using somatic embryos as explants. The later group found that while pre-culturing of explants and wounding of somatic embryos had no effect on the transformation efficiency, bacterial growth phase (O.D value=0.60), cell density (109/ml), co-cultivation periods (5 days) and pH of co-cultivation medium (5.6) enhanced the transformation efficiency significantly. These parameters were optimized on the basis of *β-glucuronidase* (*GUS*) activity evidenced by the blue spots that appeared on the somatic embryos after 48 h of co-cultivation. Eventually blue spots increased in size and became uniformly dispersed all over the embryo surface. Very strong GUS positive signals could also be detected in the leaf tissue from 1 year old tea plants recovered through germination of kanamycin resistant somatic embryos. However, no endogenous *GUS* expression was detected in the un-infected somatic embryos or tissues. Interestingly, though acetosyringone, had no effect to increase the transformation efficiency of tea as experienced by Mondal *et al.* (2001c), yet, concentration at500 μM improved the transformation efficiency in tea as reported by Matsumoto and Fukui (1999). Additionally, two more important factors affect the efficiency of transformation. They are:

- efficiency of selection of transgenic tissue on antibiotics such as kanamycin, hygromycin etc. and
- an effective use of bactericidal antibiotics.

In some *Camellia* hybrids, Tosca *et al.* (1996) detected a lethal dose of 75 mg l-1 kanamycin for selection of internode explants. A similar response was also observed by Mondal *et al.* (2001c) who reported that 50 mg l-1 kanamycin followed by an elevation of 75 mg l-1 kanamycin were optimum for the effective selection of transformants for tea somatic embryos. However, Matsumoto and Fukui (1999) found that 200 mg l-1 kanamycin was effective when leaves were used as explants. Scant systematic effort has been made on the effect of bactericidal antibiotics for tea transformation. However, Mondal *et al.* (2001b) tested the bactericidal effects of three antibiotics and found that sporidex at 400 mg l-1 not only ensured a total restriction of the *Agrobacterium tumefaciens* overgrowth but also confirmed negligible effect on the growth retardation of the somatic embryos. The next best antibiotics were found to be carbenicillin followed by cefotaxime. Therefore, sporidex in combination with carbenicillin was preferred as it was cheaper and locally available. Siswanto and Chaidamsari (1999) reported that the development of the transformed *in*

vitro leaves to calli produced a mucilage-like compound covering the calli that hinder the further development. Though the authors did not mention any further detail, yet it seems that *Agrobacterium* overgrowth, which produces a mucilage-like compound on the explants, if not controlled properly after co-cultivation stage. Further, the putative transformants were also confirmed through molecular technique. While Matsumoto and Fukui (1998, 1999) have reported stable transformations in callus after molecular characterization through PCR and southern hybridization, it was Mondal *et al.* (2001c) who confirmed the stable integration of transgene after molecular characterization through southern hybridization. This indicated that the presence of the marker gene *npt-II* was linked with that of *gus* as a single 'T-DNA strand' in the genomic DNA of the transformed plant. Luo and Liang (2000) contructed a vector containing *Bt* gene which was subsequently transformed to tea. The pGA471 plasmid containing *Bt* gene cry I A (c) was digested with *Hind III* and *Bgl II* and inserted into the vector pCAMBIA-2301. The constructed plasmid containing *Bt* gene, *GUS* intron and *npt-II* was transformed into *E. Coli* and introduced into *Agrobacterium* strains such as LBA 4404, EHA 105 and pRi15834 through triparental mating. They detected the transient expression of *GUS* gene in transgenic calli and leaves of putative transgenic tea plants. They found the optimum concentration for hygromycin and kanamycin as 20 g l-1 and 60 gml-1 for screening the tea leaves. However, no transgenic plants were produced.

2.11.2. Agrobacterium rhizogenes

Agrobacterium rhizogenes, causal agent of the 'hairy root' disease is characterized by its ability to cause root proliferation at the sites of infection of the susceptible hosts. The phenomenon of profuse root growth on hormone free media under *in vitro* conditions has been extensively exploited in a large number of plant species specially for secondary metabolite production. For the first time, in tea, Zehra *et al.* (1996), infected 35-day-old *in vitro* leaves with *Agrobacterium rhizogenes* strain A4. The explants were co-cultivated with bacterial cell (108/ml) in dark for 2 days. The excessive bacterial solution was blotted dry on sterile filter paper and leaves were then cultured on MS for 35 days for inducing hairy root. Mannopine from these roots were analysed through paper electrophoresis to confirm the stable integration of this gene. Konwar *et al.* (1998) also transformed *in vitro* tea shoots of 4–6 months old at the basal end followed by co-cultivation in liquid basal MS medium supplemented with IBA (5 mg l-1) and rifampicin (100 mg l-1). Roots were initiated after 32–45 days culture among the 66% explants from the basal end which enabled hardening of the microshoots in nursery beds. However, the technique has not been exploited commercially to produce the secondary metabolites, which will be immense useful for a crop like tea.

2.11.3. Biolistic

Biolistic mediated genetic transformation is an alternative method that has been successfully used in production of transgenics in a wide variety of plant species. Though no transgenic tea plants could be produced by this technique, yet a preliminary study on transient expression was reported by Akula and Akula (1999). Tea

somatic embryos were bombarded with gold particles (1.5–3m diameter), coated with plasmid p2k7, a vector originally derived from pBI 221. The marker and reporter gene was *npt-II* and *β-glucuronidase* respectively under the control of 35S cauliflower mosaic virus (CaMV) promotor. Various factors such as the distance between the site of delivery of the microprojectile and the target tissue, helium pressure and the state of target tissue to obtain transient expression was optimized on the basis of *β-glucuronidase* (*GUS*) assay after 30–40 h of bombardment. Following which they achieved the highest transient expression levels up to 1085 blue spots/shot. However, further details of regeneration of somatic embryo were not mentioned. Though beginning, yet the different protocols that have been standardized using both *Agrobacterium* and biolistic-mediated methods hold a tremendous potential for producing transgenic tea with useful genes.

2.12. Gene constructs and vectors

A number of gene constructs (either genomic or chimeric) have been investigated both at callus and plant level. Particular significance to achieve the reproducible transformation would depend on the avilability of selectable genes conferring resistance to antibiotics; bleomycin, chloramphenicol, hygromycin, kanamycin and streptomycin. Furthermore, several reporter genes including *CAT*, *β-glucuronidase* (*GUS*), *luciferase* and *neomycin phosphotransferase* (*npt-II*) have been employed for the analysis of precise nature of promoter activities. Some demonstrated instances of foreign gene expression are given below.

2.12.1. Herbicide tolerance genes

Transfer of aroA bacterial gene from *Salmonella typhimurium* into tobacco and a hybrid clone of *Populus* (Comai *et al* 1985). The transformed tobacco plants exhibited a high degree of tolerance to glyphosate. De Block *et al* (1987) inserted the bar gene from *Streptomyces hygroscopicus* into tobacco, tomato and potato. These transgenic plants showed an increased level of resistance to the herbicide phosphinothricin which is a potent inhibitor of glutamine synthetase in plants.

2.12.2. Insect tolerance genes

Bacillus thuringiensis is a bacterium that forms insecticidal proteinaceous crystals during sporulation. The crystal proteins are specifically toxic to lepidopteran insects. The commercial *B. thuringiensis* gene has been introduced into tomato and tobacco and the transgenic plants thus produced show an increased level of resistance to lepidopteran insects (Barton *et al* 1987). The introduction of toxin genes into plants seems to a practical method for providing protection against certain insect pests.

2.12.3. Seed storage protein genes

In legumes and most other dicots, the major storage proteins are the salt-soluble globulins. In monocots, prolamins (alcohol-soluble) and glutelins (acid- or base- soluble) are the predominant seed proteins. The tissue-specific expression and abundance of mRNA of these proteins have made it possible to clone and transfer these genes into various plant species. Some examples of transgenic plants carrying seed storage protein genes include: a 17kbp soybean DNA fragment with the *lectin* gene in tobacco (Deom *et al* 1987); the *legA* gene

of pea into *N. plumbaginifolia* petunia (Bray *et al* 1987). Transgenic tobacco plants have been obtained by introducing a 17kbp soybean DNA fragment containing the *lectin* gene and atleast four nonseed protein genes are expressed in correct tobacco developmental stages but at different quantitative levels.

2.12.4. Coat protein genes for virus protection

The practical control of plant viruses depends on methods that prevent or restrict virus infection. Recently it has been shown that the coat protein of the virus has an important role in systemic cross-protection. Insertion of a cloned coat protein (CP) gene delayed the virus disease development and subsequent systemic spread of the virus in transgenic plants. The expression of the CP gene of tobacco mosaic virus (TMV), of alfalfa mosaic virus (AIMV), of potato virus X (PVX) and of cucumber mosaic virus disease development and subsequent systemic spread of the virus in transgenic plants resulted in transgenic plants resulted in protection of these plants from infectious viruses. Abel *et al* (1986) produced transgenic tobacco plants expressed TMV mRNA and CP as a nuclear trait. Inoculation with TMV showed that seedlings expressing the CP gene delayed in symptom development. Plants accumulating AIMV-CP were highly resistant to infection with AIMV nucleoproteins.

2.12.5. *Light regulated gene*

Many light regulated genes are tissue specific in expression. Transgenic plants offer a unique opportunity for the analysis of such genes as the small subunit of ribulose-1, 5-bisphosphate carboxylase (rbcS) and chlorophyll a/b-boinding protein (Cab). The cis elements responsible for tissue-specific expression of light regulated genes have been investigated extensively. It is not known how the pathways leading to tissue specific and light-induced transcription are related. The expression of the pea rbcSW-3A gene has been studied in transgenic tobacco plants (Aoyagi *et al* 1988). This gene is expressed at low levels in dark adapted plants but after exposure of the plants to light, transcript levels increase 20-50 folds. The upstream region containing an enhancer like element and a 280bp region (-330 to-50 relative to the transcription start site) is sufficient for regulated expression. The transcription of a chimeric gene with a 1.2 kbp 5'-upstream promoter was shown to be light inducible in tobacco plants.

2.13. Phenotypic characteristics of transformed plant

Though transgenic plants have been produced in a number of plant species, more information is needed on inheritance of foreign genes and phenotypic characteristics of progenies. The effects of inserted genes on other plant characteristics have been reported in a few transgenic plants. In *B. napus,* plants carrying the gene for mouse dehydrofolate reductase had a normal morphology with good seed sets (Charest *et al* 1988). Similarly no deleterious effect on the growth and development of transgenic tobacco, showing higher levels of chitinase expression, was observed (Bedbrook *et al* 1988). Cuozzo *et al* (1988) did not observe any deleterious effect on growth, fertility or morphology of the transgenic tobacco plants and their progeny expressing CMV-CP and its antisense RNA.

Nelson *et al.* (1980) have made a detailed analysis of transgenic tomato plants carrying the *CP* gene of TMV. The plants were tested under laboratory, greenhouse and field conditions. The yield of CP^+ plants was unaffected compared to a 26-35% decrease in fruit yield of the parent tomato due to virus infection. The CP gene expression did not affect the agronomic characteristics of transgenic plants in the absence of virus inoculation. This was evident from the leaf and stem dry weight accumulation data from greenhouse grown uninoculated CP expressing and non-expressing plants which was essentially equal. The available data thus strongly supports that insertion of one or a few foreign genes do not adversely affect the agronomic characteristics of the host cultivars except for the expression of the introduced gene. Thus, breeding of crop and other plant cultivars by insertion of specific single or few genes could be a good supplement to conventional crop improvement programs. This approach could be helpful in overcoming the specific defects of otherwise high yielding and well adapted commercial cultivars (Uchimiya *et al* 1989).

Chapter 3
Material & Methods

3.1. Plant Material

Twenty six different cultivars of tea from various tea gardens in North Bengal region were collected (Fig.3.1) in July 2005 and maintained as germplasm in the tea garden of Molecular Genetics Lab., N.B.U. Tea cultivars available in North Bengal region used in this study along with their characteristics are given in Table 3.1.

3.2. DNA Fingerprinting Study

Genetic diversity of tea was studied using different PCR based methods namely RAPD, PCR-RFLP, Microsatellite markers by various workers (Refer Review of literature section 2.6). Before going to these techniques the most important work was isolation of high molecular weight DNA of tea that is suitable for digestion with restriction endonuclease which is an essential requirement for DNA fingerprinting methods like PCR-RFLP, RAPD and Microsatellite markers. The following protocol developed by Dellaporta *et al.*, 1983 worked well for tea with slight modifications as mentioned below:

3.2.1. Tea DNA extraction

- Tender leaves two and a bud shoots were taken weighing approx. 5gms in a mortar and pestle and ground into a fine powder with the help of liquid nitrogen.
- The pulverized material was taken in a 30ml Oakridge tube (Tarsons, Cat#541040) containing 15 ml of prewarmed (65°C) CTAB extraction buffer (Refer Appendix II for composition).
- The tube was then vortexed for 5 seconds and incubated in a water bath (Genei, Cat#107931) for 1 hr at 65°C with occasional mixing by gentle swirling.
- Following the 1 hr incubation an equal volume of chloroform (E Merck Ind. Ltd., Cat#822265)/Isoamyl alcohol (E Merck Ind. Ltd., Cat#8.18969.1000) (24:1) was added and the mixture was mixed gently by

Table 3.1 Description of tea cultivars used in the present study. Abbreviations: TV-Tocklai Vegetative; HV-Happy Valley; TSS-Tocklai Seed Stock; BS-Balasun; K-Kopati; P-Phoobshering; B-Bannockburn; RR-Runglee Rungliot; T-Tukdah; CP-Clonal Proving; AV-Ambari Vegetative

Sl.No.	Clone	Species	Type	Origin
1	TV29	*C.assamicasub.Lasiocalyx*	Cambod	Tocklai ExperimentalStation
2	TV17	*C.assamica*	Assam.	Tocklai ExperimentalStation
3	TV22	*C.assamicasub.Lasiocalyx*	Cambod	Tocklai ExperimentalStation
4	HV39	*C.sinensis (L.)O.Kuntze*	China	Darjeeling
5	TV20	*C.assamicasub.Lasiocalyx*	Cambod	Tocklai ExperimentalStation
6	TSS449	*Seed stock*	Seedstock	Tocklai ExperimentalStation
7	BS71A76	*C.sinensis (L.)O.Kuntze*	China	Darjeeling
8	K1/1	*C.assamica*	Assam	Darjeeling
9	TV30	*C.assamicasub.Lasiocalyx*	Cambod	Tocklai ExperimentalStation
10	P1258	*C.sinensis (L.)O.Kuntze*	China	Darjeeling
11	TV27	*C.assamicasub.Lasiocalyx*	Cambod	Tocklai ExperimentalStation
12	TV1	*C.assamica*	Assam	Tocklai ExperimentalStation
13	B777	*C.sinensis (L.)O.Kuntze*	China	Darjeeling
14	RR17/144	*C.sinensis (L.)O.Kuntze*	China	Darjeeling
15	T145	*C.assamica*	Assam	Darjeeling
16	T-78	*C.sinensis (L.)O.Kuntze*	China	Takdah T.E., Darjeeling
17	TV25	*C.assamicasub.Lasiocalyx*	Cambod	Tocklai ExperimentalStation
18	CPI	*C.assamica*	Assam	Darjeeling
19	TV18	*C.assamicasub.Lasiocalyx*	Cambod	Tocklai ExperimentalStation
20	TV28	*C.assamicasub.Lasiocalyx*	Cambod	Tocklai ExperimentalStation
21	TV14	*C.assamica*	Assam	Tocklai ExperimentalStation
22	TV23	*C.assamicasub.Lasiocalyx*	Cambod	Tocklai ExperimentalStation
23	TV26	*C.assamicasub.Lasiocalyx*	Cambod	Tocklai ExperimentalStation
24	AV2	*C.sinensis (L.)O.Kuntze*	China	Darjeeling
25	P312	*C.sinensis (L.)O.Kuntze*	China	Darjeeling
26	B157	*C.sinensis (L.)O.Kuntze*	China	Darjeeling

inverting the tube upside down.

- The extract was centrifuged (REMI make, Model No.C-24) for 10 minutes at about 10,000 rpm (room temperature) and the supernatant was carefully transferred to a fresh tube.
- The chloroform/Isoamyl alcohol step was repeated twice and 0.6 volume of ice cold Isopropanol (E Merck Ind. Ltd., Cat#17813) was added to the final supernatant.

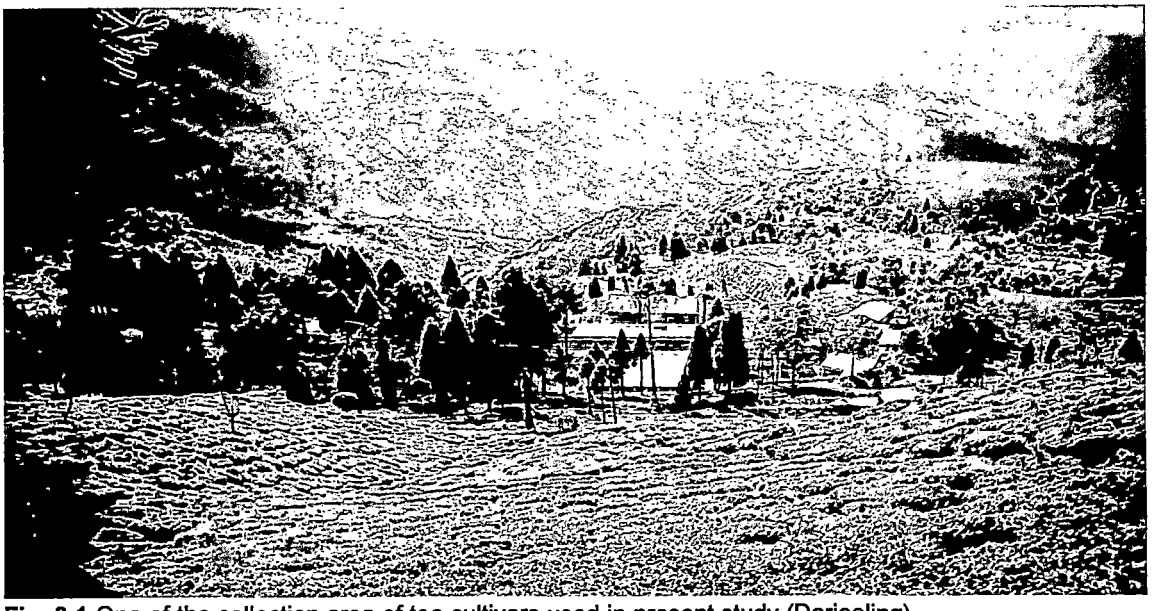


Fig. 3.1 One of the collection area of tea cultivars used in present study (Darjeeling).

- Upon gentle swirling the DNA-CTAB complex precipitated as a whitish network and was spooled out of the solution using a bent Pasteur pipette.
- It was then washed thrice in a washing solution containing 70% alcohol and 10mM ammonium acetate (SIGMA, Cat#A-7330).
- It was then dried and dissolved in 1ml of 1X TE buffer (pH 7.4) Refer Appendix II for composition.
- The dissolved DNA was extracted with an equal volume of equilibrated phenol (pH 8.0) (SRL, Cat#1624262) mixed properly and centrifuged at 10000 rpm for 15 minutes.
- The upper aqueous phase was taken in a fresh tube and to it an equal volume of chloroform-Isoamyl alcohol (24:1) was added and then centrifuged at 10000Xg for 15 minutes at room temperature.
- The upper aqueous phase was taken in a fresh tube and to it 0.1 volume of 7.5 M ammonium acetate and 2 volume of absolute alcohol (E Merck Germany, Cat#K29824783) was added and precipitated at 4°C for 30 minutes in a cooling centrifuge (REMI make, Model No.C-24) at 12000Xg.
- The pellet obtained was washed in 70% alcohol, dried with vaccum pump (Tarsons make, Model No.Rocker300) and dissolved in 500µl of 1X TE buffer (pH 7.4).

3.2.2. Purification of Tea DNA

- RNaseA (50µg/ml) (SIGMA, Cat#R-4875) was added to the genomic DNA of tea dissolved in 500µl of 1X TE buffer (pH 7.4) and it was incubated at 37°C for 1 hr in a Dry water bath (Genei make, Cat#107173).
- An equal volume of chloroform/Isoamyl alcohol (24:1) was added and mixed properly.
- Centrifuged at 10000Xg for 15 minutes at room temperature.
- The aqueous phase was then transferred to a fresh microcentrifuge tube (Tarsons, Cat#500010).

- To the aqueous phase 2 volumes of absolute alcohol and 0.1 volumes of 3M Sodium acetate (pH 5.2) (SIGMA, Cat#S-9513) was then added for DNA precipitation. It was centrifuged at 12000Xg for 30 minutes.
- The DNA pellet obtained was dried and finally dissolved in 500µl of 1XTE (pH 7.4) buffer.

3.2.3. Quantification of Tea DNA

Reliable measurements of DNA concentration is important for many applications in molecular biology including complete digestion of DNA by restriction enzymes and amplification of target DNA by polymerase chain reaction. DNA quantitation is generally carried out by spectrophotometric measurements or by agarose gel analysis. Both methods were used in the present study.

3.2.3.1. Spectrophotometric measurement

- Spectrophotometer (ThermoSpectronic, Model No.UV1 094017) was caliberated at 260nm as well as 280nm by taking 1ml TE (1X) buffer in a cuvette.
- DNA (5µl diluted in 1ml of water) was taken in the cuvette, mixed properly and the optical density (OD) was recorded at both 260 and 280 nm.
- DNA concentration was estimated by employing the following formula:
- Amount of DNA (µg/µl)

$$= \frac{OD_{260} \times 50 \times dilution \quad factor}{1000}$$

- The quality of DNA was judged from the OD values recorded at 260nm and 280 nm.

3.2.3.2. Gel Analysis

- Agarose gel (0.8%, gelling temperature 36°C) (SIGMA, Cat#A9539) was casted in 1XTBE (Tris-Borate-EDTA) buffer (Refer Appendix II for composition) on gel platform (100X70mm) (Tarsons, Cat#7024).
- Sample DNA (5µl) was loaded.
- An unknown amount of λ DNA/*HindIII* (Genei, Cat#106000) was loaded as control in the adjacent well.
- The gel was run at 50V for 1hr in a Mini Submarine Gel Electrophoresis Unit (Tarsons, Cat#7030) and made to run at a constant volt of 50 V applied with Electrophoresis Power Supply Unit (Tarsons, Cat#7090).
- After cooling it to a temperature of 40°C, the gel was stained with 0.5µg/ml Ethidium bromide (2,7-diamino-10-ethyl-9-phenylphenanthridinium bromide, $C_{12}H_{20}BrN_3$, M_r 394.33, (SIGMA Cat#E8751) for 10 min, Washed with distilled water and the gel was visualized under UV light on a UV Transilluminator (Genei, Cat#SF850).

3.2.4. RAPD (Random Amplified Polymorphic DNA) of Tea (Camellia sinensis)

A total of 34 random 10 mer primers (SIGMA, Aldrich) given in a tabular form (Table 3.2) were screened for 26 cultivars.

3.2.4.1. RAPD-PCR Amplification

- In a sterile 0.2ml thin wall PCR tube (Tarsons, Cat#500050) following components were added for PCR reaction of 25µl and mixed in the order as given below:
- Pyrogen free water- To a final volume of 25µl
- Buffer 10X -2.5µl (Refer Appendix II for composition)
- dNTPs mix -0.5µl (200µM) each (Refer

Appendix II for composition)

- Primers -1µl (0.25 µM) each
- Taq DNA polymerase -1unit (Finnzymes, Cat#F-501L).
- Template DNA -1µl (25ng)
- One negative control tube was prepared. PCR mix without DNA.
- The PCR reactions were performed on a Perkin-Elmer Thermocycler (Genei make, Model No. 9600). The amplification cycle consisted of the following specifications:

Table 3.2 Sequences of RAPD primers used in present study

Sl No.	Operon	Sequences
1	OPA01	CAGGCCCTTC
2	OPA02	TGCCGAGCTG
3	OPA03	AGTCAGCCAC
4	OPA04	AATCGGGCTG
5	OPA05	AGGGGTCTTG
6	OPA06	GGTCCCTGAC
7	OPA07	GAAACGGGTG
8	OPA08	GTGACGTAGG
9	OPA09	GGGTAACGCC
10	OPA10	GTGATCGCAG
11	OPA11	CAATCGCCGT
12	OPA12	TCGGCGATAG
13	OPA13	CAGCACCCAC
14	OPA14	TCTGTGCTGG
15	OPB01	GTTTCGCTCC
16	OPB02	TGATCCCTGG
17	OPB03	CATCCCCCTG
18	OPB04	GGACTGGAGT
19	OPB05	TGCGCCCTTC
20	OPB06	TGCTCTGCCC
21	OPB07	GGTGACGCAG
22	OPB08	GTCCACACGG
23	OPB09	TGGGGGACTC
24	OPB10	CTGCTGGGAC
25	OPB11	GTAGACCCGT
26	OPB12	CCTTGACGCA
27	OPB13	TTCCCCCGCT
28	OPB14	TCCGCTCTGG
29	OPB15	GGAGGGTGTT
30	OPB16	TTTGCCCGGA
31	OPB17	AGGGAACGAG
32	OPB18	CCACAGCAGT
33	OPB19	ACCCCCGAAG
34	OPB20	GGACCCTTAC

- Cycle 1: Denaturation at 92°C for 5min, Primer annealing at 37°C for 1min., Primer extension at 72°C for 1min.
- Cycle 2-39: Denaturation at 92°C for 1min., Primer annealing at 37°C for 1min., Primer extension at 72°C for 1min.
- Cycle 40: Denaturation at 92°C for 1min., Primer annealing at 37°C for 1min., Primer extension at 72°C for 7 min.
- The PCR products were separated on 1.5% (W/V) agarose gel run in 1X TBE buffer.
- PCR product (10µl) was mixed with 5µl of Gel loading dye (6x), (Fermentas, Cat#R0611), (Refer Appendix II for composition).
- The samples were loaded and electrophoresis was carried out at 50V for 1hr.
- The gel was stained with Ethidium bromide solution (0.5µg/ml).
- The gels were visualized with a UV transilluminator (Genei, Cat#107161) and photographed with Gel Documentation System (Vilber lourmat France, Model No. DP-001.FDC). A DNA ladder (lambda DNA *HindIII* digest) (Genei, Cat#106000) was used as a molecular size marker. All PCR reactions were run at least thrice and only reproducible and clear bands were scored and aligned by diversity data base software (NTSYSpc and POPGENE freeware).

3.2.4.2. RAPD Data Analysis

Each polymorphic band was regarded as a binary character and was scored as 1 (presence) or 0 (absence) for each sample and assembled in a data matrix. A similarity matrix on the basis of band sharing was calculated from the binary data using Dice coefficient (Nei & Li 1979). Similarities were

graphically expressed using the group average agglomerative clustering to generate dendrograms. The analysis was done using the software package NTSYSpc (version 2.0) (Rohlf 1998)

Correspondence analysis and 3D-plot of right vectors from the binary data was performed to graphically summarize associations among the varieties. Analysis was done through a batch file following the software package NTSYS-pc.

The POPGENE freeware (Yeh *et al.*, 1997) was used to partition genetic diversity among the twenty six populations. The same software was used to calculate genetic distances and similarities between populations and to draw a dendrogram based on Nei's (1972) genetic distances using the UPGMA to show the genetic relations between the different cultivars. Graphical representation of the results was done on the basis of these studies. Shannon's index was also calculated, the degree of polymorphism among the Tea varieties collected from various regions of North Bengal was calculated for each primer from the binary data matrix using Shannon's index of phenotypic diversity from the following equations as mentioned by Pattanayak *et al.*, 2002).

$H_0 = - \Sigma P_i \ln P_i$ ——————— (1)

Where P_i is the frequency of phenotype I and H_o is genetic diversity within the tea varieties detected by a particular primer.

$H_{pop} = 1/2 \Sigma [H_{0(hill)} + H_{0(plane)}]$ ———(2)

Where H_{pop} is average diversity in different varieties of tea revealed by a particular primer.

$H_{sp} = - \Sigma P_i \ln Pi$ ——————— (3)

Where H_{sp} is diversity in all the 26 varieties considered for a particular primer.

H_0, H_{pop} and $_{Hsp}$ were calculated for all the primers and the average estimate over 34 primers was calculated. Proportion of diversity present within populations, H_{pop} / H_{sp}, was compared with that between populations, $(H_{sp}-H_{pop}) / H_{sp}$. The result was given in a tabular as well as graphical form.

3.2.5. PCR-RFLP (Restriction Fragment Length Polymorphism) analysis of Tea (Camellia sinensis)

3.2.5.1. Primer used for trnL-trnF ("Taberlet") region (Taberlet et al., 1991) of the tea genome

Tab c-f in *trn*L-*trn*F ("Taberlet") region of the tea genome was amplified. The primer sequence Tab c 5'—CGAAATCGGT AGACGCTACG—3' and Tab f* 5'—ATTTGAACTGGTGACACGAG—3' was used on the basis of the known sequence from the Taberlet region of the other plant species. A schematic representation of the primer location is shown in Fig. 3.2.The primers were synthesized by SIGMA Aldrich.

3.2.5.2. PCR-RFLP (Restriction Fragment Length Polymorphism) Amplification

- The polymerase chain reaction was carried out in a 25µl volume containing the following components:
  Pyrogen free water To a final volume of 25µl
  Buffer 10X 2.5µl
  dNTPs mix 0.5µl (200µM) each
  Primers 1.25µl (0.25µM) each
  Taq DNA polymerase 1 unit
  Template DNA 1µl (25ng)
- One negative control tube was prepared.

PCR mix without DNA.

- The PCR reactions were performed on a Perkin-Elmer Thermocycler (Genei, Model No. 9600). The amplification cycle consisted of the following specifications:
  Cycle 1: Denaturation at 94°C for 5min, Primer annealing at 54°C for 1min., Primer extension at 72°C for 1min.
  Cycle 2-34: Denaturation at 94°C for 1min., Primer annealing at 54°C for 1min., Primer extension at 72°C for 1min.
  Cycle 35: Denaturation at 94°C for 1min., Primer annealing at 54°C for 1min., Primer extension at 72°C for 5 min.
- The PCR products were separated on 1.5% (W/V) agarose gel run in 1X TBE buffer (pH-8.0)
- Gel loading dye 6X (5µl) was mixed with 10µl of PCR product.
- The samples were loaded and electrophoresis was carried out at 50V for 1hr
- The gel was stained with Ethidium bromide solution (0.5µg/ml).
- The gels were visualized with a UV transilluminator (Genei, Cat#107161) and photographed with Gel Documentation System (Vilber Lourmat France, Model No. DP-001.FDC). A DNA ladder (1 KB ladder) (Genei, Cat#105998) was used as a molecular size marker.

3.2.5.3. PCR-RFLP product Restriction digestion

The PCR products were subjected to restriction digestion with 5 different restriction endonucleases, namely *AluI, TaqI, HinfI, MboI, MspI* all 4-base cutters. The list of the enzymes along with their cutting sites and other informations are given in a tabular form (Table 3.3)

Protocol:

- The restriction digestion reaction was done in total of 20µl volume containing the following components for each sample:
- Restriction Enzyme buffer 2µl, Refer Appendix II for composition.
- Sterile water To make up the volume
- Enzyme 5 units.
- The restriction digestion mix was spin for a moment.
- The mix was then incubated at 37°C for 1 hr in a Dry water bath.
- After 1 hr the restriction digestion mix product was separated on a 2% (W/V) agarose gel and run in 1X TBE buffer (pH-8.0).
- 5. Gel loading dye 6X (5µl) was mixed with 20µl of Restriction digestion product.
- The samples were loaded and electrophoresis was carried out at 50V for 2hrs.
- The gel was stained with Ethidium bromide solution (0.5µg/ml).
- The gels were visualized with a UV transilluminator (Genei make, Cat#107161) and

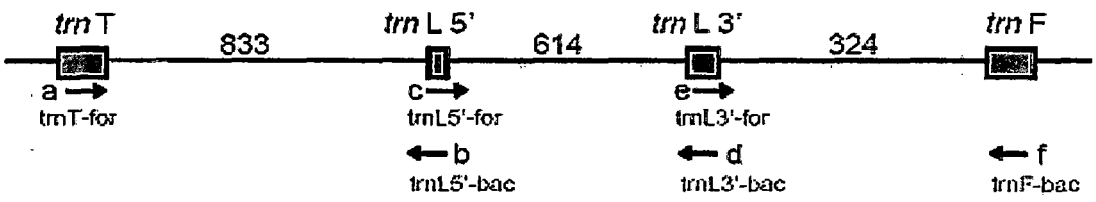


Fig. 3.2 Diagram showing the amplified Tab c-f (938bp) in trnL-trnF region of tea genome.

Table 3.3 Restriction Enzymes showing their respective cutting sites

Restriction Enzymes	Cutting Site	Temperature	Units
AluI	AG↓CT	37°C	5
TaqI	T↓CGA	65°C	5
HinfI	G↓ANTC	37°C	5
MboI	↓GATC	37°C	5
MspI	C↓CGG	37°C	5

photographed with Gel Documentation System (Vilber Lourmat France, Model No. DP-001.FDC). A DNA ladder (1 KB ladder) (Genei, Cat#105998) was used as a molecular size marker.

3.2.5.4. PCR-RFLP Data analysis

The markers were scored as diploid data. Each polymorphic band was regarded as a binary character and was scored as 1 (presence) or 0 (absence) for each sample and assembled in a data matrix. A similarity matrix on the basis of band sharing was calculated from the binary data using Dice coefficient (Nei & Li 1979). Similarities were graphically expressed using the group average agglomerative clustering to generate dendrograms. The analysis was done using the software package NTSYSpc (version 2.0) (Rohlf, 1998)

Correspondence analysis and 3D-plot of right vectors from the binary data was performed to graphically summarize associations among the varieties. Analysis was done through a batch file following the software package NTSYS-pc.

The POPGENE freeware (Yeh *et al.*, 1997) was used to partition genetic diversity among the twenty six populations. The same software was used to calculate genetic distances and similarities between populations and to draw a dendrogram based on Nei's (1972) genetic distances using the UPGMA to show the genetic relations between the different cultivars. Graphical representation of the results was done on the basis of these studies. Shannon's index was also calculated, the degree of polymorphism among the Tea varieties collected from various regions of North Bengal was calculated for each primer from the binary data matrix using Shannon's index of phenotypic diversity from the following equations as mentioned by Pattanayak *et al.*, 2002).

$H_0 = - \Sigma P_i \ln$ ——————————(1)

Where P_i is the frequency of phenotype I and H_o is genetic diversity within the tea varieties detected by a particular primer.

$H_{pop} = 1/2 \Sigma [H_{0(hill)} + H_{0(plane)}$ ——————(2)

Where H_{pop} is average diversity in different varieties of tea revealed by a particular primer.

$H_{sp} = - \Sigma P_i \ln$ ——————————(3)

Where H_{sp} is diversity in all the 26 varieties considered for a particular primer.

H_0, H_{pop} and H_{sp} were calculated for all the primers and the average estimate over 34 primers was calculated. Proportion of diversity present within populations, H_{pop} / H_{sp}, was compared with that between populations, $(H_{sp}-H_{pop}) / H_{sp}$. The result was given in a tabular as well as graphical form.

3.2.6. Sequencing of PCR-RFLP amplification products

To confirm the identity of the PCR bands generated by the primer pair, the corresponding amplification products from ten cultivars of tea showing good amount of polymorphism (Table 3.4) were purified at first using SIGMA PCR Clean-up kit (Refer Ap-

pendix II for composition) and the purified products were sent for sequencing to Bangalore Genei, India.

3.2.6.1. Purification of 10 PCR products for sequencing

Procedure

- A GenElute Miniprep Binding Column (with a blue o-ring) into a provided collection tube, if not already assembled. 0.5ml of column preparation solution was added to each miniprep column and centrifuged at 12000Xg for 1minute. The flow through liquid was discarded.
- Binding solution 5volumes was added to 1 volume of the PCR reaction and mixed. The solution was then transferred into the binding column. The column was centrifuged at maximum speed (12000-16000Xg) for 1 minute. The flow through liquid was discarded, but the collection tube was retained.
- The binding column was replaced into the collection tube. To the column 0.5ml of diluted wash solution was applied and centrifuged at maximum speed for 1 minute. Flow through liquid was discarded and the collection tube was retained.
- The column was replaced into the collection tube. The column was centrifuged at maximum speed for 2 minutes, without any additional wash solution, to remove excess ethanol. Any residual flow through was discarded along with the collection tube.
- The column was transferred to a fresh 2ml collection tube. Elution solution 50µl was applied to the center of each column. Incubated at room temperature for 1 minute.
- DNA was eluted by centrifuging the column at the maximum speed for 1 minute. The PCR amplification product was thus present in the eluate and ready to be used immediately or storage at -20°C.
- The eluate thus containing the purified PCR products were sent to Bangalore Genei for sequencing.

3.2.6.2. Sequence analysis and GenBank submission

Multiple sequence alignment was performed using Clustal W (Thompson *et al.* 1994). A cladogram was constructed with Phylip and visualized with the help of Treeview software (Phylogeny inference package) version 3.2 (Felsenstein 1989). From this cladogram the similarity and dissimilarity be-

Table 3.4 List of cultivars sequenced

Sl No.	Cultivars	Species	Type	Origin
1	TV29	*C.assamica*sub.Lasiocalyx	Cambod	Tocklai ExperimentalStation
2	TV22	*C.assamica*sub.Lasiocalyx	Cambod	Tocklai ExperimentalStation
3	BS71A76	*C.sinensis* (L.)O.Kuntze	China	Darjeeling
4	K-1/1	*C.assamica*	Assam	Darjeeling
5	TV27	*C.assamica*sub.Lasiocalyx	Cambod	Tocklai ExperimentalStation
6	B777	*C.sinensis (L.)O.Kuntze*	China	Darjeeling
7	TV25	*C.assamica*sub.Lasiocalyx	Cambod	Tocklai ExperimentalStation
8	CP1	*C.assamica*	Assam	Darjeeling
9	P312	*C.sinensis* (L.)O.Kuntze	China	Darjeeling
10	B157	*C.sinensis* (L.)O.Kuntze	China	Darjeeling

tween the ten cultivars of tea were observed and the results were compared with RFLP data analysis done by POPGENE software.

The raw sequences of all ten cultivars were documented with the help of Sequin Application Version 6.25 Standard Release [May19 2006] for Database submission to GenBank providing necessary informations like, size of the DNA, definition of the sequence (i.e., the specific region of the genome), source of the sequence (*Camellia sinensis* chloroplast DNA in this case; name of the organism along with its taxonomic position etc.).

3.2.7. Amplification of rbcL-ORF106, chloroplast DNA (all of rbcL plus spacer between it and ORF106 exon)

A RFLP study was done for rbcl gene (Chloroplast DNA) of tea. The primer sequence *rbc*L 5'-ATGTCACCACAAAC AGAAACTAAGCAAGT-3'and ORF106*5'-ACTACAGATC TCATACTACCCC-3' for amplifying the Chloroplast DNA was obtained on the basis of the known sequence based on Sequencer restriction maps from GenBank Solanaceae sequences. The primers were synthesized by Sigma Aldrich. (USA).

3.2.7.1. PCR Amplification

- The polymerase chain reaction was carried out in a 25µl volume containing the following components:
- Pyrogen free water To a final volume of 25µl
- Buffer 10X 2.5µl (Refer Appendix II for composition)
- dNTPs mix 0.5µl (200µM) each (Finnzymes, Cat#F-560L)
- Primers 1.25µl (0.25µM) each
- Taq DNA polymerase 1unit (Finnzymes, Cat#F-501L).
- Template DNA 1µl (25ng)
- One negative control tube was prepared. PCR mix without DNA.
- The PCR reactions were performed on a Perkin-Elmer Thermocycler (Genei, Model No. 9600). The amplification cycle consisted of the following specifications:
- Cycle 1: Denaturation at 94°C for 5min, Primer annealing at 50°C for 1min., Primer extension at 72°C for 1min.
- Cycle 2-34: Denaturation at 94°C for 1min., Primer annealing at 50°C for 1min., Primer extension at 72°C for 1min.
- Cycle 35: Denaturation at 94°C for 1min., Primer annealing at 50°C for 1min., Primer extension at 72°C for 5 min.
- The PCR products were separated on 1.5% (W/V) agarose gel run in 1X TBE buffer (pH-8.0)
- Gel loading dye 6x (5µl) was mixed with 10µl of PCR product.
- The samples were loaded and electrophoresis was carried out at 50V for 1hr
- The gel was stained with Ethidium bromide solution (0.5µg/ml).
- The gels were visualized with a UV transilluminator (Genei, Cat#107161) and photographed with (Vilber Lourmat France, DP-001.FDC). A DNA ladder (1 KB ladder) (Genei, Cat#105998) was used as a molecular size marker.

3.2.7.2. PCR product Restriction digestion and analysis

The PCR products were subjected to restriction digestion with 3 different restriction endonucleases, namely *AluI, HinfI, HhaI* given

in tabular form (Table 3.5).

Protocol

- The restriction digestion reaction was done in total of 20µl volume containing the following components for each sample:
- Restriction Enzyme buffer 2µl
- Sterile water To make up the volume
- Enzyme 5 units
- The restriction digestion mix was spin for a moment.
- The mix was then incubated at 37°C in a Dry bath (Genei, Cat#107173) for 1 hr.
- After 1 hr the restriction digestion mix product was separated on a 2% (W/V) agarose gel run in 1X TBE buffer (pH-8.0)
- 5. Restriction digestion product (20µl) was mixed with 5µl of 6X Gel loading dye.
- 6. The samples were loaded and electrophoresis was carried out at 50V for 2hrs
- 7. The gel was stained with Ethidium bromide solution (0.5µg/ml).
- 8. The gels were visualized with a UV transilluminator (Genei, Cat#107161) and photographed with Gel Documentation System (Vilber Lourmat France, Model No. DP-001.FDC). A DNA ladder (lambda DNA *HindIII* digest) (Genei, Cat#106000) was used as a molecular size marker.

3.2.8. Microsatellite Markers study

3.2.8.1. Primers Used

Microsatellite markers were developed by Ueno *et al.*, 1999 for *Camellia japonica* by screening random amplified polymorphic DNA (RAPD) profiles to avoid time consuming screening of genomic libraries. The objective was to analyse seed dispersal and parentage of *C. japonica* (factors expected to have strong influences on genetic structure of the species) in an old growth on Tsushima Island, Nagasaki (Ueno *et al* 1999). Cross-species amplification with the three microsatellite primers showed that these primer pairs worked well for *Camellia sinensis*. Therefore, the present study was conducted using these 3 microsatellite primer pairs (Table 3.6) for genotyping 26 cultivars of *Camellia sinensis* found in North Bengal.

3.2.8.2. PCR- Amplification

- The polymerase chain reaction was carried out in a 25µl volume containing the following components:
- Pyrogen free water To a final volume of 25µl
- Buffer 10X 2.5µl (Refer Appendix II for composition)
- dNTPs mix 0.5µl (200µM) each (Finnzymes, Cat#F-560L)
- Primers 1.25µl (0.25µM) each
- Taq DNA polymerase 1unit (Finnzymes, Cat#F-501L).
- Template DNA 1µl (25ng)
- One negative control tube was prepared. PCR mix without DNA.
- The PCR reactions were performed on a Perkin-Elmer Thermocycler (Genei, Model No.9600). The amplification cycle consisted of the following specifications:
- Cycle 1: Denaturation at 94°C for 5min, Primer annealing at 60°C for 1min., Primer

Table 3.5 Restriction Enzymes showing their respective cutting sites

Resriction Enzymes	Restriction Site	Temperature	Units
AluI	AG↓CT	37°C	5
HinfI	G↓ANTC	37°C	5
HhaI	GC↓GC	37°C	5

extension at 72°C for 1min.

- Cycle 2-34: Denaturation at 94°C for 1min., Primer annealing at 60°C for 1min., Primer extension at 72°C for 1min.
- Cycle 35; Denaturation at 94°C for 1min., Primer annealing at 60°C for 1min., Primer extension at 72°C for 5 min.
- The PCR products were separated on 1.5% (W/V) agarose gel run in 1X TBE buffer (pH-8.0)
- PCR product (10µl) was mixed with 5µl of 6X Gel loading dye.
- The samples were loaded and electrophoresis was carried out at 50V for 1hr
- The gel was stained with Ethidium bromide solution (0.5µg/ml).
- The gels were visualized with a UV transilluminator (Genei, Cat#107161) and photographed with Gel Documentation System (Vilber Lourmat France, Model No. DP-001.FDC). A DNA ladder (1 KB ladder) (Genei, Cat#105998) was used as a molecular size marker.

3.3. *In vitro* culture studies

In vitro culture study was done prior to Genetic transformation of tea to standardize the protocol for developing an *in vitro* grown whole plantlet by using different explants and various hormone combinations in MS medium (Murashige and Skooge medium) Hi-media, Cat#PT0018 (Refer Appendix I for composition). The photographs were taken for all stages of *in vitro* grown plantlets of tea with (Canon Camera, Model No. EOS35OD).

3.3.1. Establishment of callus cultures

- Green, healthy stems were collected from greenhouse grown seedlings (var. *sinensis,* cultivar TV-26) with three or four leaves. Leaves were detached from the stems.
- Stems were washed in 2% Extran (E Merck India Cat#MA02) for 15 minutes and then rinsed several times with double distilled water.
- The stems were then surface sterilized with 70% alcohol for 5 minutes and then with 0.1% $HgCl_2$ (E Merck India Cat#17524)
- The stems were washed several times with sterile water and blot dried with sterile tissue paper.
- The stems were taken in a petriplate containing MSO (MS medium without hormones) (Hi-media, Cat#PT0018) and the segments of size 5-6mm were sampled from the first, second and third nodes of seedlings.
- The epidermal layers were stripped off with a sterile microscalpel.
- Three types of explants were inoculated:

Table 3.6 Characteristics of three polymorphic microsatellite loci in *Camellia sinensis*. Microsatellite motifs are sequenced alleles. 26 cultivars of tea were genotyped. GenBank Accession numbers are listed under their respective locus.

Locus	Motif	Allele Size(bp)	Sequence (5'-3')	Annealing temp. (°C)
MSCja38 AB016190	$(GA)_{14}$	348-378	F:GCTGAGCTTGGAGATTTTGTT R:CCTATTGCCTACGACCATTTC	55
MSCjaF37 AB016191	$(AG)_{13}(GAA)_7$	335-367	F:CGAGCCTTCCCTTTTCCCATTC R:CGCTCGACGTAATGCCACACT	60
MSCjaH46 AB016192	$(GA)_{16}$	444-464	F:CATCGTCCTAATCCACTTCAC R:AGAGAGCATTATGAGTCGTCT	60

epidermal layers, intact stem segments (stem segments) and without epidermal layers (stripped segments). These explants were blot dried and inoculated on callus induction medium (Table 3.7) containing 1% agar (Hi-media Cat#RM201), 100mg/l Myo-inositol (SIGMA, Cat#I5125), 30gm sucrose (Hi-media, Cat#RM1158) and 0.332mg/l $CaCl_2$ (Hi-media, Cat#MB034) with pH of 5.6.

3.3.2. Regeneration of plants from callus tissues

- Callus tissues were cultured on the callus induction medium (Table 3.7) for two months till formation of embryos in it and thereafter the embryogenic calli were transferred to MS medium containing 0.5mg/l IBA (SIGMA, Cat#I5386) and 10mg/l BA (SIGMA, Cat#B-3408) with pH of 5.6 for shooting.
- The shoot buds from embryogenic calli were transferred to the semi liquid rooting medium (1/2 MS+0.5mg/l IBA), (1/2 MS+1mg/l IBA) and (1/2 MS+1μM TDZ SIGMA, Cat#P-6186) containing 0.5% agar and pH of 5.6 after 2 months.
- After 6 weeks in rooting medium the plantlet was transferred to the pot containing a mixture of vermiculite and soil (1:1).
- After 4 weeks the plantlets were transferred to the Greenhouse in Sikkim Council of Science and Technology for a cooler atmosphere as per its requirement.

3.3.3. Formation of Somatic embryos and whole plantlet from cotyledon explants on Murashige and Skooge medium containing various hormones

- The mature green fruits of *Camellia* (cultivar TV26) were collected and their fruit coat was removed to expose the seeds containing the two large cotyledons.
- The seeds were washed with 2% Extran for 15 minutes and then rinsed several times with double distilled water.
- The seeds were surface sterilized with 70% alcohol for 5 minutes and then with 0.1% $HgCl_2$ for 7 minutes.
- The seeds were rinsed properly with autoclaved distilled water and blot dried on sterile tissue papers.
- The seeds were then cut open to take out the cotyledons.
- The cotyledons were then cut into 4-5 pieces and the embryo axis was removed from it.
- The cotyledon pieces were inoculated on MS medium (pH 5.6) containing four different hormone compositions. (Table 3.8)
- After 15 days embryos developed on the swollen portion of the cotyledons.
- These were then sub cultured on to the same medium and histological studies were performed on the 15-30 days old cotyledons producing somatic embryos which were maintained by regular transfer onto fresh medium.
- After 1 month, Somatic embryos produced were, subcultured on same medium for the formation of shoots till 2 months.
- The green shoots thus produced after 2 months of inoculation were transferred to the semi liquid rooting medium (1/2MS+0.5mg/l IBA), (1/2MS+1mg/l IBA) and (1/2MS+1μMTDZ) containing 0.5% agar with pH of 5.6..
- After 6 weeks in rooting medium the plant-

let was transferred to the pot containing a mixture of vermiculite and soil (1:1).

- After 4 weeks the plantlets were transferred to the Greenhouse in Sikkim Council of Science and Technology for a cooler atmosphere as per its requirement.

3.4. Histological observations of the cultured cotyledons

- The swollen cotyledons cultured for 15 days, 1month and 2 month were taken for preparation of the sections which were cut both transversely and longitudinally.
- Sections were dehydrated in graded Ethanol solutions (30 to 95%) and stained with Safranin (Hi-media, Cat#RM1315) and light green (Hi-media, Cat#RM386) (Santra *et al.*, 1989).
    a. The tissues were dipped in 30% alcohol for 5 minutes.
    b. Dipped in 1% solution of Safranin (in 50% alcohol) for 30 minutes.
    c. Dehydrated in 70%, 80%, and 90% alcohol, keeping 5 minutes in each.
    d. Counterstained in 1% solution of Light green (in 95% alcohol) for 1 minute.
    e. Dehydrated in absolute alcohol (E Merck India, Cat#101076HBD) for 5 minutes.
    f. Passed through alcohol xylol (1:1) mixture keeping the sections in it for 5 minutes.
    g. Cleared in xylol (Hi-media, Cat#RM1877) for atleast 10 minutes.
    h. Mounted in DPX (Hi-media, Cat#RM655) and dried.
- Sections both transversely cut and longitudinally cut were than observed under phase contrast microscope and photographed (Olympus Phase Contrast Microscope, Model No.CH-20i).

3.5. Genetic transformation study

Trial of different gene transfer methods to evaluate the suitability of their use in tea

3.5.1. Agrobacterium mediated transformation of Tea

3.5.1.1. Bacterial Strains and vector used

Agrobacterium tumefaciens strain LBA4404, harbouring binary vector pCAMBIA-2301 was used in the transformation experiments which was kindly provided by Dr. Sampa Das of Bose Instititute, kolkata. The constructs contain the *uid*A gene (interrupted by catalase intron), encoding the enzyme glucuronidase, under the control of the CaMV35S promoter and the *nos* terminator. Plasmid pCAMBIA-2301 has the neomycin phosphotransferase II (*npt* II) gene, driven by the CaMV35S promoter and terminated by the CaMV35S polyA sequence. (Fig.3.3). For routine use, bacterial cultures were grown in the liquid YEM medium. Refer Appendix I for composition, supplemented with kanamycin monosulphate (SIGMA, Cat#K4378) 50mg/

Table 3.7 Stem explants (TV-26) inoculated in induction medium containing various hormones

Growth regulator mg/l	Epidermal layers	Stem segments	Stripped Segments
Kinetin (0.2)+NAA (2)	100	100	100
IBA (2)+BA (4)	100	100	100
BA(0.5)+NAA(0.1)+GA_3(0.1)	100	100	100
BA (4)+IBA (2)	100	100	100

l and Rifampicin 75mg/l for 2 days in a Cooling Orbital Shaker (REMI make, Model No. CIS-24BL) at 28 ± 2°C (150 rpm).

3.5.1.2. Optimization of parameters required for genetic transformation of Somatic embryos from cotyledonary explants used in the present study

- Determination of phytotoxic levels of selective and bactericidal antibiotics

  Somatic embryos formed from cotyledon pieces (Table 3.8) were inoculated in MS-104 medium (Refer Appendix I for composition) containing MS+ +0.5mg/lIBA+10mg/lBA augmented with different levels of kanamycin monosulfate (25, 30, 35, 40, 45, 50, 60, 65 and 100mg/l) (SIGMA, Cat#K-4378). In another study, two bactericidal antibiotics, namely Cephalexin (Sporidex; Ranbaxy India) and Cefotaxime (Hi-media, Cat#RM1193) were tested, each at different levels (200, 300, 400, 600, 800 and 1,000mg/l. All antibiotics were filter sterilized and added to the autoclaved medium after the latter had been cooled to 45°C prior to solidification. Each experiment had three replicates, each containing ten somatic embryos each. All experiments were repeated three times and subculturing was carried out in the same medium at regular intervals of 15 days until the tissue became necrotic. Data were recorded with respect to the increase or decrease in the fresh weight of the embryos, together with the extent of the necrosis.

2. Determination of bacterial growth phase (OD values)

   Somatic embryos formed from cotyledon pieces (Table 3.8) were inoculated in MS-104 medium containing MS+0.5mg/l IBA+10mg/l BA inoculated with *Agrobacterium* strain (LBA4404) having different growth phase (OD values of 0.4, 0.6. 0.8, 1.0 at 600nm) by a Colorimeter (Monozyme make, Model No. innova-1000).

3. Determination of wounding level of embryogenic tissues by ultra sonication

   Somatic embryos formed from cotyledon pieces (Table 3.8) were placed in a 1.5ml microcentrifuge tube (Tarsons, Cat#500010) along with 0.5ml of the *Agrobacterium* suspension (LBA4404). Tissues were SAAT (Sonication Assisted *Agrobacterium*-mediated Transformation) treated for different timings (0.5second, 1.0second, 1.5second and 2seconds) in an Ultra Sonicator (Branson make) using the method of Finer and Trick, 1997.

4. Determination of length of co-cultivation period

   Somatic embryos formed from cotyledon pieces (Table 3.8) were inoculated with *Agrobacterium* strain (LBA4404) having different lengths of co-cultivation period (2, 3, 4, 5, 6, 7 days).

5. Determination of pH of co-cultivation

Table 3.8 Cotyledonary explants (TV-26) inoculated in embryogenesis medium containing various hormones

Growth regulators	Cotyledon Slices inoculated
BA (10mg/l)+IBA (0.5mg/l)	100
BA(0.5mg/l)+NAA(0.1mg/l)+GA(0.1mg/l)	100
BA (2mg/l)+ IBA (4mg/l)	100
TDZ (1μM)	100

medium

Somatic embryos formed from cotyledon pieces (Table 3.8) were inoculated with *Agrobacterium* strain (LBA4404) having different pH (5.4, 5.5, 5.6, 5.7, 5.8, 5.9) of the co-cultivation medium (Liquid MS-104 medium containing MS+0.5mg/l IBA+10mg/l BA.

6. Determination of acetosyringone concentration

Somatic embryos formed from cotyledon pieces (Table 3.8) were augmented with different concentration of acetosyringone in co-cultivation medium (Liquid MS-104 medium containing MS+0.5mg/l IBA+10mg/l BA) containing *Agrobacterium* strain LBA4404 (10, 30, 40, 50, 60µM). The phenolic compound acetosyringone (Fluka, Cat#38766) was dissolved in ethanol, and the stock volume made up in autoclaved distilled water. The appropriate aliquot of the filter-sterilized stock solution was added to autoclaved co-cultivation medium (Liquid MS-104 medium containing MS+0.5mg/l IBA+10mg/l BA containing *Agrobacterium* strain LBA4404) to make the required final concentration.

3.5.1.3. Transformation protocol

- Single bacterial colonies of *Agrobacterium* containing the plasmid were prepared by serial dilutions, and each colony was then inoculated in liquid YEM medium (20ml) (Refer Appendix I for composition) containing 50mg/l Kanamycin (SIGMA, Cat#K-4378).
- The bacteria were then allowed to grow in the dark at 28°C for 16-18 hrs at 180 rpm in a Cooling Orbital Shaker (REMI make, Model No.CIS-24BL)
- Bacterial cells corresponding to OD_{600}=0.6 were pelleted by centrifugation (6000Xg,

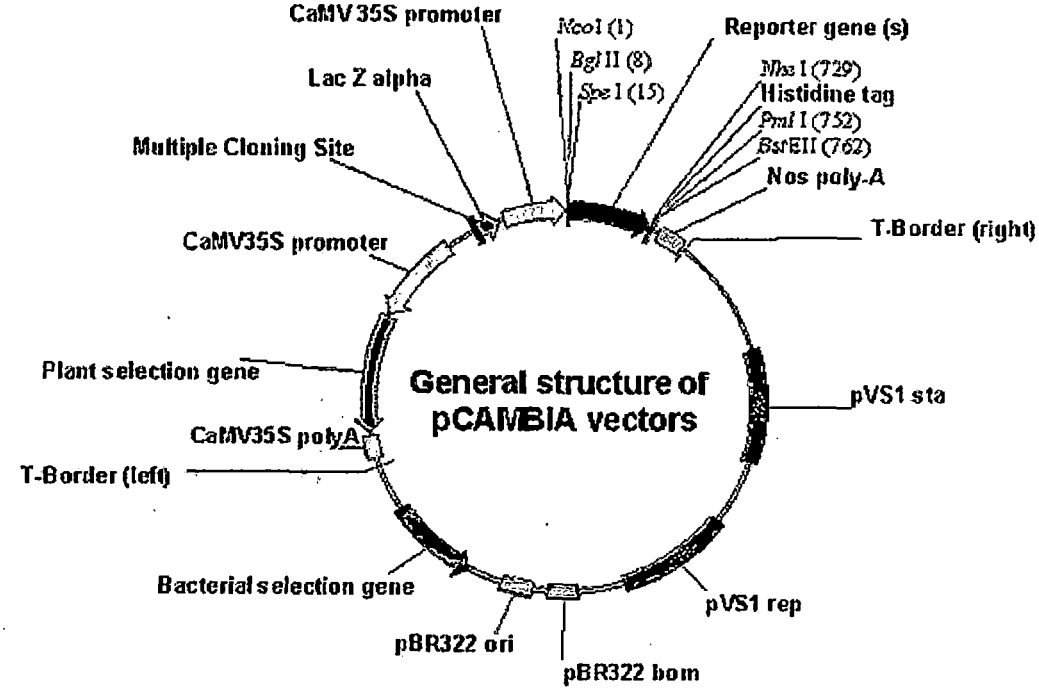


Fig. 3.3 Map showing general structure of pCAMBIA vector used in transformation study

10min.) followed by washing twice with liquid YEM.

- The final cell density was adjusted to 10^9 cells/ml with liquid MS-104 (See Appendix I for composition) medium containing 0.5mg/l IBA+10mg/l BA
- Somatic embryos formed from cotyledon pieces (Table 3.8 at the end of this chapter) were placed in a 1.5ml microcentrifuge tube along with 0.5ml of the *Agrobacterium* suspension and few of them were SAAT (Sonication Assisted *Agrobacterium*-mediated Transformation) treated.
- Tissues were SAAT treated in an Ultrasonicator (BRANSON make) for 2 seconds.
- After 20 minutes the excess *Agrobacterium* was blot dried from SAAT treated and untreated tissues on sterile filter paper and were then placed upside down in petriplate containing MS 104 medium (MS+BA-10mg/ml+IBA-0.5mg/ml) sealed with parafilm (Hi-media, Cat#LA045) and incubated for two days in dark at 28°C in an incubator (REMI make, Model No.CIS-24BL) (Binns *et al.* 1992).
- After two days the plant tissues were washed several times with sterile distilled water and then with liquid MSO (See Appendix I for composition) medium containing Cefotaxime (300mg/ml) to remove the *Agrobacterium* adhering to the surface of tissue and blot dried with sterilized filter papers.
- The tissues were then incubated for 2-4 days on a nonselective MS-104 (Solid) medium containing 300mg/ml cefotaxime (See Appendix I for composition).
- After 2-4 days tissues were transferred to selective medium MS 104 containing 50mg/ml Kanamycin and 300mg/ml cefotaxime (See Appendix I for composition) and incubated in culture room at 25°±2°C under a 12/12h (day/night) photoperiod with light provided by cool fluorescent tubes at a photon flux density of 52μmol $m^{-2}s^{-1}$.
- After 8 week of culture period tissues were transferred to MS-104 medium containing MS+0.5mg/lIBA+10mg/lBA augmented with an elevated level of Kanamycin (75mg/l).
- After about 3weeks-4weeks many embryos appeared on the surface of the cotyledon pieces which transformed into green shoots.
- Control explants (non-transformed with *Agrobacterium)* were also cultivated on the selective medium which should bleach.
- The green shoots were maintained on MSO (MS medium without hormones) medium with antibiotics (Kanamycin 75mg/ml) for 8-12 weeks.
- For rooting the green shoots were transferred to a semi liquid (0.5% agar) ½ MS-medium containing 1% sucrose and 0.5 mg/ml IBA.
- After 6 weeks the plantlets showing good amount of rooting were transferred for hardening in pots containing mixture of vermiculite and soil (1:1).
- After 4 weeks the plantlets were transferred to the Greenhouse in Sikkim Council of Science and Technology for a cooler atmosphere as per its requirement.

3.5.2. Particle Bombardment- Mediated Transformation of tea

The ultimate goal of plant genetic engineering is to produce novel transgenic plants with improved agronomic traits. The production of transgenic plants depends among other factors on the stable introduction of foreign gene into the plant genome. A large number of gene transfer methods are currently available. However, the method of gene transfer using the particle Delivery System (PDS/1000He, Biorad, Inc., USA) is the most versatile (Christou, 1996) and has proven successful in introducing foreign genes not only to the nucleus but also to the chloroplasts in plants (Svab & Maliga, 1993). A number of parameters have been standardized in this experiment involving transformation through particle bombardment in callus and leaf tissue of tea. The experiment was performed in the National Research Centre on Plant Biotechnology, Indian Agriculture Research Institute, PUSA, New Delhi-110012, India.

3.5.2.1. Bacterial Strain and vector

pZP200KC gene used in this experiment was engineered for nuclear expression from the 35S promoter (Fig. 3.4) and kindly provided by Dr. K.C. Bansal of IARI, New Delhi. For routine use, strains of pZP200KC were grown at 37°C in agar solidified LB medium See Appendix I for composition, supplemented with 50mg/l Spectinomycin (Sigma Cat#S-9007).

3.5.2.2. Plasmid Isolation from the strain pZP200KC using SIGMA miniprep isolation kit according to the instructions manual

- Single bacterial colony of pZP200KC containing the plasmid was inoculated in liquid LB medium (20ml) (Refer Appendix I for composition) containing 50mg/l Spectinomycin.
- The bacteria were then allowed to grow at 37°C overnight in an Orbital Shaker (REMI make, Model No.RIS-24BL).
- The overnight grown culture (1.5ml) was pelleted at =12000xg for 1 minute. The supernatant was discarded.
- The bacterial pellet was completely resuspended in 200µl of resuspension solution. The cells were then vortexed and thoroughly resuspended in the solution.
- The resuspended cells were lysed by adding 200µl of the lysis solution. The contents were then immediately mixed by gentle inversion (6-8 times) until the mixture becomes clear and viscous. Keep it for 5 minutes in room temperature.
- The cell debris was precipitated by adding 350µl of the Neutralization/Binding solution. The tube was gently inverted 4-6 times. The cell debris was pelleted by centrifuging at 12000xg for 10 minutes.
- To the miniprep column 500µl of column preparation solution was added and centrifuged at 12000xg for 1 minute. The flow through liquid was discarded and column was kept.
- To the column cleared lysate prepared in step 6 was loaded and centrifuged at 12000xg for 1 minute. The flow through liquid was again discarded.
- To the column 750µl of diluted wash solution was added and centrifuged at 12000 x g for 1 minute. The flow through liquid was discarded and the centrifugation step was repeated without adding any more wash solution.
- The column was transferred to a fresh col-

lection tube. To it 100µl of Elution solution was added and centrifuged at 12000 x g for 1 minute. The eluate containing the plasmid was stored at -20°C for further use.

3.5.2.3. Transformation Protocol (Christou 1996)

A. Preparation of Microprojectiles

- Tungsten powder (50mg) (BioRad Tungsten M-17) was used for 100 shots.
- Freshly opened absolute alcohol (1ml) was added.
- Centrifuged for 10 secs. in a microcentrifuge tube.
- Replaced with 1ml of absolute ethanol and centrifuged for 10 secs.
- Ethanol was removed. 1ml water was added, tungsten was resuspended and centrifuged. The step was repeated thrice.
- Finally tungsten particles resuspended in 1ml of water.
- Preparation of samples for 6 shots: 50µl tungsten suspension
- (Important: added in order)-
- pZP200KC plasmid DNA (10µl) in TE buffer (atleast 1µg/µl)
- 2.5M $CaCl_2$ (50µl) (Hi-media, Cat# MB034)
- 0.1M Spermidine (20µl) (free base) (SIGMA, Cat#S9389)
- Ethanol (200µl) was added.
- Centrifuged for 10 secs in a microcentrifuge tube.
- Supernatant removed. 200µl ethanol added and resuspended well, making sure that all clumps are dispersed. Repeated thrice.
- Final pellet resuspended in 30µl ethanol.
- Taken 5µl on to each disk for shooting.

B. Preparation of callus and leaf tissues from *in vitro* grown tea plantlets for shooting

Several pieces of 15-20 days old embryogenic callus formed on MS+Kin.0.2mg/ml+NAA2mg/ml and leaf tissue from *in vitro* grown tea plantlets were placed on the top of a sterile filter disc on MS medium containing sterile plate. Callus and leaf tissues were placed in centre to maximize the surface receiving the shot.

C. Shooting the callus and leaf tissues

For each shot one 1100p.s.i. rupture disc (the small dark brown discs), one flying disc (the large orange discs), and one stopping screen (the wire mesh screens) are required. All were supplied with the BioRad shooting kit.

D. Sterilization Process

- The number of discs and stopping screens needed were sterilized by placing them for 5-10 minutes in a petridish containing absolute alcohol.
- The discs and screens were then dried by standing them up along the side of a sterile petridish and allowing them to air dry under sterile conditions in the tissue culture hood.
- While sterilizing the disposable shooting components, the gun parts were also surface sterilized by wiping with absolute alcohol. Three components that can be removed from the gun vacuum chamber were surface sterilized. Those were the holder for the rupture discs, the stage and holder assembly for the flying discs, and the platform for the callus samples.
- After removing these and wiping with ethanol, the interior of the vaccum chamber and

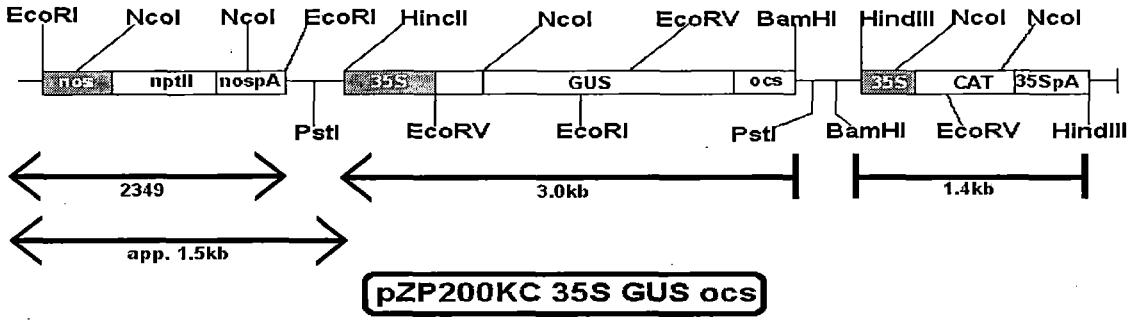


Fig. 3.4 Construct of pZP200KC used in transformation

door were also wiped with absolute ethanol.

- Finally the five ring holders for the flying discs should also be surface sterilized.

3.5.2.4. Ready to shoot

- The gun was turned on (first red button on the left)
- The pump was turned on
- The gas cylinder was turned on. First, screw bar counter was turned clockwise until it feels loose. Then the knob on top of the cylinder counter was turned counterclockwise (the pressure valve on the right should read at least 1300 p.s.i.). Then the screw bar was turned clockwise slowly until the pressure valve on the left reads 1100 p.s.i.
- Five flying disc holders were placed in a petriplate and into each one a flying disc was placed (the large orange ones). The discs were pressed down with the red plastic "capplug", so that they fit snugly into the holders.
- On to the surface of each disc, 5µl of DNA –coated tungsten was pipetted.
- The gun is ready to shot. All the following steps were done in the same order for each shot.
- A rupture disc was placed into the rupture disc holder, using forceps. The disc was properly seated in the holder by tapping the holder a few times gently. The holder was screwed into the vacuum chamber. It was screwed tightly.
- A stopping screen was placed on to the diaphragm in the flying disc holder assembly. On top of this a metal ring was placed upside down containing a flying disc with DNA on it. All the components were screwed securely on the assembly ring. The stage was placed in the second slot from the top in the vacuum chamber.
- In a Petri plate the callus to be shot was placed on the sample platform (lid off!). The sample platform should be positioned in the chamber in the fifth slot from the top (second from the bottom).
- Vacuum button was pressed (middle red button, up position) to begin pumping air out of the shooting chamber. The vacuum pressure was allowed to reach at least 25 inches of Hg. Then the shooting button was pressed continuously (red button on the right, up position). When the pressure reached 1100 p.s.i., the rupture disc inside the gun broke (a pop sound was heard and the Petri plate jumped). The shooting but-

ton was then released and the vacuum was removed by setting the vacuum button to vent (middle position).

3.6. Assay of putative transformants achieved by both types of gene transfer methods

3.6.1. Assay of GUS activity

Transient *GUS* activity of pCAMBIA LBA4404 was detected by characteristic blue spots which appeared on the somatic embryos and leaf tissues 48h following co-cultivation. Also, transient expression of the construct (pZP200KC) was monitored in the callus and leaf tissues, 24-48 hours after shooting, by staining the shot callus and leaf tissue for *GUS* activity (Bansal *et al.*, 1992).The histochemical assay of *GUS* activity shown by both strains were carried with the help of the ß-Glucuronidase (*GUS*) reporter Gene Staining kit (SIGMA, product No. *GUS-S)*

- Stained transformed somatic embryos, embryogenic Callus and leaf tissues from the putative transgenic plants and control plants (Non-transformed tissues) were taken in to two separate 3-5ml clear vials and *GUS* staining solution (See appendix II for composition) was added till the tissues were covered completely.
- The vials were vaccum infiltrated (2minutes) in a vaccum desiccator (Tarsons make, Model No.402010) until bubbles appeared. This step serves to remove air from the plants tissue and facilitate uptake of the staining solution.
- The vials were properly sealed with parafilm (Hi-media, Cat#LA045) incubated at 37°C upto 24 hours. A blue stain develops with time. When expression is high the solution becomes blue due to leakage of the blue reaction product from the tissue.
- The green chlorophyll was removed by destaining the sample with ethyl alcohol. The tissues were left in contact with the ethyl alcohol for 1-3 hours.
- The *GUS* stained tissues (transformants) and unstained tissues (Non-transformants) were then photographed with Camera (Canon, EOS35OD).
- The tissues were then stored in ethyl alcohol.

3.6.2. Isolation of transformed plant DNA

DNA was isolated from young unfolded leaves of putative transformed (By both the methods) and untransformed control plants using the following CTAB procedure (Doyle and Doyle 1987).

- CTAB isolation buffer, 7.5ml (Refer Appendix II for composition) was taken in a 30 ml Oakridge tube (Tarsons, Cat3541040) and preheated at 60°C in a water bath (Genei, Cat#107931).
- Fresh transformed leaf tissues (*In vitro* grown transformed plants by both methods), 1.0 g was taken and grind to fine powder in 60°C CTAB isolation buffer taken in a preheated mortar.
- The samples were incubated at 60°C for 30 minutes with occasional gentle swirling.
- The samples were then extracted once with equal volume of chloroform-Isoamyl alcohol (24:1), mixing gently but thoroughly.
- The samples were spin in a centrifuge (Remi make, Model No. RM12C) at room temperature to concentrate phases at 6000Xg for 10 minutes.

- The aqueous phase was removed with wide bore pipette, and transferred to a clean Oakridge tube, to it 2/3 volumes cold Isopropanol was added, and mixed gently to precipitate nucleic acids.
- The DNA was spooled out with the help of a glass hook and transferred to 10-20 ml of wash buffer (76% EtOH, 10 mM ammonium acetate) for 20 minutes at room temperature.
- The DNA was resuspended in 1ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4).
- RNA was removed by adding RNAase A to a final concentration of 10 *u*g/ml and incubated for 30 min at 37°C in a Dry Bath (Genei make, Cat#107173).
- To the samples ammonium acetate (7.5 M stock, pH 7.7) was added to a final concentration of 2.5 M, and 2 volumes of ice cold absolute alcohol (E-Merck, Cat#101076HBD) was added and gently mixed to precipitate DNA.
- The samples were spin in the centrifuge at 10000 RPM for 30 minutes.
- The pellet obtained was air dried and resuspended in 500µl of TE buffer.

3.6.3. Polymerase chain reaction characterization

The forward and reverse primer (Oligonucleotide) sequences (Sigma) used for PCR amplification of the *nptII* gene were 5'-CCA TCG GCT GCT CTG ATG CCG CCG T-3' and 5'-AAG CGA TAG AAG GCG ATG GC TGC-3' respectively. The primers were so designed as to give amplification products of the internal sequence of the *nptII* gene of 693bp. The DNA isolated from transformed plants by both the methods of transformation was amplified using the *nptII* specific primers for confirmation of the transgene.

- The polymerase chain reaction was carried out in a 25µl volume containing the following components:
- Pyrogen free water To a final volume of 25µl
- 10X buffer 2.5µl (Refer Appendix II for composition)
- dNTPs mix 0.5µl (200µM) each (Finnzymes, Cat#F-560L)
- Primers 2.5 µl (0.5µM) each
- Taq DNA polymerase 1unit (Finnzymes, Cat#F-501L).
- Template DNA (transformed tea DNA) 1µl (50ng)
- One positive control tube each was prepared. PCR mix with plasmid DNA (LBA4404 and pZP200KC).
- One negative control tube was prepared (non transformed tea DNA).
- The PCR reactions were performed on a Perkin-Elmer Thermocycler (Genei make, Model No.9600). The amplification cycle consisted of the following specifications:
- Cycle 1: Denaturation at 94°C for 5min, Primer annealing at 54°C for 1min., Primer extension at 72°C for 1min.
- Cycle 2-34: Denaturation at 94°C for 1min., Primer annealing at 54°C for 1min., Primer extension at 72°C for 1min.
- Cycle 35; Denaturation at 94°C for 1min., Primer annealing at 54°C for 1min., Primer extension at 72°C for 7 min.
- The PCR products were separated on 1.5%.(W/V) agarose gel run in 1X TBE buffer.
- PCR product (10µl) was mixed with 5µl of

6X Gel loading dye.

- The samples were loaded and electrophoresis was carried out at 50V for 1hr.
- The gel was stained with Ethidium bromide solution (0.5μg/ml).
- The gels were visualized with a UV transilluminator (Genei, Cat#107161) and photographed with Gel Documentation System (Vilber Lourmat France, Model No. DP-001.FDC). A DNA ladder (λDNA/ *HindIII* digest) was used as a molecular size marker.

Chapter 4
Results & Discussion

4.1. DNA fingerprinting study

4.1.1. Tea DNA extraction, purification and quantification

4.1.1.1. Tea DNA extraction

Tea DNA was extracted using the method of Dellaporta *et al.*, 1983 and DNA-CTAB step gave a very good network of whitish precipitate of nucleic acid which was used in further experiments after purification. The agarose gel run after extraction showed a bright band of DNA in all the samples.

4.1.1.2. Tea DNA purification

Major contaminants in crude DNA preparation were RNA, protein and polysaccharides. Inclusion of CTAB in DNA extraction buffer helped in elimination of polysaccharides from DNA preparations to a large extent. The RNA was removed by treating the sample with RNase. Extraction with phenol:chloroform following RNase treatment eliminated most of the RNA and proteins from the sample.

4.1.1.3. Tea DNA quantification

Tea DNA was quantified using two methods, through spectrophotometric analysis the DNA showing A_{260}/A_{280} ratio around 1.8 was chosen for further PCR-based methods. Using the method of gel analysis the DNA quality was judged by presence of a single compact band at the corresponding position to λ DNA/ *HindIII* (Genei, Cat#106000) indicating high molecular weight of the DNA. The quantity of the DNA was estimated by comparing the sample DNA with the control by eye judgment.

Tea DNA extraction, purification and quantification steps allowed extraction of sufficient DNA from tea leaves. However, the modification that we have made in incorporating the RNase treatment followed by one more purification step allowed us to obtain substantial amount of sufficiently pure DNA for PCR amplification.

4.1.2. RAPD analysis

4.1.2.1. RAPD PCR product agarose gel analysis

Thirty-four oligonucleotide primers were used

Table 4.1 Genetic polymorphism of tea cultivars detected by RAPD markers

Sequences	Bands Generated	Polymorphic Bands	%age of polymorphism
OPA01(CAGGCCCTTC)	11	9	81.8
OPA02(TGCCGAGCTG)	10	8	80
OPA03(AGTCAGCCAC)	11	10	90.9
OPA04(AATCGGGCTG)	16	14	87.5
OPA05(AGGGGTCTTG)	9	8	88.88
OPA06(GGTCCCTGAC)	NO AMPLIFICATION		
OPA07(GAAACGGGTG)	14	13	92.8
OPA08(GTGACGTAGG)	17	15	88.2
OPA09(GGGTAACGCC)	10	9	90
OPA10(GTGATCGCAG)	12	12	100
OPA11 (CAATCGCCGT)	8	7	87.5
OPA12(TCGGCGATAG)	15	14	93.3
OPA13(CAGCACCCAC)	12	9	75
OPA14 (TCTGTGCTGG)	10	9	90
OPB01 (GTTTCGCTCC)	16	15	93.75
OPB02 (TGATCCCTGG)	8	7	87.5
OPB03 (CATCCCCCTG)	NO AMPLIFICATION		
OPB04(GGACTGGAGT)	7	7	100
OPB05 (TGCGCCCTTC)	12	12	100
OPB06 (TGCTCTGCCC)	10	8	80
OPB07(GGTGACGCAG)	16	12	75
OPB08(GTCCACACGG)	17	14	82.3
OPB09(TGGGGGACTC)	7	7	100
OPB10(CTGCTGGGAC)	8	6	75
OPB11(GTAGACCCGT)	5	4	80
OPB12 (CCTTGACGCA)	9	9	100
OPB13 (TTCCCCCGCT)	15	15	100
OPB14(TCCGCTCTGG)	9	8	88.88
OPB15(GGAGGGTGTT)	10	8	80
OPB16 (TTTGCCCGGA)	NO AMPLIFICATION		
OPB17(AGGGAACGAG)	12	11	91.6
OPB18(CCACAGCAGT)	16	15	93.75
OPB19(ACCCCCGAAG)	12	11	91.6
OPB20 (GGACCCTTAC)	15	14	93.3
TOTAL	359	321	89.4

to determine genetic divergence among 26 cultivars of tea which revealed their clear identification. Moreover, in this study molecular techniques like RAPD have been tagged with the statistical analysis using newly developed software's like POPGENE and NTSYSpc which helped in characterization and identification of plant germplasm in a more improved way. A total of 359 major scorable fragments ranging from 500–3000 bp were generated from 31 primers as the other 3 primers could not amplify any of the samples. Out of the 359 scorable bands 321 were polymorphic. Each primer was tried thrice and the results were reproducible. The number of polymorphic bands per primer ranged from four in OPB-11 to fifteen in OPA-08 (Fig. 4.1a), OPB-01 (Fig. 4.1b), and OPB-18 (Fig.4.1c), with an average of 10.3 polymorphic bands per primer (Table 4.1). Selection of polymorphic alleles was carried out in a very careful manner and only clear, repeatable and polymorphic bands were scored and used in further statistical analysis.

Table 4.2 Nei's analysis of gene diversity in multipopulations as a whole (RAPD)

Sample size	Nei's gene diversity (h)	Shannon's Information index (I)	Total gene diversity (Ht)	Coefficient of gene differentiation (Gst)
26	0.2893	0.4394	0.2893	1.00

The percentage of polymorphic bands ranged from 75% to 100%. OPA-10 (Fig. 4.1d), OPB-04, OPB-05, OPB-09, OPB-12 and OPB-13 revealed 100% polymorphism while, OPA-13, OPB-07 (Fig. 4.1e), and OPB-10 generated 75% polymorphism. An intermediate polymorphism (87.5%) was revealed by OPA-4 (Fig. 4.1f), OPA-11 and OPB-02 (Table 4.1). The percentage of polymorphism averaged at 89.42% when all the primers were considered together, whereas few primers also showed a 100% polymorphism among the varieties owing to their self incompatibility which makes them highly heterogeneous and consequently shows broad genetic variation among themselves.

4.1.2.2. RAPD PCR product data analysis using POPGENE freeware software

Diversity measures were calculated by Nei's (1973) index and ranged from h= 0.07 to h= 0.50 (Fig. 4.2). Averaged over all markers, primer OPB-14 showed least diversity (0.1920) among the populations and primers OPB-04 revealed the highest diversity (0.3850) among the tea populations. An intermediate diversity is revealed by primers OPB-08 (0.2961) and OPB-17 (0.2823) at few loci for all the populations. The mean genetic diversity index (h) was 0.2893 (Table 4.2).

The mean value for the total diversity (Ht) among all populations was calculated to be 0.2893. The mean level of genetic differentiation (Gst) among populations over all loci was 1.00 (Table 4.2). This indicates that 100% polymorphism is observed among populations. Genetic distances calculated from the Nei's original measure of genetic distance (Nei, 1972) averaged 0.28 and varied from 0.16, lowest between cultivars TV17 and TV22 and 0.52, highest between the cultivars BS71A76 and TV23 (Table 4.3).

A dendrogram constructed on the basis of Nei's genetic distances and the UPGMA method (Fig. 4.3) showed three main clusters: Cluster I, cluster II and cluster III. The clus-

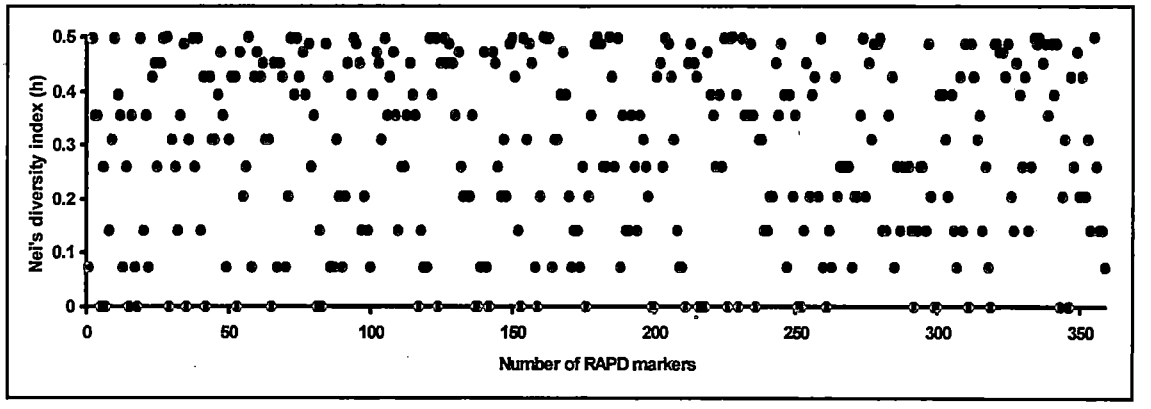


Fig. 4.2 The figure shows genetic diversity among tea cultivars estimated by Nei's (1973) diversity measure for 359 RAPD markers.

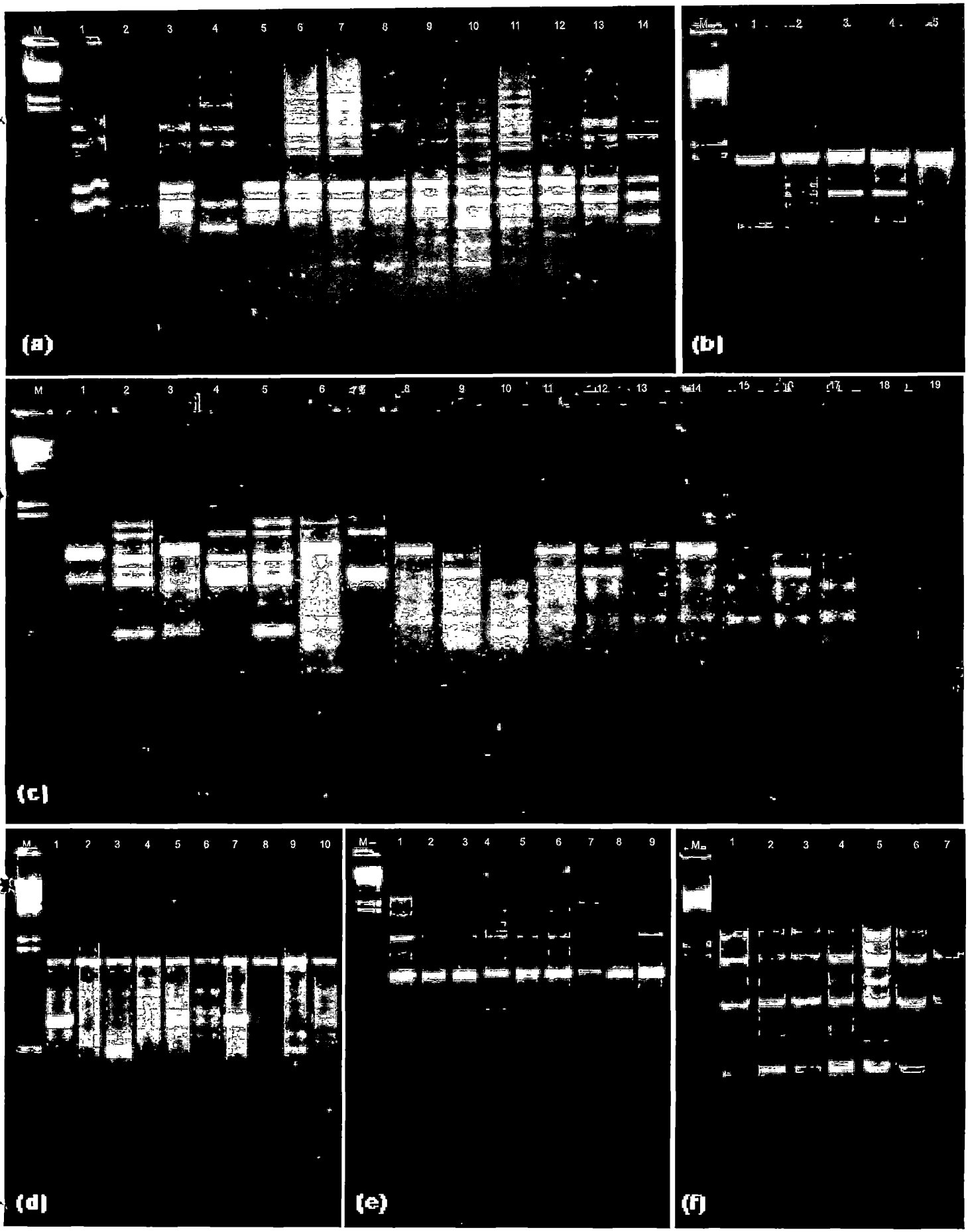


Fig 4.1 PCR amplification of different cultivars of tea DNA using various RAPD markers. Lane M in fig a-f: Molecular marker (λ–DNA/*HindIII* digest). For tea cultivar samples in different lanes refer to Table 3.1 in Materials & Methods chapter. **(a)** Primer OPA-08, **(b)** Primer OPB-01, **(c)** Primer OPB-18, **(d)** Primer OPA-10, **(e)** Primer OPB-07 and **(f)** Primer OPA-04

Table 4.3 Nei's measure of genetic distance (RAPD)
For popID codes (1-26) refer Table 3.1 in Material & Methods chapter.

popID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	0																									
2	0.252	0																								
3	0.256	0.16	0																							
4	0.3	0.263	0.224	0																						
5	0.256	0.186	0.231	0.238	0																					
6	0.296	0.245	0.278	0.263	0.263	0																				
7	0.252	0.366	0.394	0.362	0.346	0.319	0																			
8	0.3	0.323	0.274	0.334	0.358	0.346	0.292	0																		
9	0.346	0.285	0.319	0.327	0.245	0.378	0.378	0.252	0																	
10	0.358	0.296	0.263	0.323	0.33	0.296	0.374	0.234	0.292	0																
11	0.296	0.304	0.285	0.33	0.315	0.334	0.342	0.241	0.285	0.252	0															
12	0.323	0.315	0.296	0.374	0.334	0.323	0.37	0.304	0.289	0.292	0.263	0														
13	0.296	0.311	0.33	0.37	0.362	0.327	0.399	0.292	0.292	0.319	0.217	0.256	0													
14	0.358	0.327	0.338	0.362	0.362	0.342	0.39	0.315	0.346	0.296	0.274	0.307	0.245	0												
15	0.346	0.354	0.374	0.358	0.358	0.378	0.42	0.399	0.366	0.33	0.285	0.327	0.33	0.263	0											
16	0.407	0.35	0.386	0.394	0.362	0.358	0.513	0.362	0.386	0.342	0.296	0.37	0.252	0.267	0.3	0										
17	0.394	0.292	0.334	0.39	0.327	0.354	0.463	0.415	0.35	0.37	0.338	0.327	0.263	0.338	0.289	0.22	0									
18	0.358	0.374	0.362	0.338	0.378	0.304	0.39	0.338	0.346	0.382	0.374	0.386	0.319	0.319	0.346	0.304	0.354	0								
19	0.415	0.327	0.346	0.403	0.403	0.374	0.467	0.37	0.437	0.415	0.334	0.411	0.311	0.358	0.315	0.319	0.27	0.259	0							
20	0.407	0.334	0.37	0.403	0.386	0.374	0.476	0.411	0.37	0.407	0.342	0.403	0.366	0.382	0.362	0.407	0.411	0.382	0.374	0						
21	0.407	0.39	0.42	0.37	0.428	0.39	0.467	0.437	0.463	0.424	0.382	0.445	0.382	0.358	0.411	0.424	0.42	0.35	0.39	0.304	0					
22	0.407	0.415	0.437	0.499	0.437	0.399	0.522	0.454	0.499	0.45	0.374	0.428	0.424	0.467	0.445	0.424	0.472	0.45	0.476	0.289	0.327	0				
23	0.437	0.394	0.45	0.382	0.415	0.362	0.437	0.407	0.485	0.463	0.42	0.415	0.42	0.378	0.415	0.403	0.441	0.386	0.428	0.378	0.285	0.37	0			
24	0.37	0.33	0.358	0.358	0.407	0.315	0.428	0.39	0.415	0.411	0.378	0.415	0.37	0.338	0.432	0.411	0.441	0.33	0.386	0.285	0.315	0.33	0.327	0		
25	0.42	0.394	0.415	0.399	0.458	0.362	0.463	0.432	0.467	0.437	0.386	0.399	0.472	0.378	0.432	0.445	0.467	0.362	0.378	0.354	0.338	0.33	0.327	0.334	0	
26	0.42	0.354	0.358	0.358	0.415	0.338	0.463	0.382	0.399	0.403	0.378	0.366	0.42	0.346	0.399	0.386	0.399	0.323	0.378	0.394	0.338	0.386	0.334	0.281	0.289	0

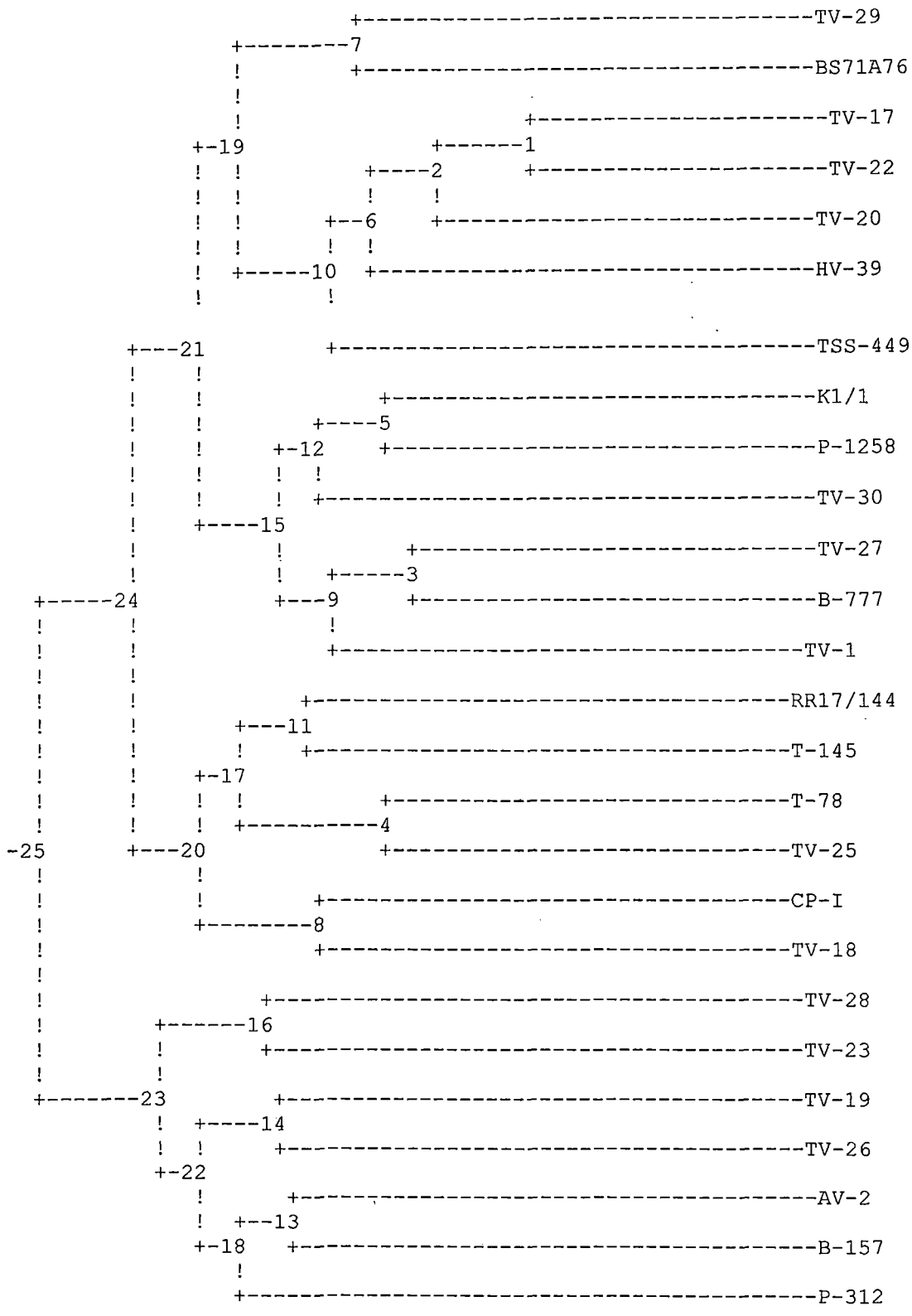


Fig. 4.3 Dendrogram based on Nei's (1972) Genetic distance: Method = UPGMA Modified from NEIGHBOR procedure of PHYLIP Version 3.5(RAPD markers)

ters revealed the level of relatedness between the 26 cultivars studied (Refer Materials and Methods section, Table 3.1). Measure of similarity ranged from a minimum 46% between TV-17, TV-22 and P-312 to a maximum 100% between TV-28 and TV-23. Cluster I consist of thirteen cultivars which is the largest cluster comprising of Assam type which are divided into two main groups: The first group comprises of seven cultivars TV29, BS71A76, TV17, TV22, TV20, HV39, TSS-449 and within this group there are two subgroups. In the first subgroup two completely different cultivars TV-29 (Assam) and BS71A76 (China) revealed height similarity of 100% which does not match with the existing knowledge on the systematic of tea (*C. sinensis*) .The reason for their clustering is not known presently and the possible explanation may be due to the fact that traditionally tea is classified on the basis of morphological features which are subjected to substantial environment changes coupled with extensive hybridization , the other two cultivars TV-17 and TV22 although one Assam and other Cambod respectively also revealed 100% similarity, this may be due to the reason that traditionally cambod is a subgroup of Assam type or as it is sometimes reffered to as a subspecies of *assamica* (ssp. Lasiocalyx) (Wight 1962). The other three cultivars TV-20 (Cambod), HV-39 (China) and TSS449 (Seed stock) formed subgroups of their own. 2nd group comprises of six cultivars Kopati-1/1 (Assam type), P-1258 (China type), TV30 (Assam type), TV27 (Cambod), B777 (China type), TV1 (Assam/China type), among this group K-1/1 revealed 100% similarity with P-1258 and TV-27 revealed 100% similarity with B-777 again the pairs exhibited a lot of variation from the previous results TV30 and TV-1 formed their own subgroups. Cluster II comprises of six cultivars RR 17/144 (China hyb.), T-145 (Assam hyb.), T-78 (China hyb.), TV-25 (Cambod), CP-I (Assam hyb.), TV-18 (Cambod) with most of the Assam and cambod types and two exceptions of China type. Cluster IIIA includes all Cambod cultivars TV-19, TV-28, TV-23, TV-26 with a height similarity of 100% between TV-28 and TV-23 as well between TV-19 and TV-26 .The second group of cluster IIIB comprises of all China cultivars AV-2, B-157, P-312.

Based on RAPD profile of each primer, measures of Shannon's index of diversity was from 0.28 to 0.56, with average of 0.44 (Fig. 4.4). This result shows that a relatively high level of polymorphism was determined by these ran-

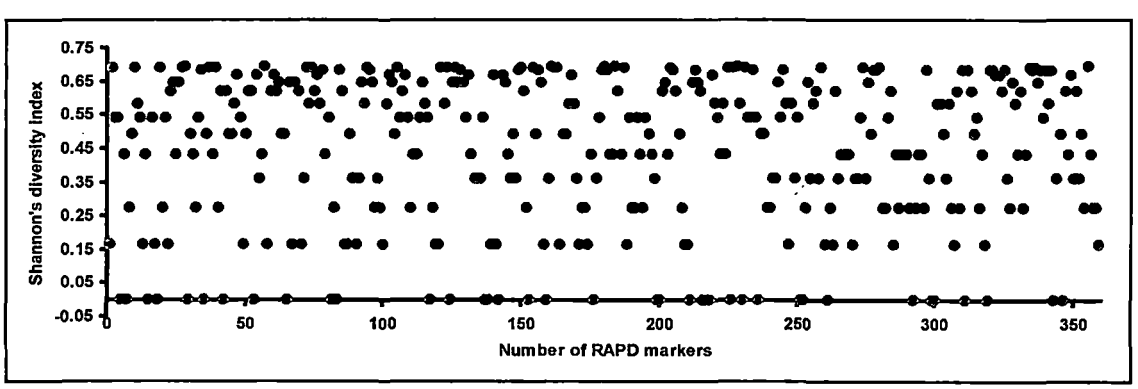


Fig. 4.4 Shannon's index of diversity (RAPD markers)

dom primers in the cultivars of tea. However, because the sample size and total amount of bands could influence the percentage of polymorphic bands value, other parameters that based on the frequency of polymorphic bands, such as Shannon's index of diversity and Simpson's index of diversity, are more suitable in estimating genetic variability (Cruzan 1998). The average of Shannon's index of diversity in the cultivars as studied is 0.44 (Table 4.2) indicating a relatively high degree of diversity among the tea population. The result is in agreement with the high percentage of polymorphism as detected by Nei's genetic diversity index.

4.1.2.3. RAPD PCR product data analysis using NTSYSpc software

The same clusters were obtained in the dendrogram when the data was analyzed using NTSYSpc (Fig. 4.5). Dendrograms constructed using both the software's POPGENE as well as NTSYSpc revealed the same result (Fig. 4.3 & Fig. 4.5) and is in complete agreement with the previous knowledge of tea taxonomy. Three main clusters have been formed and within these three clusters three types of tea can be easily differentiated Assam type, Cambod type and China type. The degree of polymorphism revealed in the present study was found to be 100% for few primers. And for the first time a large number of individuals could be analysed from the total available diversity in *Camellia sinensis*.

Correspondence analysis of the data was also in agreement with the dendrogram clustering as seen in a 2D and a 3D view of the plots (Fig. 4.6 & Fig. 4.7), it also revealed three main clusters, Cluster I on the right of the plane includes Assam and Cambod varieties namely TV-20, TV-22, TV29, TV-30, TV-1, TV-27, at a correlation coefficient of 0.18, TV17 clustered very near to TV-22 and TV-20 which was also seen in the Dendrograms generated by UPGMA method. Cluster II in the middle includes Cambod varieties TV-18 and CP-I between a correlation coefficient of 0.27 and 0.18 with exceptions of RR 17/144, B-777, T-78. Cluster IIIA on left corner consists of all Cambod varieties namely TV-19, TV-26, TV-28 and TV-23. Cluster IIIB also on left comprises of China varieties most of which are Darjeeling clones namely P-312, B-157, AV-2 between a correlation coefficient of 0.71 and 1.16. One Chinese variety namely HV-39 which is from Darjeeling and a TRA released variety clustered alone at the extreme right corner with a correlation coefficient of around 0.40. Thus, Correspondence analysis revealed a clear picture of genetic variability as revealed by dendrograms constructed on the basis of both the software's. The Assam varieties clustered on right of the plane and the China ones clustered on left and few Cambod cultivars clustered in the middle of the plane thus confirming their ancestral differences.

This study demonstrates that RAPD offers a suitable means for the detection of genetic variability and molecular study of tea genotypes. Although the studies have been done on few varieties of tea found in India by various workers using RAPD technique but a complete study of twenty six varieties using 34 random deca-mer primers have been done for the first time in the present study revealing as high as 89.42% of polymorphism.

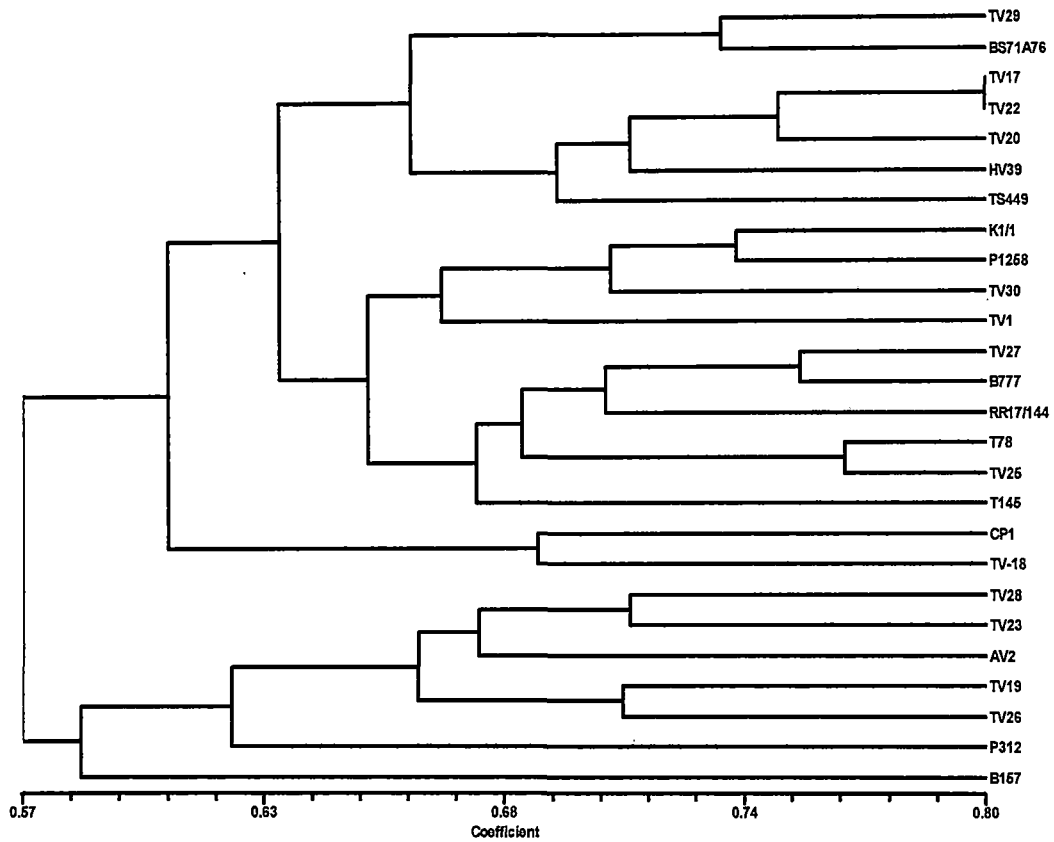


Fig. 4.5 Dendrogram based on NTSYS illustrating the relationships among twenty six tea cultivars (RAPD markers)

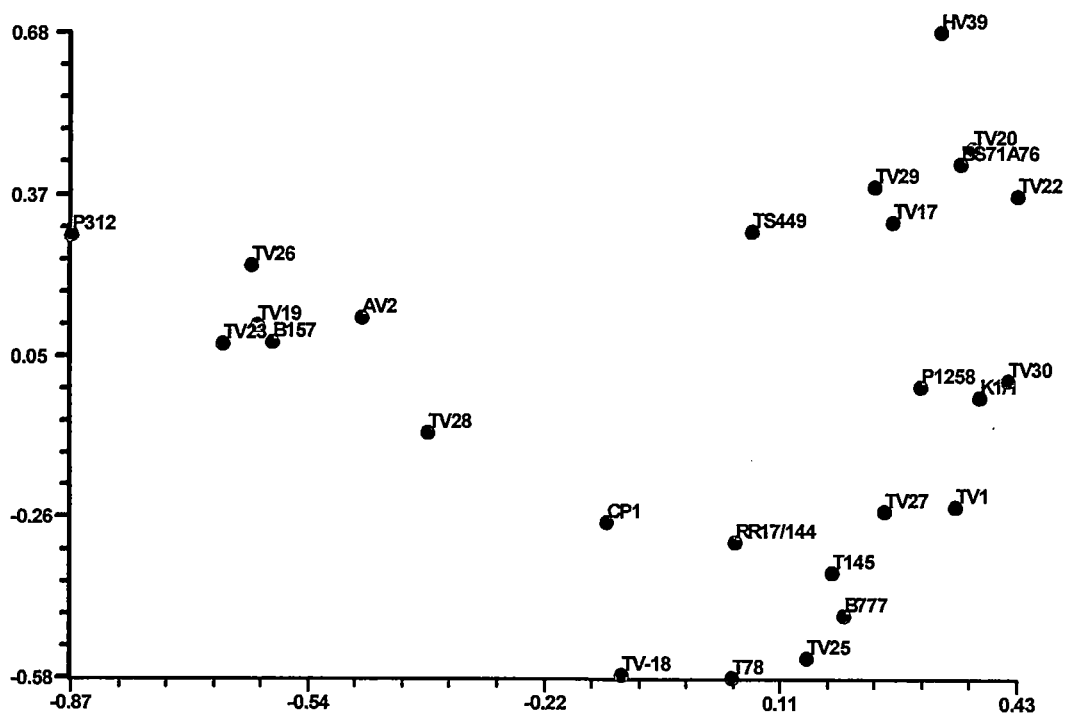


Fig. 4.6 Correspondence analysis (2D plot) of twenty six tea cultivars (RAPD markers)

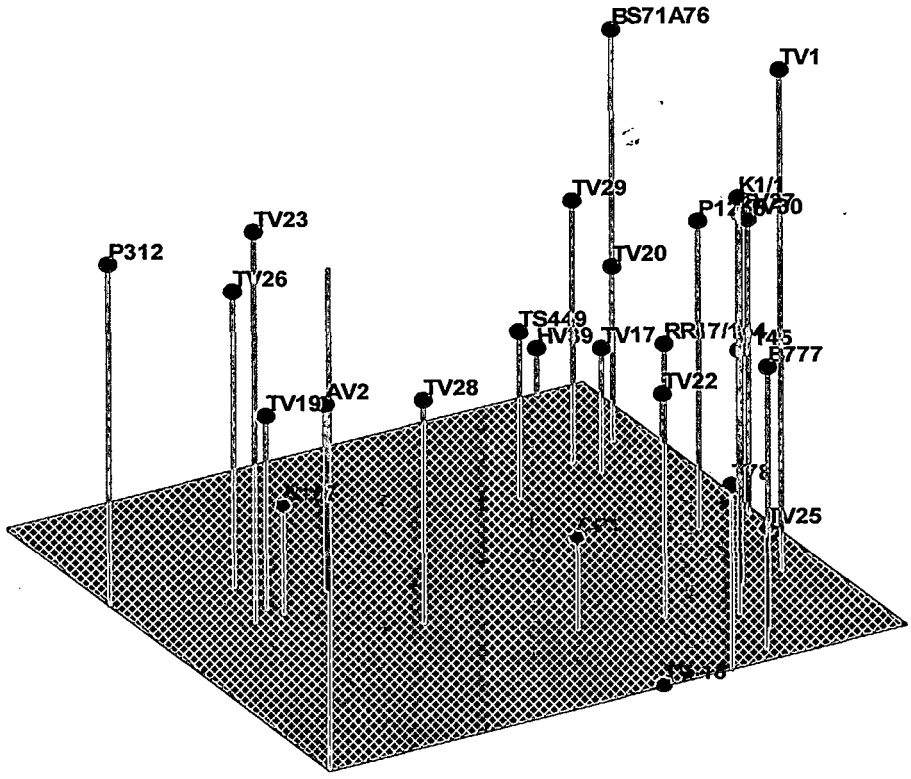


Fig. 4.7 Correspondence analysis (3D plot) of twenty six tea cultivars (RAPD markers)

4.1.3. PCR-RFLP analysis

PCR-RFLP is a simple and inexpensive method capable of authenticating tea and thus has become of prime importance in genetic diversity studies of different plant species. In this study twenty six varieties of tea cultivars most commonly grown in North Bengal and Darjeeling (Refer Materials and Methods section, Table 3.1) were fingerprinted using the restriction fragment length polymorphism (RFLP) methodology. Marker development was based on Tab e-f in *trn*L-*trn*F ("Taberlet") region of the tea genome for which the nucleotide information was available in other plant species. One primer pair was used to target the Tab e-f in trnL-trnF ("Taberlet") region as shown in Fig.3.2 (Refer Material and Methods section 3.2). The primer pair amplified the Tab e-f in trnL-trnF (Taberlet) region of tea genome. The amplification was carried out on DNA obtained from fresh tea leaves of different tea cultivars.

4.1.3.1. RFLP PCR product agarose gel analysis

Using template DNA from different tea cultivars, the primer pair generated a single band following PCR. The primer targeting the Tab e-f in trnL-trnF ("Taberlet") region amplified a band of expected length (938bp) as deduced from cDNA sequences (Fig. 4.8).

4.1.3.2. PCR-RFLP product restriction digestion agarose gel analysis

The PCR products were subjected to restriction digestion with 5 different restriction endonucleases, namely *AluI, TaqI, HinfI, MboI, MspI* all 4-base cutters (Refer Material and Methods section, Table 3.3). This procedure generated polymorphism by restricting known PCR-amplified DNA sequences. Upon restriction digestion with *AluI* most of the cultivars

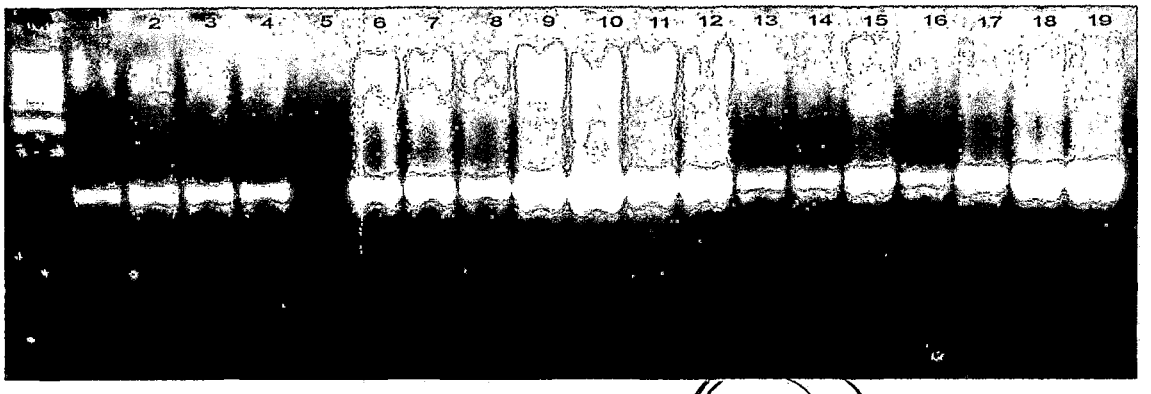


Fig. 4.8 Amplification of tea cultivars with primer trnL-trnF (Tab e-f) (RFLP markers) Lane M=Molecular marker-1Kb ladder. For tea cultivar samples in different lanes refer to Table 3.1 in Materials & Methods chapter.

generated three bands except TV-1 that produced only single band and B-157 cultivar was individualized by showing no site for digestion by *AluI* (Fig. 4.9a). Restriction enzyme *MboI* revealed no polymorphism generating three bands of same molecular weight in each of the cultivars (Fig. 4.9b). When the PCR products were subjected to *MspI* enzyme it produced a single band in few of the cultivars like TV-29, P-1258, TV-27, TV-1, B-777, R-144 and in others two bands were produced (Fig. 4.9c). Restriction enzyme *TaqI* generated three bands in all except cultivars TV-17, TV-20, TSS-449, BS71A76, K-1/1, and TV-30 in which all total six bands were generated. In cultivars TV-28, TV-14, TV-23, TV-1, B-777 five bands were produced, two bands were generated in AV-2, P-312 and four bands in cultivars T-78, TV-25, CPI and TV-18 (Fig. 4.9d). Digestion with *HinfI* revealed maximum polymorphism generating as many as eight bands in cultivars TV-17, TV-22, HV-39, TV-20, TSS-449, TV-30 , six bands were produced in cultivar BS71A76, five in K-1/1, three bands in all others and no site was observed in TV-29 (Fig. 4.9e).

4.1.3.3. PCR-RFLP product data analysis using POPGENE freeware software

A total of 24 major scorable fragments ranging from 500-3000bp were generated by Tab e-f in trnL-trnF ("Taberlet") region. Out of the 24 scorable bands 21 were polymorphic. The primer was tried thrice and the results were reproducible. The number of polymorphic bands per enzyme ranged from one in *MboI* to eight in *HinfI* and *TaqI*, with 3an average of two polymorphic bands per enzyme. Restriction enzyme *MspI* had no site in any of the cultivars. These clear, repeatable and polymorphic bands were scored and used in further statistical analysis.

The percentage of polymorphic bands ranged from 25% to 100%. Restriction digestion enzyme *MboI* revealed 25% polymorphism while, *TaqI* generated 100% polymorphism. An intermediate polymorphism (88.8%) was revealed by *HinfI* (Table 4.4). The overall percentage of polymorphic bands was 87.50. Diversity measures were calculated by Nei's (1973) index and ranged from h= 0.07 to h= 0.48 (Fig. 4.10). Averaged over all enzymes, *AluI* showed least diversity (0.1193) among the populations and enzyme *TaqI* revealed the highest diversity (0.2799) among the tea populations. An intermediate diversity is revealed by enzyme *HinfI* (0.2514) for all the

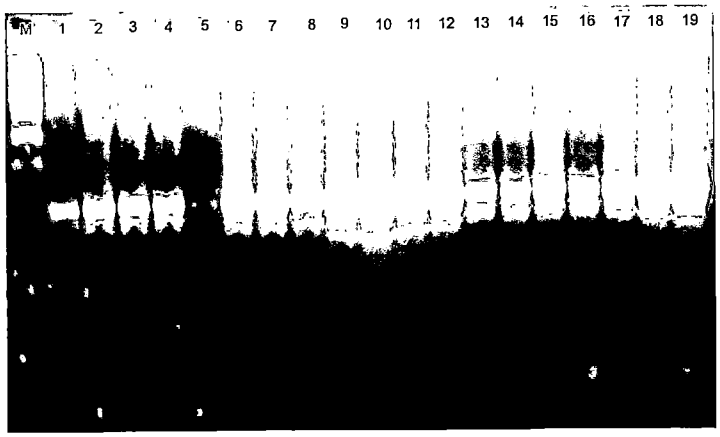
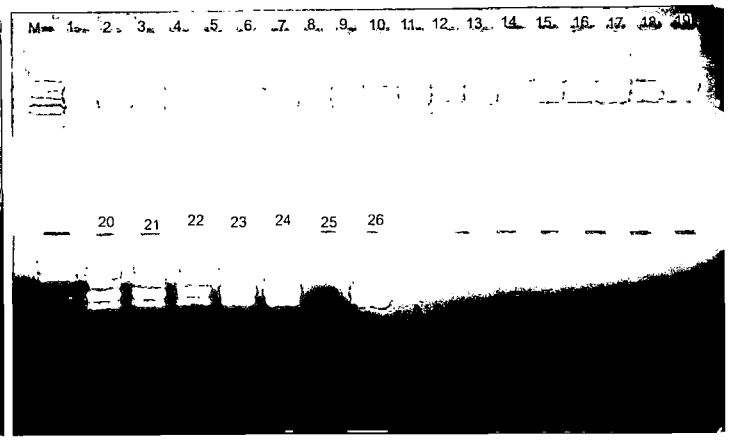
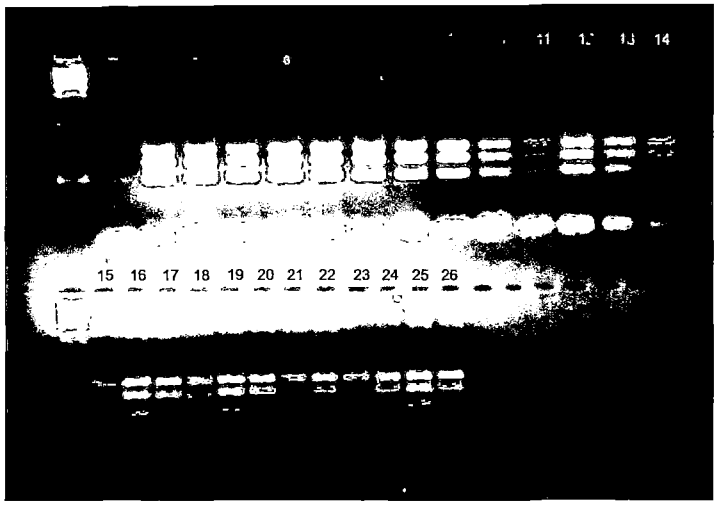


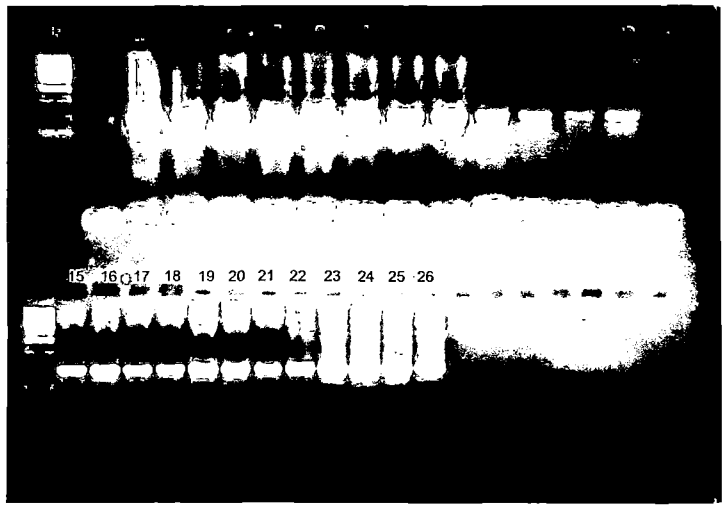
Fig. 4.8 PCR amplification of various tea cultivars using trnL-trnF primer.
Lane M=Molecular marker- 1Kb ladder. For tea cultivar samples in different lanes refer to Table 3.1 in Materials & Methods chapter.



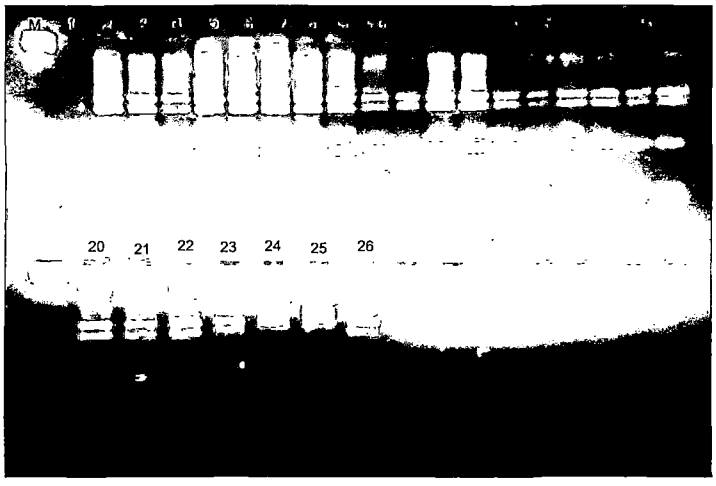
(a) Restriction digestion product using *AluI* enzyme.
Lane M=Molecular marker- 1Kb ladder. For tea cultivar samples in different lanes refer to Table 3.1 in Materials & Methods chapter.



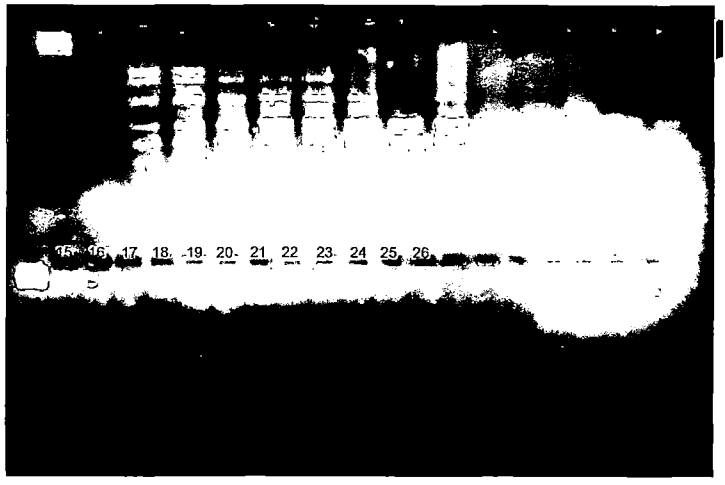
(b) Restriction digestion product using *MboI* enzyme.
Lane M=Molecular marker- 1Kb ladder. For tea cultivar samples in different lanes refer to Table 3.1 in Materials & Methods chapter.



(c) Restriction digestion product using *MspI* enzyme.
Lane M=Molecular marker- 1Kb ladder. For tea cultivar samples in different lanes refer to Table 3.1 in Materials & Methods chapter.



(d) Restriction digestion product using *TaqI* enzyme.
Lane M=Molecular marker- λ-DNA/*HindIII* digest. For tea cultivar samples in different lanes refer to Table 3.1 in Materials & Methods chapter.



(e) Restriction digestion product using HinfI enzyme.
Lane M=Molecular marker- λ-DNA/*HindIII* digest. For tea cultivar samples in different lanes refer to Table 3.1 in Materials & Methods chapter.

Fig. 4.9(a-e) Restriction digestion products of trnL-trnF amplified PCR products using various enzymes.

Table 4.4 Genetic polymorphism of tea cultivars detected by RFLP marker by various enzymes

Enzymes used	Bands Generated	Polymorphic Bands	%age of polymorphism
MboI	4	2	25
HinfI	9	8	88.8
AluI	3	3	100
TaqI	8	8	100
MspI	No site		
TOTAL	24	21	87.5

populations. The mean genetic diversity index (h) was 0.2253 (Table 4.5).

The mean value for the total diversity (Ht) among all populations was calculated to be 0.2253.The mean level of genetic differentiation (Gst) among populations over all loci was 1.00 (Table 4.5). This indicates that 100% polymorphism is observed among populations. Genetic distances calculated from the Nei's original measure of genetic distance (Nei, 1972) averaged 0.43 and varied from 0, lowest between cultivars TV22 and HV-39, TV-25 and TV-18, TV25 and TV-19, TV-25 and TV-23, TV-18 and TV-19, TV-18 and TV-23 to 0.876, maximum between the cultivars TV-29 and TV-17 (Table 4.6).

A dendrogram constructed on the basis of Nei's genetic distances and the UPGMA method (Fig. 4.11) showed three main clusters: Cluster I, cluster II and cluster III. The clusters revealed the level of relatedness between the 26 cultivars studied (Refer Material and Methods section, Table 3.1). Measure of similarity ranged from a minimum 0% between TV-25, TV-18, TV-19, TV-23, TV-22, HV-39 and P-312 to a maximum 100% between TV-25, TV-18, TV-19, TV-23 TV-22 and HV-39. Cluster I consist of twelve cultivars which is the largest cluster comprising of China type which are divided into two main groups: The first group comprises of three cultivars TV29, AV-2 and B-157, in this group TV-29 a cambod variety clustered with AV-2 with a similarity measure of 4.35057 whereas the other China variety B-157 showed a similarity measure of 9.17866 far away from AV-2. 2nd group comprises of nine cultivars P-1258, TV-27, RR17/144, TV-26, T-145, CPI, B-777, TV-28 and T-78. Cluster II comprises of all Cambod cultivars TV-25, TV-18, TV-19 and TV-23 with a height similarity of 0.0000 that means they showed no genetic difference among themselves which is obvious as all are cambod varieties. Cluster III includes four Assam cultivars TV-17, TSS-449, K1/1, TV-1 with three Cambod cultivars TV-20, TV-30 and TV-22 and three China cultivars BS71A76, HV-39 and P-312. In this cluster TV-22 showed a height similarity with a China cultivar namely HV-39.

Based on RFLP profile of each enzyme, measures of Shannon's index of diversity were from 0.20 to 0.43, with average of 0.35 (Table 4.5 and Fig. 4.12). This result shows that a relatively high level of polymorphism was determined by the RFLP primer in the cultivars

Table 4.5 Nei's analysis of gene diversity in multipopulations as a whole (RFLP)

Sample size	Nei's gene diversity (h)	Shannon's Information index (I)	Total gene diversity (Ht)	Coefficient of gene differentiation (Gst)
26	0.2253	0.3565	0.2253	1.00

Table 4.6 Nei's measure of genetic distance (RFLP)
For popID codes (1-26) refer Table 3.1 in Material & Methods chapter.

Pop. ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	0																									
2	0.876	0																								
3	0.693	0.087	0																							
4	0.693	0.087	0	0																						
5	0.78	0.043	0.134	0.134	0																					
6	0.78	0.134	0.134	0.134	0.087	0																				
7	0.693	0.087	0.182	0.182	0.043	0.134	0																			
8	0.47	0.234	0.234	0.234	0.182	0.182	0.134	0																		
9	0.693	0.087	0.087	0.087	0.043	0.043	0.087	0.134	0																	
10	0.234	0.613	0.47	0.47	0.539	0.539	0.47	0.288	0.47	0																
11	0.234	0.613	0.47	0.47	0.539	0.539	0.47	0.288	0.47	0.087	0															
12	0.406	0.406	0.288	0.288	0.345	0.345	0.288	0.134	0.288	0.234	0.234	0														
13	0.288	0.406	0.288	0.288	0.345	0.345	0.288	0.134	0.288	0.134	0.134	0.087	0													
14	0.134	0.613	0.47	0.47	0.539	0.539	0.47	0.288	0.47	0.087	0.087	0.234	0.134	0												
15	0.182	0.539	0.406	0.406	0.47	0.47	0.406	0.345	0.406	0.134	0.134	0.288	0.182	0.134	0											
16	0.345	0.345	0.234	0.234	0.288	0.288	0.234	0.288	0.234	0.182	0.182	0.234	0.134	0.182	0.134	0										
17	0.288	0.406	0.288	0.288	0.345	0.345	0.288	0.234	0.288	0.134	0.134	0.182	0.087	0.134	0.087	0.043	0									
18	0.234	0.47	0.345	0.345	0.406	0.406	0.345	0.288	0.345	0.087	0.087	0.234	0.134	0.087	0.043	0.087	0.043	0								
19	0.288	0.406	0.288	0.288	0.345	0.345	0.288	0.234	0.288	0.134	0.134	0.182	0.087	0.134	0.087	0.043	0	0.043	0							
20	0.345	0.345	0.234	0.234	0.288	0.288	0.234	0.182	0.234	0.182	0.182	0.134	0.043	0.182	0.134	0.087	0.043	0.087	0.043	0						
21	0.288	0.406	0.288	0.288	0.345	0.345	0.288	0.234	0.288	0.134	0.134	0.182	0.087	0.134	0.087	0.043	0	0.043	0	0.043	0					
22	0.288	0.406	0.288	0.288	0.345	0.345	0.288	0.234	0.288	0.134	0.134	0.182	0.087	0.134	0.087	0.043	0	0.043	0	0.043	0	0				
23	0.182	0.539	0.406	0.406	0.47	0.47	0.406	0.345	0.406	0.134	0.134	0.288	0.182	0.043	0.087	0.134	0.087	0.043	0.087	0.134	0.087	0.087	0			
24	0.087	0.693	0.539	0.539	0.613	0.613	0.539	0.47	0.539	0.234	0.234	0.406	0.288	0.134	0.087	0.234	0.182	0.134	0.182	0.234	0.182	0.182	0.087	0		
25	0.406	0.693	0.693	0.693	0.613	0.78	0.539	0.613	0.693	0.47	0.345	0.406	0.539	0.47	0.288	0.47	0.406	0.345	0.406	0.47	0.406	0.406	0.406	0.288	0	
26	0.234	0.78	0.613	0.613	0.693	0.693	0.613	0.539	0.613	0.288	0.288	0.47	0.345	0.182	0.134	0.288	0.234	0.182	0.234	0.288	0.234	0.234	0.134	0.134	0.47	0

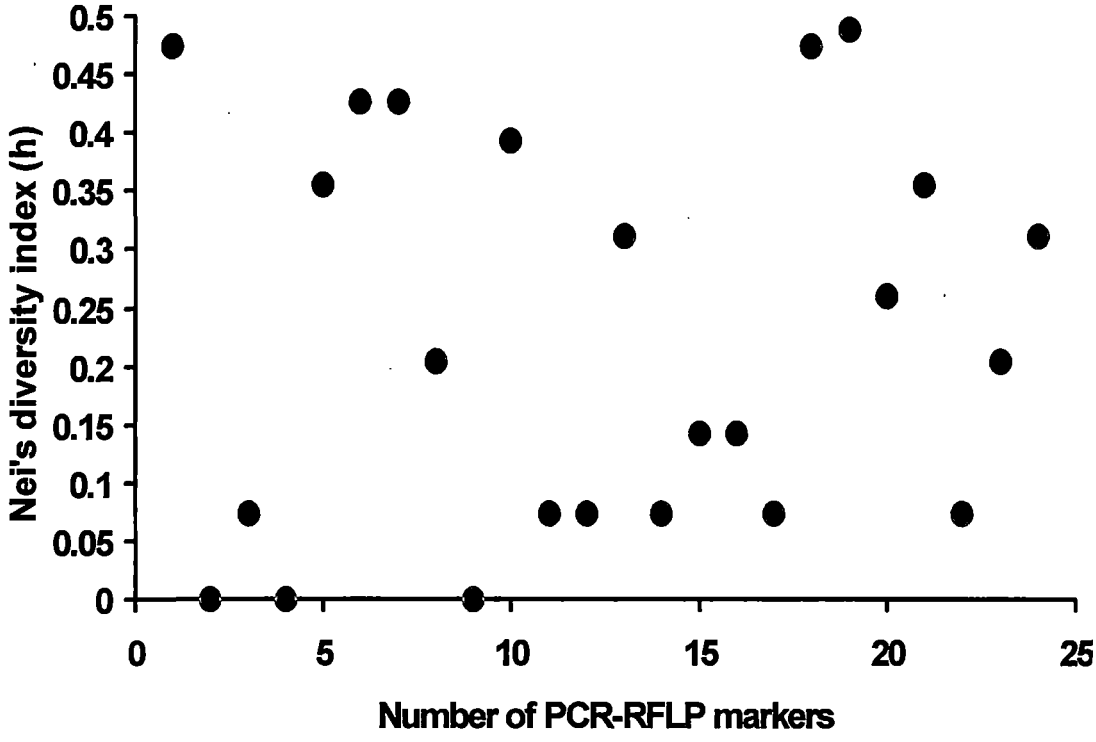


Fig. 4.10 The figure shows genetic diversity among tea cultivars estimated by Nei's (1973) diversity measure for 21 RFLP markers.

of tea.

4.1.3.4. PCR-RFLP product data analysis using NTSYSpc software

The same clusters were obtained in the dendrogram when the data was analyzed using NTSYSpc (Fig. 4.13) and a 2D and 3D plot of correspondence analysis of the data was also in agreement with the dendrogram clustering (Fig. 4.14 & Fig. 4.15) it also revealed three main clusters, Cluster I on the right of the plane includes Cambod cultivars namely TV-20, TV-22, TV17, TV-30 at a correlation coefficient of 0.5, HV-39 and TV-22 overlapped as a result of their similar characteristic which was also seen in the Dendrograms generated by UPGMA method. One more cultivar TSS-449 clustered alone at a correlation coffecient of 0.60. Cluster II in the middle includes Assam cultivars TV-1 and K-1/1 between a correlation coefficient of 0.05 and 0.34 with exceptions of B-777, T-78, TV-28. Cluster III on left corner consists of two Assam cultivars T-145, CPI, four China cultivars RR17/144, AV-2, B-157, P-1258 and rest Cambod cultivars namely TV-19, TV-25, TV-18 and TV-23. One Darjeeling clone namely P-312 clustered alone at the upper middle portion with a correlation coefficient of around 0.50.

4.1.3.5. Sequence analysis using Clustal W (Thompson et al., 1994) and GenBank submission

One of the ten sample tea cultivars (Bannockburn-157 cultivar) sent for sequencing to the Genbank is given in Fig. 4.16a and finally the sequences of all ten cultivars (Refer section 3.3.3, Table 3.4 of Materials and Methods) were aligned using Clustal W (Thompson *et al.*, 1994), and aligned sequences generated a Cladogram for 10 culti-

vars sequenced. Analysis of the Cladogram revealed (Fig.4.16) three major clades, first clade comprises of two cambod cultivars namely TV-29 and TV-22, a Assam cultivar namely K-1/1 and one China cultivar BS71A76. Among these four cultivars highest similarity was observed between TV-22 and BS71A76. In the second clade there are three cultivars B-777, TV-29 and CP1, among these cultivars the highest similarity was obtained between TV-29 and CP1 cultivars as TV-29 is a cambod type and CP1 Assam type cultivar. Third clade comprises of again three cultivars of which P-312 and B-157 are both China type revealing maximum similarity between them and TV-27 a cambod type cultivar.

These results when compared to the RFLP data analysis done by POPGENE software revealed lots of similarities among themselves. As in the second clade TV-29 and CP1 are very close to each other exactly same result was observed by POPGENE Dendrogram analysis as mentioned in section 4.1.2.3 of Result and Discussion. Similarly in third clade also cultivars P-312 and B-157 were close to each other as found in POPGENE dendrogram analysis in section 4.1.2.3 of Result and Discussion. Therefore, RFLP technique can be used to differentiate various cultivars of tea. Further sequencing of tea cultivars using RFLP markers revealed their molecular structure which can help conserve the best quality tea varieties and also help selection from wide variability in the commercial tea populations and select elite mother bushes with desirable attributes in a practical plant improvement programme (Richards 1960; Bezbaruah 1975). Therefore, it needs to be preserved and utilized judiciously.

The sequences of the ten cultivars thus submitted to the GenBank were allotted with their specific accession numbers (Table 4.7).

4.1.4. rbcL-ORF106, chloroplast DNA (all of rbcL plus spacer between it and ORF106 exon) analysis

4.1.4.1. Chloroplast DNA PCR product agarose gel analysis

A RFLP study was done for *rbcl* gene (Chloroplast DNA) of tea. The primer sequence *rbcL* 5'-ATGTCACCACAAACAGAAA CTAAGCAAGT-3' and ORF106*5'-ACTACAGATCTCATACTACCCC-3' for amplifying the Chloroplast DNA was obtained on the basis of the known sequence based on Sequencer restriction maps from GenBank Solanaceae sequences. Among 26 tea cultivars studied the primer pair could amplify only

Table 4.7 Tea cultivars with GenBank accession numbers

Cultivar	GenBank Accession number
B157F	EF057736
B777F	EF057737
BS71A76	EF057738
CPIF	EF057739
K-1/1F	EF057740
P312F	EF057741
TV22F	EF057742
TV25F	EF057743
TV27F	EF057744
TV29F	EF057745

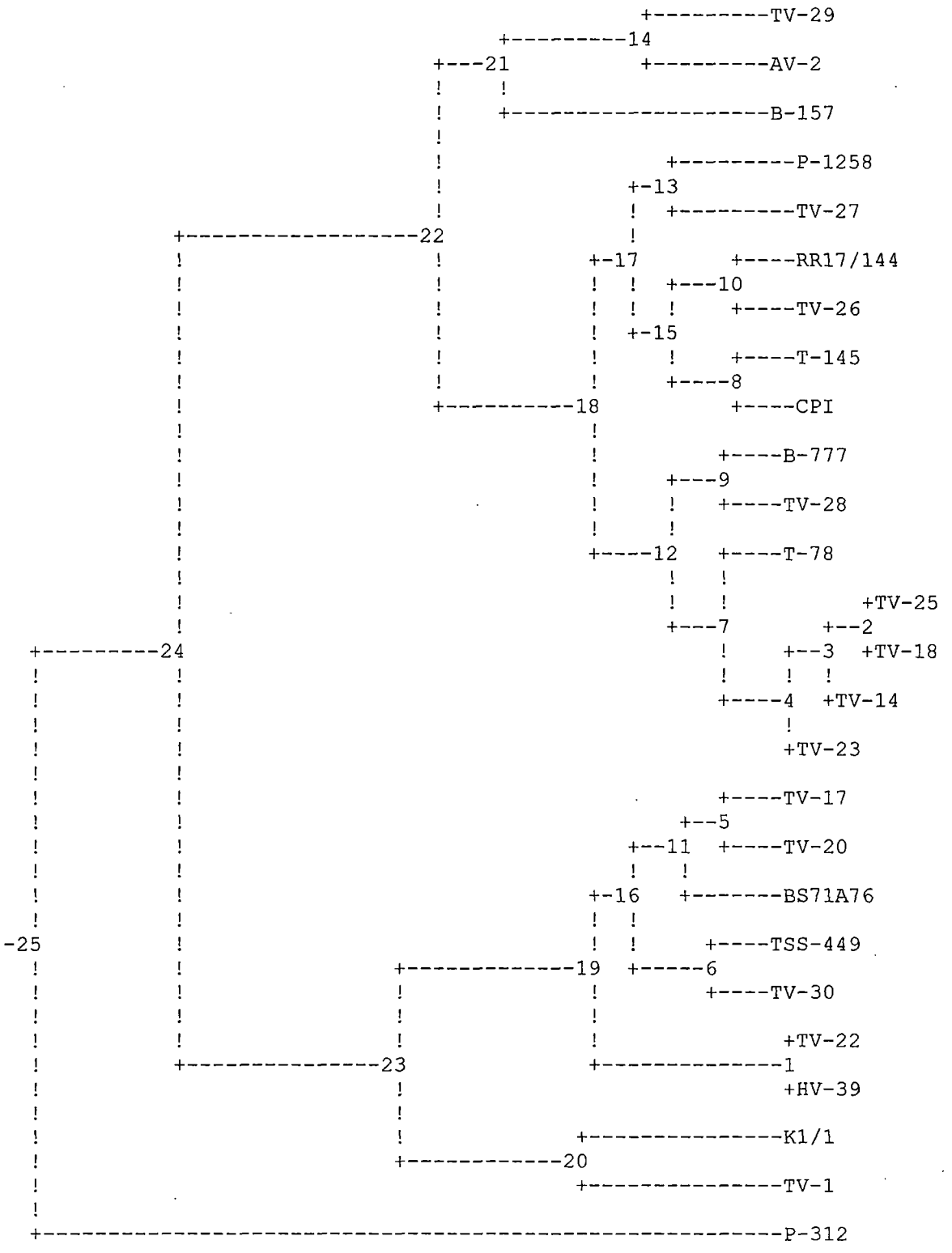


Fig. 4.11 Dendrogram based on Nei's (1972) Genetic distance: Method = UPGMA Modified from NEIGHBOR procedure of PHYLIP Version 3.5 (RFLP markers)

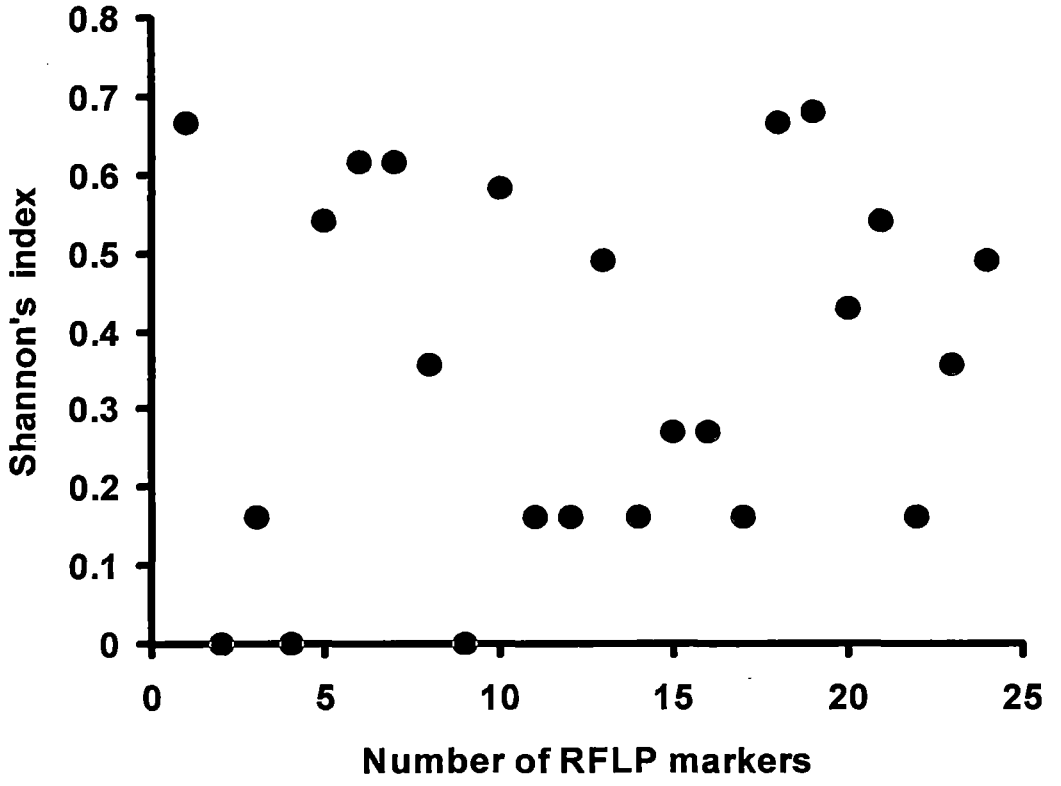


Fig. 4.12 Shannon's index of diversity (RFLP markers)

50% of the tea cultivars. Cultivars amplified successfully are given in the Fig.4.17. The amplified products when subjected to restriction digestion by *AluI* generated six bands in each of the cultivar revealing no polymorphism (Fig. 4.17a). Digestion by *HhaI* showed no restriction site in any of the cultivars (Fig. 4.17b) and upon digestion with *HinfI* though four bands were produced but again there appeared no polymorphism in any of the cultivars (Fig. 4.17c). In some previous studies Prince and Parks (1997) analyzed the evolutionary relationship in tea subfamily Theoideae based upon two cp DNA regions, namely *rbcL* and *mat*K sequence data, for 4 species of subfamily Ternstroemioideae and 24 species from Theoideae. Later on, the same workers also examined the same cp DNA region (*rbcL* and *mat* K) to confirm the family Theaceae, a natural group as well as to evaluate the validity of circumscription of tribes and genus of its subfamily Theoideae (Prince and Parks, 2000). The nucleotide sequences of *rbcL* gene in chloroplast DNA are determined on the native tea varieties of Japan, Korea, China, South East Asia, Sri Lanka and India. Direct sequencing of the amplified cp DNA products were carried out. The nucleotide sequences of the *rbcL* gene in cpDNA of China and Assam type were presented. Alignments were obtained by assuming two substitutions, at nucleotide position 40 (adenine in China tea) and 948 (guanine in China tea). The nucleotide sequences of the *rbcL* gene in China and Assam were 99.8% similar. On the other hand, the 1370 nucleotide sequences of *rbcL* gene among *C. irrawadiensis C. taliensis* and Assam tea were the same except a different

base at position 627. At this position in *C. irrawadiensis* and *C. taliensis*, thymine and adenine were observed, respectively, as specific bases (Kato, 2001). Thus the present result is in confirmation with these studies as in present study also no polymorphism was detected among the Assam and China cultivars.

4.1.5. Microsatellite markers study analysis

Recently microsatellite markers are in focus mainly for genome mapping of crop plants. In the present study 3 pairs of microsatellite markers were used for genotyping the 26 cultivars of *Camellia sinensis*. Microsatellite markers were developed for *Camellia japonica* by screening random amplified polymorphic DNA (RAPD) profiles to avoid time consuming screening of genomic libraries. The objective was to analyse seed dispersal and parentage of *C. japonica* (factors expected to have strong influences on genetic structure of the species) in an old growth on Tsushima Island, Nagasaki (Ueno *et al* 1999). Cross-species amplification with the three microsatellite primers showed that these primer pairs worked well for *Camellia sinensis* (Ueno *et al* 1999). Therefore, the present study was conducted using these 3 microsatellite primer pairs (Refer Materials and Methods section, Table 3.6) for genotyping 26 cultivars of *Camellia sinensis* found in North Bengal.

After PCR optimization the three primer pairs yielded polymorphic and single locus amplification products (Fig. 4.18). Primer pair for locus MSCjaH38 yielded single locus amplification product of the expected size (348-378bp) for all 26 cultivars. Cultivars TV-29, TV-22, TV-20, TSS-449, BS71A76, TV-28, TV-26 and AV-2 revealed strong bright bands

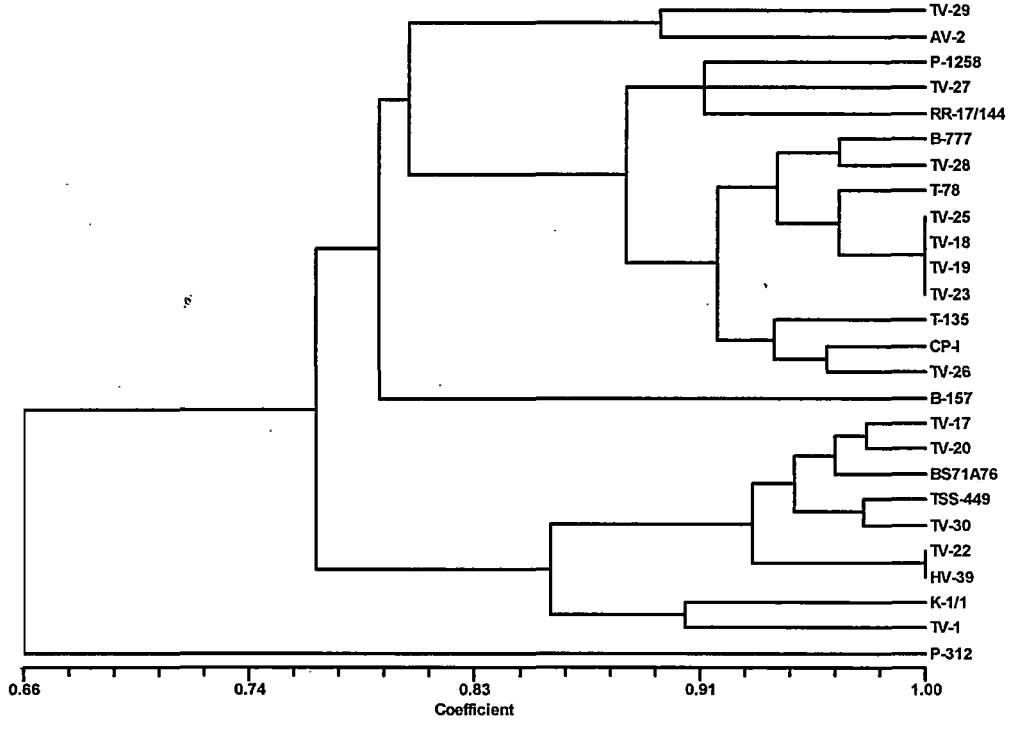


Fig. 4.13 Dendrogram based on NTSYS illustrating the relationships among twenty six tea cultivars.(RFLP markers)

whereas cultivar P-1258 produced a faint amplification product (Fig. 4.18a).

Primer pair for locus MSCjaF37 yielded single locus amplification product of expected size (335-367bp) in cultivars TV-29, TV-17, TV-22, TV-20, K-1/1, T-145, T-78, TV-25, TV-18, TV-28, TV-19, TV-23 and B-157. One cultivar B-777 showed an unexpected size of amplification product revealing polymorphism in it. And few cultivars HV-39, TSS-449, BS71A76, TV-30, P-1258, TV-27, TV-1, RR17/144, CPI, TV-19 and TV-26 had no site for microsatellite repeats $(AG)_{13}(GAA)_7$ motif as there was no amplification, hence, revealing polymorphism in the cultivars (Fig. 4.18b).

Primer pair for locus MSCjaH46 yielded multiple banding patterns in all the 26 cultivars. Cultivars TV-17, TV-22, TV-20, TSS-449, BS71A76, K-1/1, TV-30, B-777, T-78 and TV-18 revealed 2 bands in each. Cultivars TV-29, HV-39, P-1258, TV-1, RR17/144, T-145, CPI, TV-19 and B-157 produced single locus specific band of the expected size of (444-464 bp). Cultivar TV-27 revealed four bands and cultivars TV-26, AV-2 and P-312 produced three bands in each exhibiting their polymorphic state (Fig. 4.18c).

4.1.6. Comparative account of DNA fingerprinting study

A detailed study of DNA fingerprinting showed that among various techniques used in this study like RAPD, PCR-RFLP (trnL-trnF gene and *rbcl* gene), and microsatellite markers, RAPD revealed the maximum polymorphism among the twenty six tea cultivars, RAPD technique revealed a quick and effective means to establish the genetic relationships between the tea cultivars without any prior knowledge of genomes or the use of polyacrylamide gels and radioactivity for resolution. Different cultivars can be conserved on the basis of their

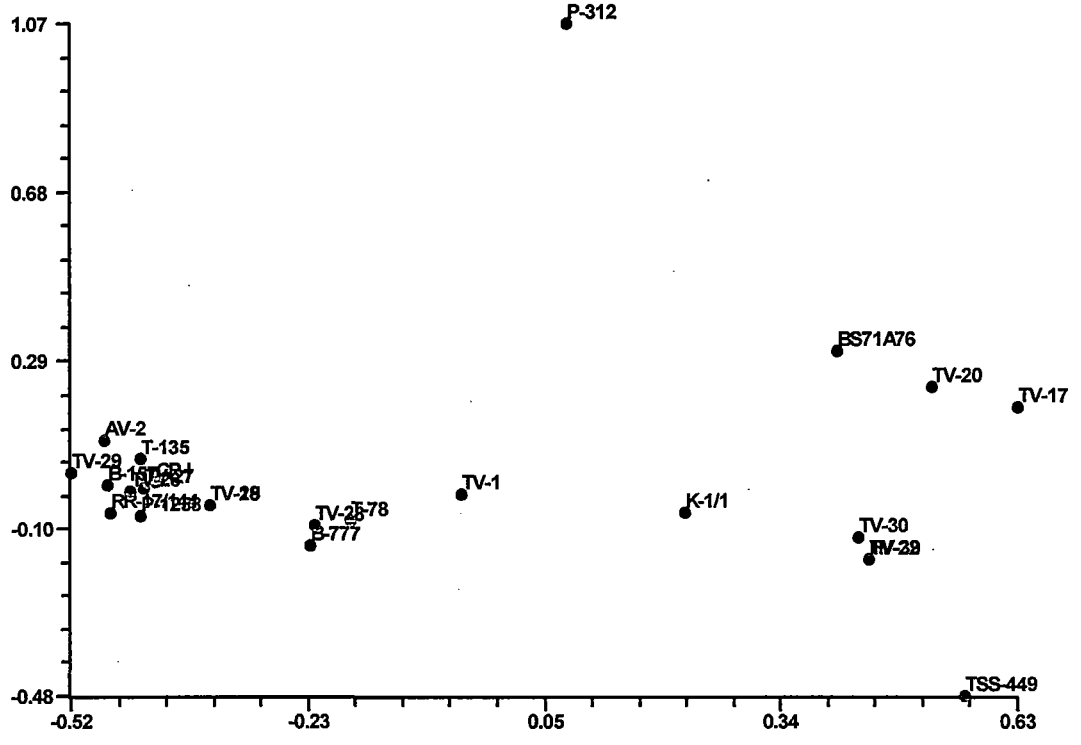


Fig. 4.14 Correspondence analysis (2D plot) of twenty six tea cultivars (RFLP markers)

molecular differences under the light of the statistical analysis using newly developed software's like POPGENE and NTSYSpc. The six primers OPA-10, OPB-04, OPB-05, OPB-9, OPB-12 and OPB-13 revealing 100% polymorphism may prove to be the most efficient markers obtained from this study along with OPA-04, OPA-11 and OPB-02 primers revealing as high as 87.5% polymorphism. Shannon's information index also showed an average of 0.44 diversity among the cultivars using thirty four primers. These markers may provide a cheap, rapid and effective means to evaluate the genetic diversity among a large number of tea varieties and help devise sampling strategies to compliment classical morpho-agronomic descriptors. PCR-RFLP technique using trnL-trnF region also revealed a good amount of polymorphism among the cultivars as it showed an average of 0.35 diversity among the cultivars as revealed by Shannon's information index. The only disadvantage of this method is that it requires locus specific primers for studying various gene locations in the genome and also it requires very pure DNA for restriction digestion studies. A tedious work done on PCR-RFLP of trnL-trnF region have provided us with a very good data i.e., sequences of ten cultivars of tea and their GenBank accession numbers has been obtained which can be helpful in future for conservation of tea Germplasm and will also help various workers working in this field. PCR-RFLP of *rbcl* gene in tea could not reveal any good amount of polymorphism among the cultivars in the present study, thus a further effort in this field is required. Microsatellite markers also gave an average

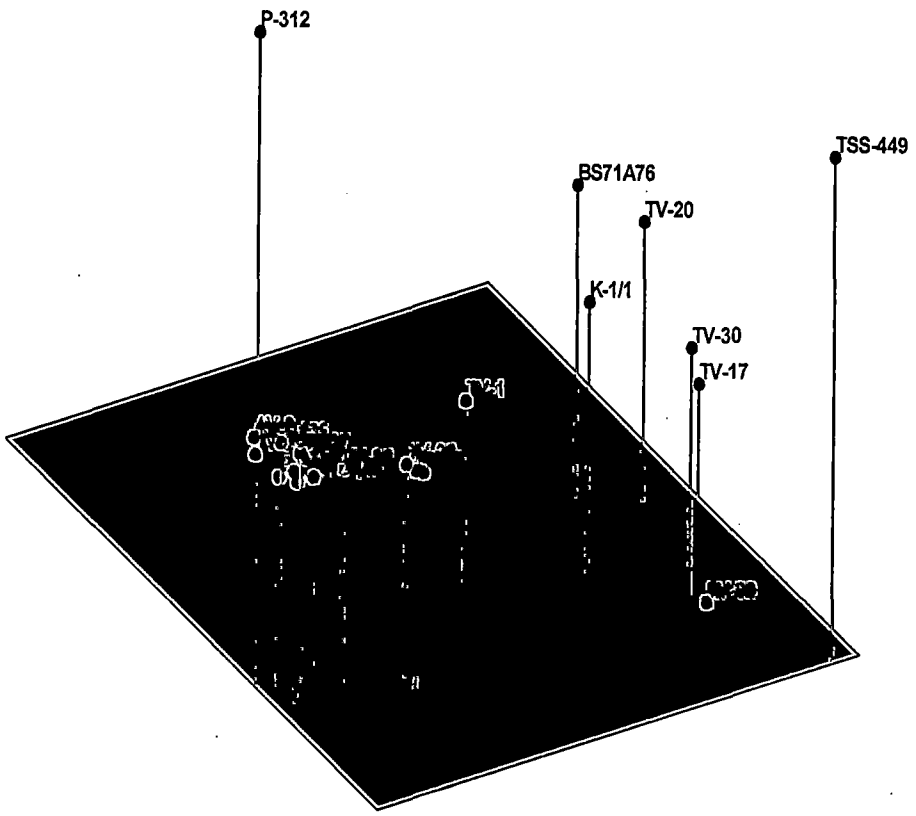


Fig. 4.15 Correspondence analysis (3D plot) of twenty six tea cultivars (RFLP markers)

```
LOCUS       EF057736 876 bp    DNA     linear   PLN 08-NOV-2006
DEFINITION  Camellia sinensis isolate B157F tRNA-Leu (trnL) gene and trnL-trnF
            intergenic spacer, partial sequence; chloroplast.
ACCESSION   EF057736
VERSION     EF057736
KEYWORDS    .
SOURCE      chloroplast Camellia sinensis
  ORGANISM  Camellia sinensis
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            asterids; Ericales; Theaceae; Camellia.
REFERENCE   1  (bases 1 to 876)
  AUTHORS   Sen,A. and Gill,M.
  TITLE     Diversity of Camellia sinensis by intergenic spacer sequences
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 876)
  AUTHORS   Sen,A. and Gill,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-OCT-2006) Department of Botany, North Bengal
            University, Molecular Genetics Laboratory, RajaRamMohunpur,
            Siliguri, West Bengal 734013, India
FEATURES             Location/Qualifiers
     source          1..876
                     /organism="Camellia sinensis"
                     /organelle="plastid:chloroplast"
                     /mol_type="genomic DNA"
                     /isolate="B157F"
                     /db_xref="taxon:4442"
     misc_feature    <1..>876
                     /note="contains tRNA-Leu (trnL) and trnL-trnF intergenic
                     spacer"
ORIGIN
        1 ccgatttgag ctcgtgtcac ccgtttcaaa taggaaacct ctctaagtga taactttcaa
       61 attcagagaa acccctggaa ttaataaaaa tgggcaatcc tgagccaaat cctgtttttc
      121 taaaacaaac aaagattccg aaagcgaaaa taaaaaaagg ataggtgcag agactcaatg
      181 gaagctgttc taacaaatag agttgactgc gttagtagag gaatccttct attgaaactt
      241 cagaaaggat gaaagagaaa cctatataca tacgcatacg tactgaaata ctatatcaaa
      301 tgattaatga cgaccccaat ctgtatccgt attttttttt atatgaaaaa tgaaaaatgg
      361 aagaattgtt gtgaatcgat tccacgttga ataaagaatt gaatattcat tgatcacatc
      421 atttactcca tagtttagtt tgatagatct tttgaagaac tgattaatcg gacgagaata
      481 aagatagagt cccattctac atgtcaatac cgacaacaat gaaatttata gtaagaggaa
      541 aatccgtcga ctttagaaat cgtgagggtt caagtccctc tatccccaaa agcccatttg
      601 actacttaac tatttatcct atccgttttt cattagcagt tccaaattag ttatctttct
      661 cactcactct actctttcac aaacagatct gagcagaaat gcttttctct tatcccaagt
      721 cttgcgatat gtgatgtgat atatagtaca cgtacaatga acatctttga gcagaaatcc
      781 ccatttgaat ttgatgattc acggtccata tcatatcgta ctgaaactta caaagtttct
      841 ttgaaatcca gaattccagg gcctggatag actgga
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Fig. 4.16a Sequence of Cultivar B-157 (Bannockburn-157) along with its accession number provided by the Genbank.

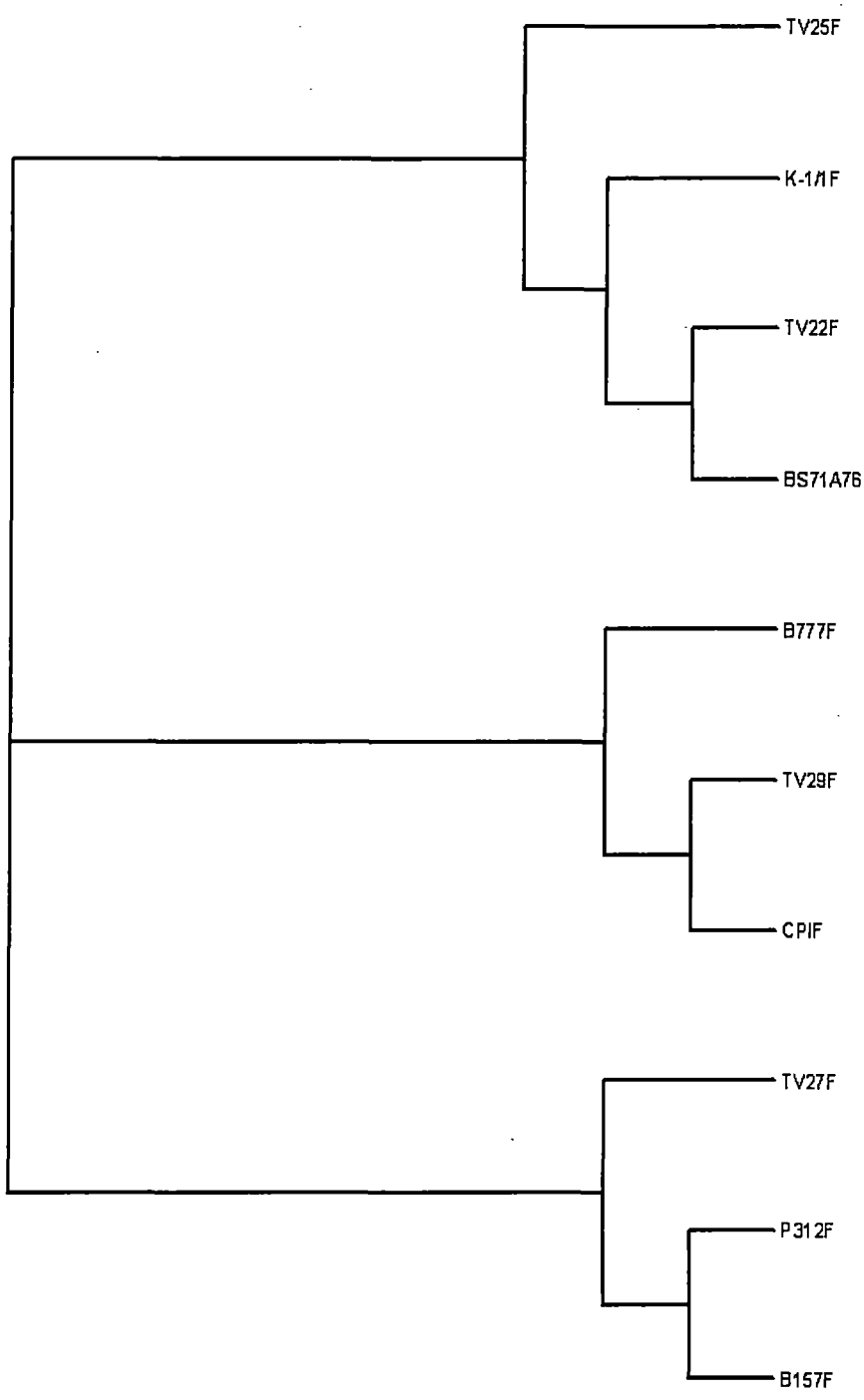


Fig. 4.16b Cladogram for ten tea cultivars sequenced using Clustal W (Thompson *et al.*, 1994)

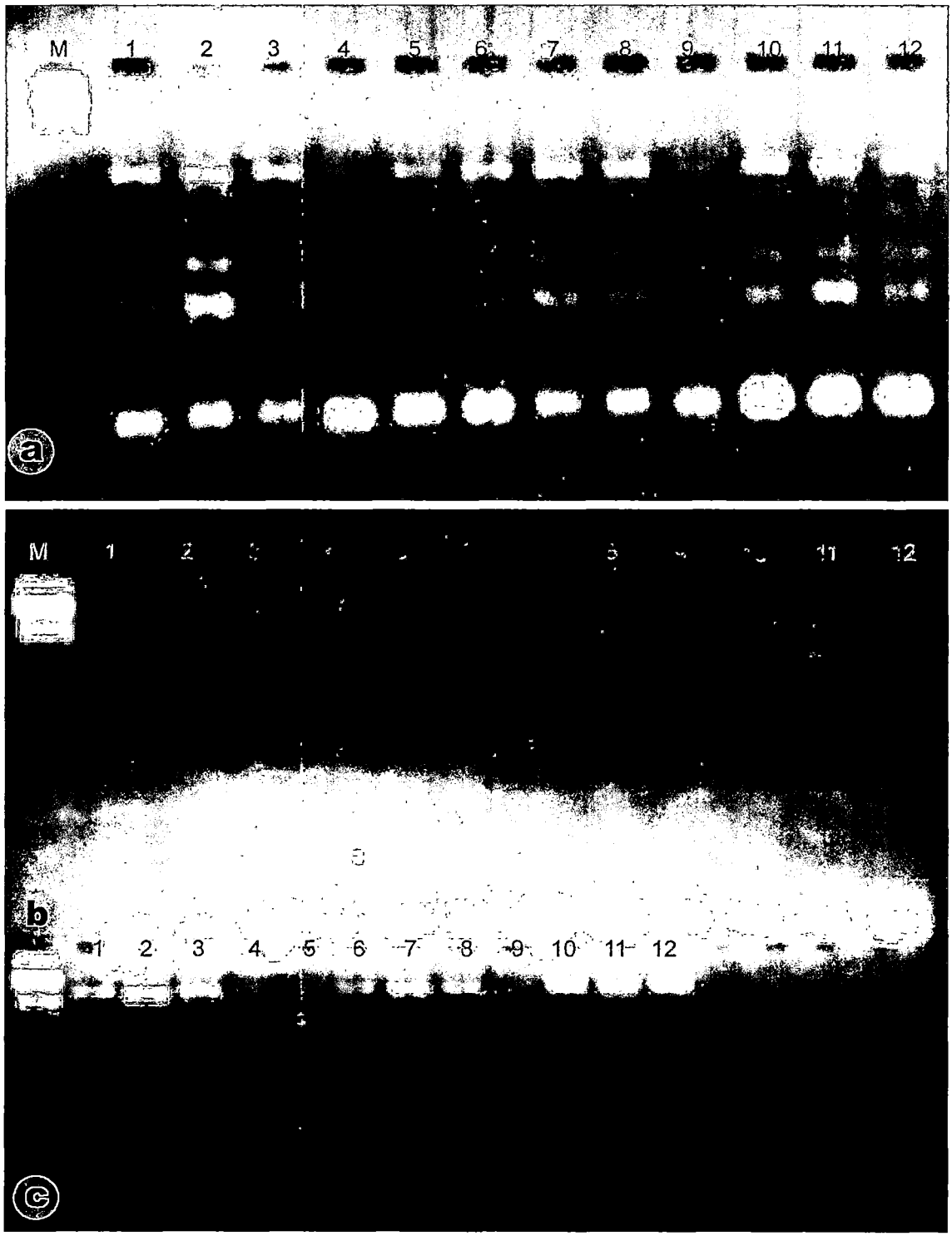


Fig. 4.17 Restriction digestion gels of PCR-Amplified *rbcl* chloroplast tea DNA using various enzymes. Lane M=Molecular marker (λDNA/*HindIII* digest), Lane 1-12: Refer Table 3.1 of Material & Methods chapter. **(a)** Restriction digestion using enzyme *AluI*. **(b)** Restriction digestion using enzyme *HhaI*. **(c)** Restriction digestion using enzyme *HinfI*.

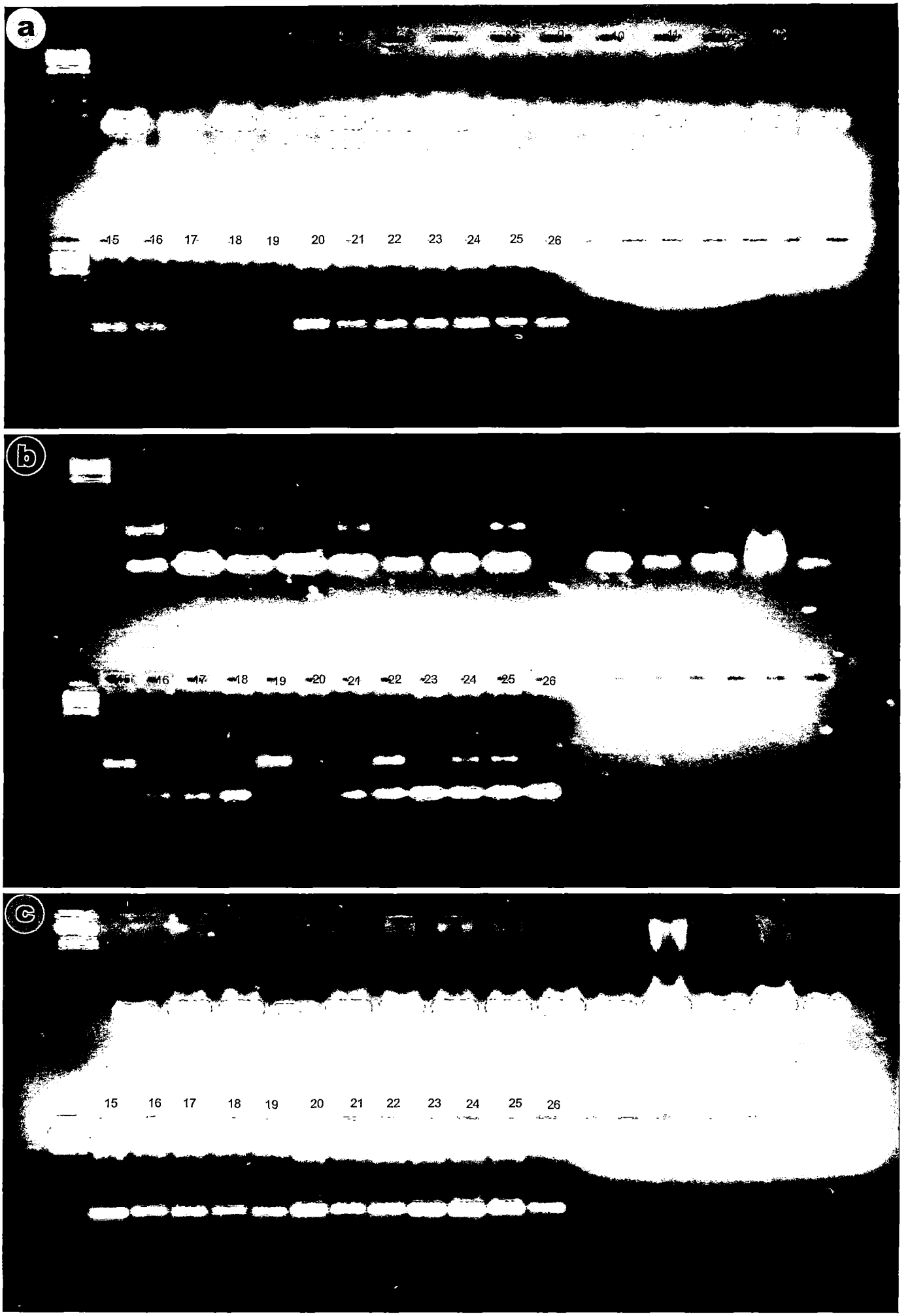


Fig. 4.18 PCR amplification products using microsatellite markers **(a)** Primer MSCjaH38, **(b)** Primer MSCjaH37 and **(c)** Primer MSCjaH46. Molecular marker(Lane M) in fig. a&b =1Kb ladder. Molecular marker (Lane M) in fig. c=λ-DNA/*HindIII* digest. Lane 1-26 in all gels refer Table 3.1 in Materials & Methods chapter.

result but proved to be good primers for amplification of *Camellia sinensis* DNA, though they were initially designed for *Camellia japonica*. The microsatellite marker study done can be further utilized by other workers interested in this field. Thus, overall DNA fingerprinting study of tea cultivars have generated lots of new markers for identification of tea and can prove to be a promising result for conservation of tea cultivars found in North Bengal.

4.2. *In vitro* culture studies

In vitro culture study was done prior to Genetic transformation of tea to standardize the protocol for developing an *in vitro* grown whole plantlet by using different explants and various hormone combinations in MS medium (Murashige and Skooge medium).

4.2.1. Establishment of callus cultures

Three types of explants were inoculated: epidermal layers, intact stem segments (nodal and internodal) and without epidermal layers (stripped segments) using cultivar TV-26 (Refer Materials and Methods section *in vitro* culture studies). The epidermal layers contained epidermis and cortex including three to four cell layers, while intact stem segments had epidermal layers. The basal medium contained MS inorganic salts supplemented with vitamins (Hi-media make) see appendix I for composition, 100mg/l Myoinositol, 3% sucrose, 1% agar and 500mg/l of MES buffer was added to the MS medium. Four combinations of hormones including IBA (indol-3-butyric acid), BA (N6-benzylaminopurine), Kinetin, NAA, GA_3, were added to the basal medium as plant growth regulators. The pH was adjusted to 5.6 before autoclaving. The cultures were maintained at 25°C±2°C under a 12/12 h (day/night) photoperiod with light provided by cool fluorescent tubes at a photon flux density of 52μmol $m^{-2}s^{-1}$. Callus initiation from the three explants was observed after 15 days of culture on the induction medium (Refer Material and Methods section, Table 3.7). Callus formation, which occurred 1 month after inoculation is given in (Table 4.8 and Fig. 4.19a). The percentage of callus formation from epidermal layers was lower than that from other explants after 1 month of culture, but after 2 months of culture, most of the epidermal layers formed callus and the multiplication of the callus was identical in the three types of explants (Table 4.8).

4.2.2. Regeneration of plant from callus cultures

Callus tissues were cultured on the callus induction medium (Refer Materials and Methods section, Table 3.7) for 2 months and thereafter transferred to shooting medium containing 0.5mg/l IBA and 10 mg/l BA and to 1μM TDZ. After 2 months the callus from the epi-

Table 4.8 Percentage of callus formation from the stem (1 month) in cultivar TV-26

Growth regulator mg/l	Epidermal layer	Stem segments	Stripped segments
Kinetin (0.2)+NAA (2)	80.8	100	88.5
IBA (2)+BA (4)	68	70.5	82.4
BA (0.5)+NAA (0.1)+GA (0.1)	79.5	84.4	90.6
BA (4)+IBA (2)	78	90.2	89.5

Table 4.9 Differences of bud formation in three types of explants. Callus tissues were cultured on MS medium supplemented with 0.5mg/l IBA and 10mg/l BA for 6 months

Type of explant	Number of inoculated calli	Percentage of bud formation
Epidermal layers	20	30
Stem segments	20	5
Stripped segments	20	0

dermal layers, Stem segments and stripped segments began to form adventitious buds (Fig. 4.19b). These buds which formed in 20% of the calli, were subcultured monthly on the same medium until they grew sufficiently to be transferred to the rooting medium (Table 4.8). Buds observed from the callus derived from the epidermal layers continued to grow during subcultures. The callus derived from the stem segments differentiated very small buds and failed to grow in subcultures (Table 4.9). These buds formed many buds or reverted back to the callus state in subcultures. Buds did not develop further to enable transfer to the rooting medium even when maintained for 1 year in subcultures. The callus from the stripped segments did not form any buds. The shoot buds from the callus of the epidermal layers were transferred for rooting in to the semi liquid medium containing half strength MS salts and supplemented with 1 and 0.5mg/l IBA and 1μM TDZ. Root initiation was seen to occur 15 days after inoculation on the rooting medium. The number of shoots which formed roots was larger on the medium with 0.5mg/l IBA and also in 1μM TDZ than on that with 1mg/l IBA (Fig. 4.19c). After 1month and 15 days in the rooting medium, the plantlets were transplanted to pots containing a mixture of vermiculite and soil (Fig. 4.19d). Although the dissection of the stem was a complex procedure, the callus of epidermal layers was softer than that of stem segments and stripped segments. Generally pre-cultured explants induced softer callus than intact explants. In tea plants, stem segments from micropropagated shoots on the medium supplemented with 0.5mg/l IBA and 10mg/l BA induced callus, adventitious buds were regenerated on these calli (Kato 1986c). When the precultured explant was used, the callus of the epidermal layers regenerated adventitious buds at a higher percentage than that of stem segments or stripped segments (Table 4.10).

4.2.3. Formation of Somatic embryos and whole plantlet from cotyledon culture

4.2.3.1. Somatic embryo formation

The seed of a mature fruit of *Camellia sinensis* contains two large cotyledons (Fig.4.20a). Somatic embryo formation is performed through cotyledon culture (Refer Material and Methods section, Table 3.8). The cotyledons were cut into four to five pieces after removing embryos from the seeds, and these slices of cotyledons were inoculated as explants (Fig.4.20b). The basal medium and growth conditions were the same as in callus culture. BA, NAA, IBA and GA_3 were added as plant

Table 4.10 Differences of bud formation in three types of pre-cultured explants. Callus tissues were cultured on MS medium supplemented with 0.5mg/l IBA and 10mg/l BA for 3 months

Type of explant	Number of inoculated calli	Percentage of bud formation
Epidermal layers	20	30
Stem segments	20	10
Stripped segments	20	0

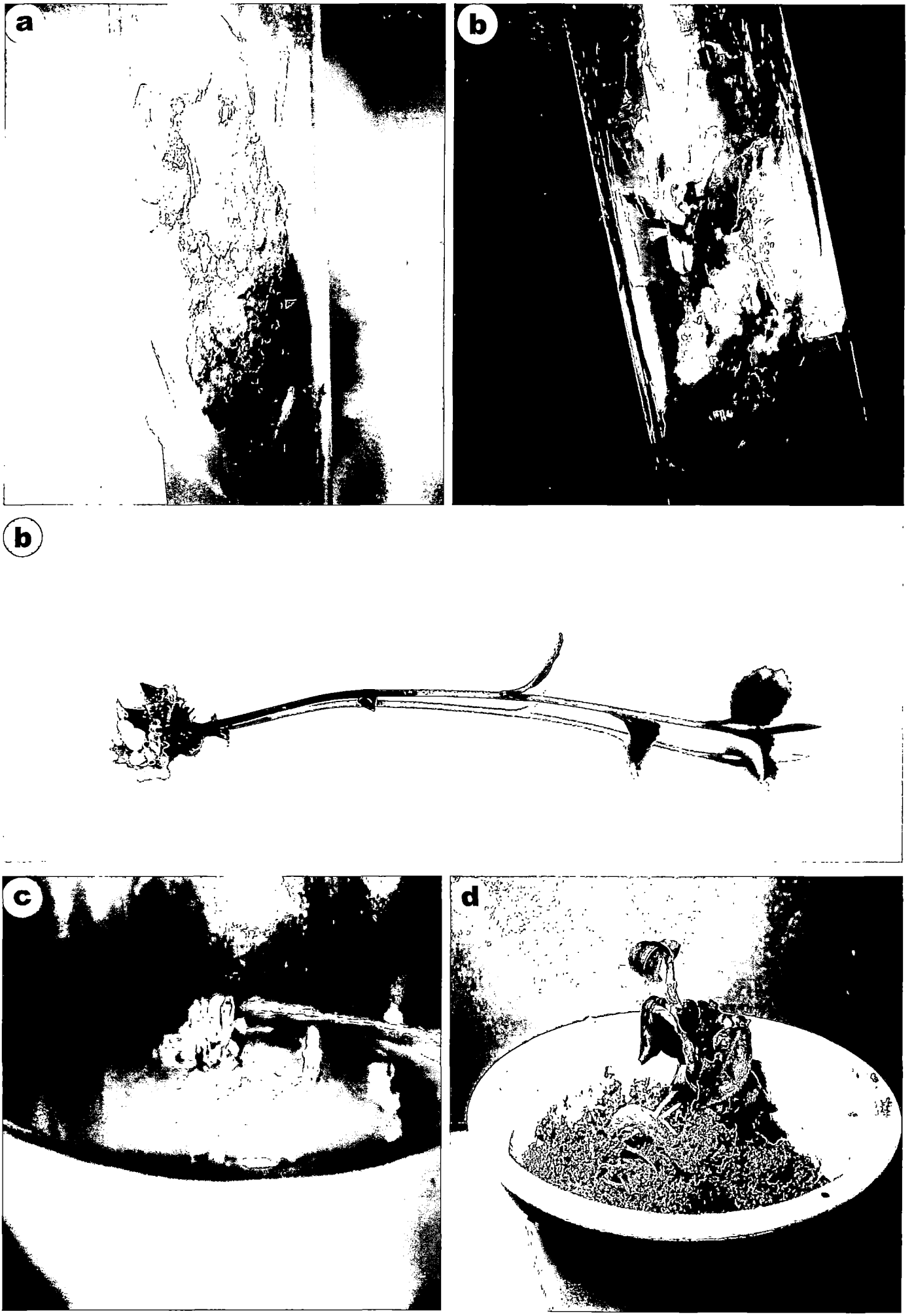


Fig. 4.19 Stages of callus initiation and regeneration from stem segment of TV-26 cultivar **(a)** Callus formation after 1 month of explant inoculation **(b)** Formation of adventitious buds from callus after 2 months **(c)** Formation of roots from *in vitro* grown shoots **(d)** Transfer and hardening of *in vitro* grown plantlet into pot containing soil:vermiculite.

growth regulators. Slices of cotyledon of *Camellia sinensis* (TV-26) swelled gradually 1 week after inoculation. Many adventitious embryos were formed directly on the swollen parts, which did not form callus from the surface of cotyledons (Fig. 4.20c). The formation of many adventitious embryos extended to the whole swollen parts after 2 months of inoculation (Table 4.11). The number of adventitious embryos increased during the subcultures in the same medium as for the induction of adventitious embryos with the exception of the medium supplemented with 2mg/l IBA and 4mg/l BA (Table 4.11), these embryos were transferred to the medium supplemented with 1mg/l GA_3 for the development of the shoots and roots. After 1 month of culture on the GA_3 medium it was observed that shoots and roots did not grow in spite of the swelling of the embryos on the GA_3 medium. The additional embryos induced embryogenesis with subcultures in the same medium for cotyledon inoculation (Table 4.11).

4.2.3.2. Plantlet regeneration from embryos formed

The embryos so formed were transferred to the medium containing 0.5mg/l IBA and 10mg/l BA; 0.5mg/l BA, 0.1mg/l NAA and 0.1mg/l GA_3 where shoot growth and rooting were promoted to the extent of enabling their transplantation to pots. Some embryos differentiated many adventitious buds as axillary buds (Fig. 4.20d). These shoot buds were transferred for rooting into the semi liquid medium containing half strength MS salts and supplemented with 0.5mg/l IBA and 1μM TDZ. It was observed that 1μM TDZ containing semi liquid MS medium increased the number of roots (Fig. 4.20e). Numerous plantlets were obtained from one seed. Plantlets from somatic embryos were established almost 8-10 months after inoculation of cotyledon slices.

4.2.4. Transfer of plants to Soil

Plantlets were transplanted to pots containing a mixture of vermiculite and soil (Fig. 4.20f). They were maintained for 2 or 3 weeks under plastic covers in order to avoid their dessication in the growth chamber or in the laboratory. It is possible to maintain plantlets in flasks for several months in a cold chamber under light before transplanting them. Four weeks after transplantation, pots were transferred to the Greenhouse in Sikkim Council of Science and Technology. Plantlets derived from cotyledons exhibit healthy growth and their growth is the same as that of seedlings under natural conditions.

4.3. Histological observations of the cultured cotyledons

Through histological observations of cotyledons it was noted that callus did not develop on the cotyledon surface.

A transverse section of embryogenesis on the surface of the cotyledon slices in *Camellia*

Table 4.11 Embryo formation on slices of cotyledons (cultured for 2 months) as induced by various hormones in cultivar TV-26

Growth regulator mg/l	Inoculated slices	Slices of differentiated embryos
BA(10)+IBA(0.5)	100	95
BA(0.5)+NAA(0.1)+GA(0.1)	100	90
BA(2)+IBA(4)	100	60
TDZ(1μM)	100	40

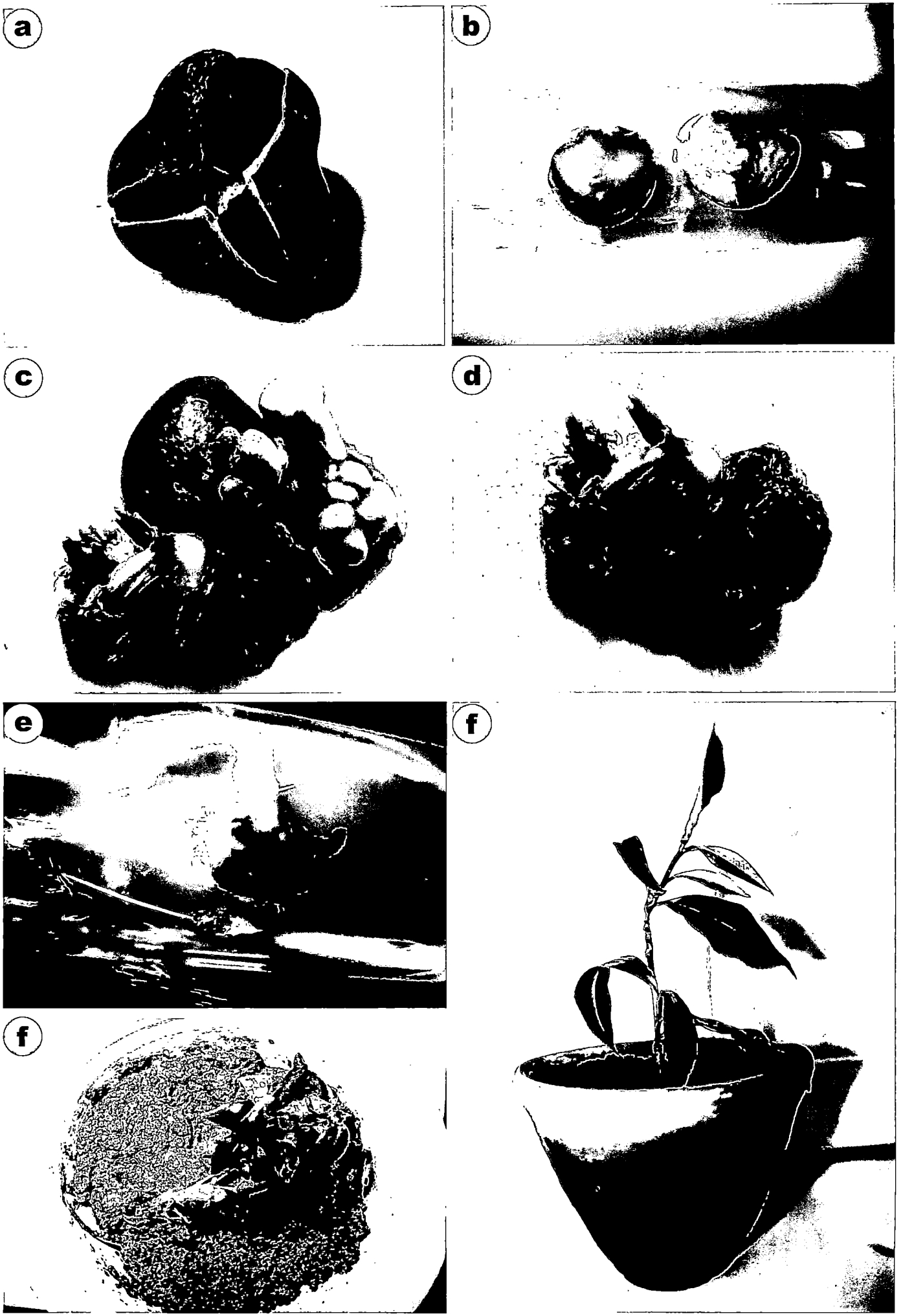


Fig. 4.20 Stages of embryo formation and regeneration from cotyledonary explant of TV-26 cultivar. **(a)** A mature fruit of TV-26 containing two large cotyledons **(b)** Slices of cotyledon inoculated on embryo induction medium **(c)** Formation of adventitious embryos on cotyledon **(d)** Shooting from embryos **(e)** Rooting from *in vitro* grown shoots of TV-26 **(f)** Transfer and hardening of *in vitro* grown plantlets into pot containing soil and vermiculite.

sinensis (TV-26) revealed the following structure under 10X power in a phase contrast microscope (Fig. 4.21).

- Anthocyanin pigmentation on the edges of the section observed in the form of red spots as stained by safranin (Fig. 4.21a-1).
- Primordium of embryo development from the surface of the cotyledon slices observed in the form of mass of compact cells arranged sequentially on the edge of section (Fig. 4.21a-2).
- Proembryonic globules differentiation and separation (Fig.4.21a-3).
- Proembryonic globules with well differentiated epidermis (Fig. 4.21a-4).
- Details of epidermal cell divisions like periclinal, anticlinal and mitotically active cells (Fig.4.21a-5).

Longitudinal section of 1 month old embryo on the surface of cotyledon revealed the following structure under 10X power in a phase contrast microscope (Fig. 4.21b).

- Embryonic axis with shoot meristem, cortical tissues, central cylinder and procambial strands were clearly visible.

4.4. Genetic transformation study

Analysis of the studies by different gene transfer methods to evaluate the suitability of their use in tea

4.4.1. Agrobacterium mediated transformation of Tea

This is the most commonly used procedure so far used in the present study for introducing alien genes into a majority of dicots and, of late, some monocots is mediated by the vectors based on the soil bacterium *Agrobacterium tumefaciens*. This bacterium carries a large plasmid (of around 200 Kb in size) called the tumor inducing plasmid (Ti plasmid). One of the most significant achievements in the area of plant biology is the elucidation of the mechanism by which *A. tumefaciens* delivers the transfer DNA (T-DNA) to the plant cell through a cascade of events, which include induction of vir gene, formation of the T-DNA intermediates and T strand and the transfer of the latter to the plant cell (Hooykaas & Schilperoort, 1992; An *et al*, 1988). The method was standardized for transforming the somatic embryos formed from cotyledonary explants of cultivar TV-26 (Table 4.11) of *Camellia sinensis* by optimizing the various parameters required for *Agrobacterium* mediated gene transformation (Using the strain LBA4404 in pCAMBIA vector) as given below.

- Evaluation of the antibiotic effect on somatic embryos and *Agrobacterium*

Increased level of phytotoxic antibiotic kanamycin showed a gradual reduction in the fresh weight of somatic embryos. Bleaching of tissue occurred due to the loss of chlorophyll pigmentation and arrest of growth at 35mg/l kanamycin. All somatic embryos were necrotic at 45mg/l kanamycin within 6-8 weeks. Kanamycin is generally used in the range of 30mg/l to 150mg/l for selecting transformed plant cells (Manders *et al.* 1994). In *Camellia*, 75mg/l of was identified to be the lethal dose of kanamycin for *in vitro* internodes (Tosca *et al.* 1996) The optimum bactericidal concentration was 300mg/l regardless of the antibiotic used. Of the two antibiotics tested, Cefotaxime (300mg/l) had a negligible effect on the growth and organogenic response of the somatic em-

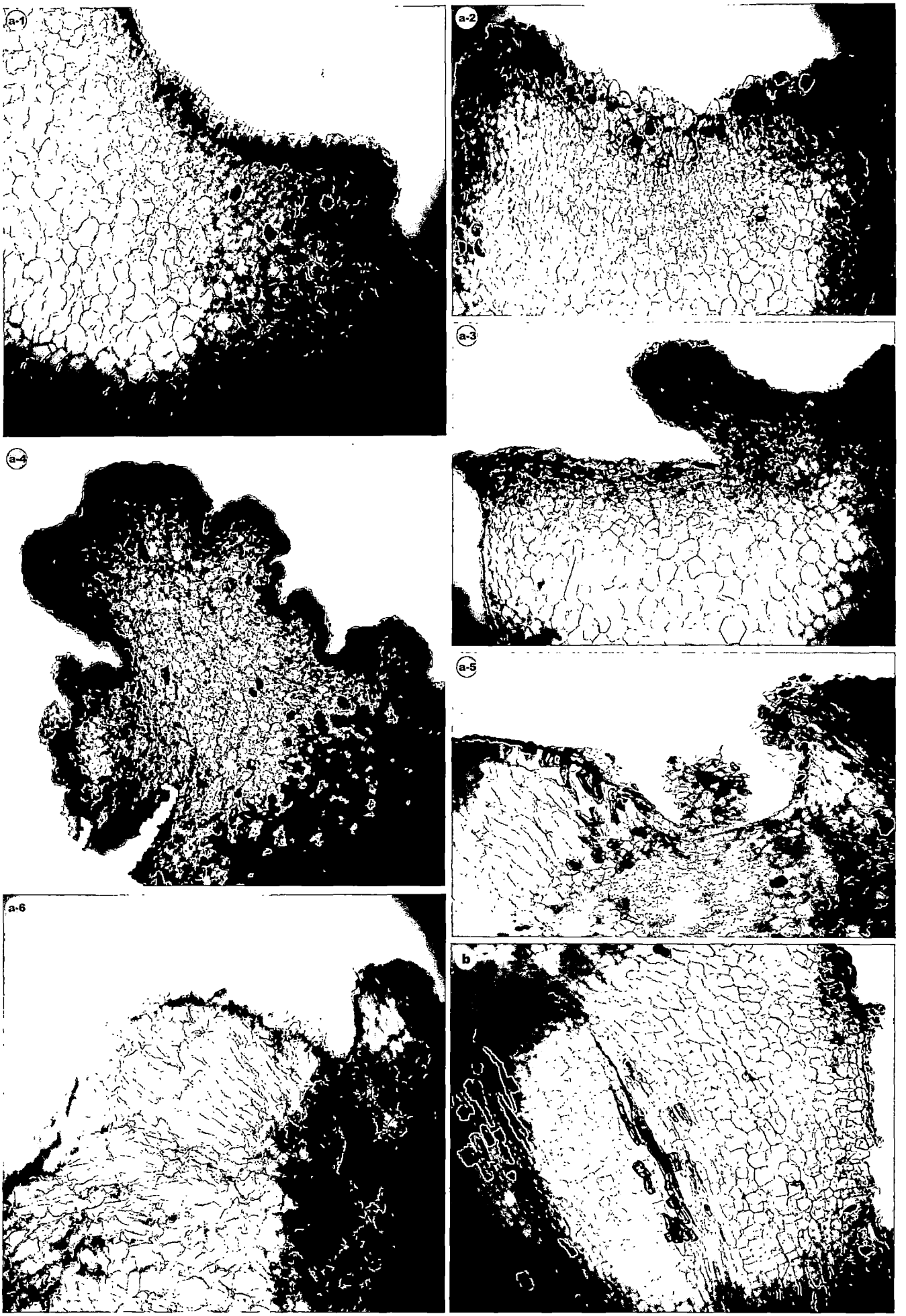


Fig. 4.21 Histology of various stages of embryogenesis from cotyledonary explant of TV-26 as observed under 10X of phase contrast microscope. **(a)** Transverse section of embryogenic tissues. **(a-1)** Anthocyanin pigmentation on the edges of the section. **(a-2)** Formation of primordium of embryo from the surface of cotyledon. **(a-3)** Differentiation and separation of proembryonic globules. **(a-4)** Formation of epidermis on proembryonic globules. **(a-5)** Periclinal and anticlinal divisions of epidermis. **(b)** Longitudinal section showing embryonic axis with cortical tissues and procambial strands.

bryos while being the most effective (followed by Cephalexin) in controlling overgrowth of *Agrobacterium.*

- Bacterial growth phase (OD value)

At a log phase corresponding to OD_{600}=0.6, maximum transformation as measured by the *GUS* assay was achieved (Fig. 4.22a). LBA4404 showed a *GUS* expression of 50%. An increase or decrease in the OD value resulted in a decrease in transformation efficiency. OD values greater than 0.8 were not suitable for transformation, and extensive tissue damage occurred at OD values greater than 1.0 due to bacterial overgrowth. At a higher OD, the regeneration of plant tissue in Citrus was inhibited by bacterial induced stress, and it also became difficult to control the overgrowth of bacteria following co-cultivation (Pena *et al.* 1995). A late log phase corresponding to an OD of 0.6 was the most effective for obtaining high rates of transformation in almond (Archilleti *et al.* 1995).

Of the range of bacterial cell densities tested at OD~0.6, 10^9 cells/ml was the optimum. The requirement for an ideal bacterial density has been shown to vary with plant species. In Citrus, maximum transformation was achieved with $4X10^7$ cells/ml (Pena *et al.* 1995), while in hybrid poplars 10^7 cfu/ml gave the maximum transformation efficiency (Howe *et al.* 1994). In the case of poplars, transformation frequencies varied greatly with the genotype as well as the *Agrobacterium* strain used (Confalonieri *et al.* 1997).

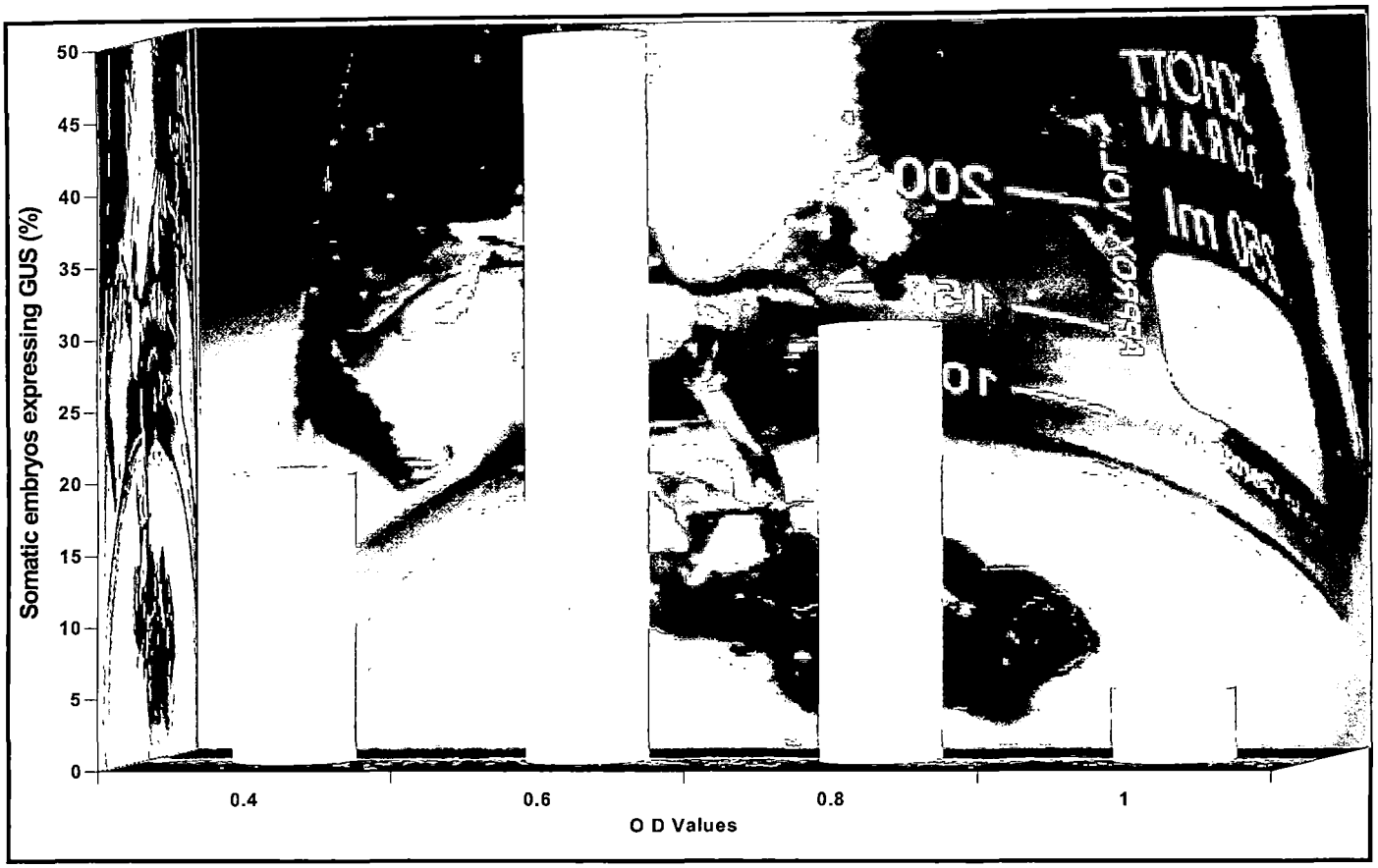
- Wounding effect

The maximum number of transformation events (approx. 50%) were scored using a 2 second SAAT treated somatic embryos (Fig. 4.22b). When somatic embryos were not wounded by SAAT treatment, a lower number of transformation events (45%) were noticed. Thus, SAAT treatment was found to be effective for inducing transformation in tea embryos. This was also observed in several species including soybean, cowpea, maize, wheat and white spruce (Finer and Trick 1997)

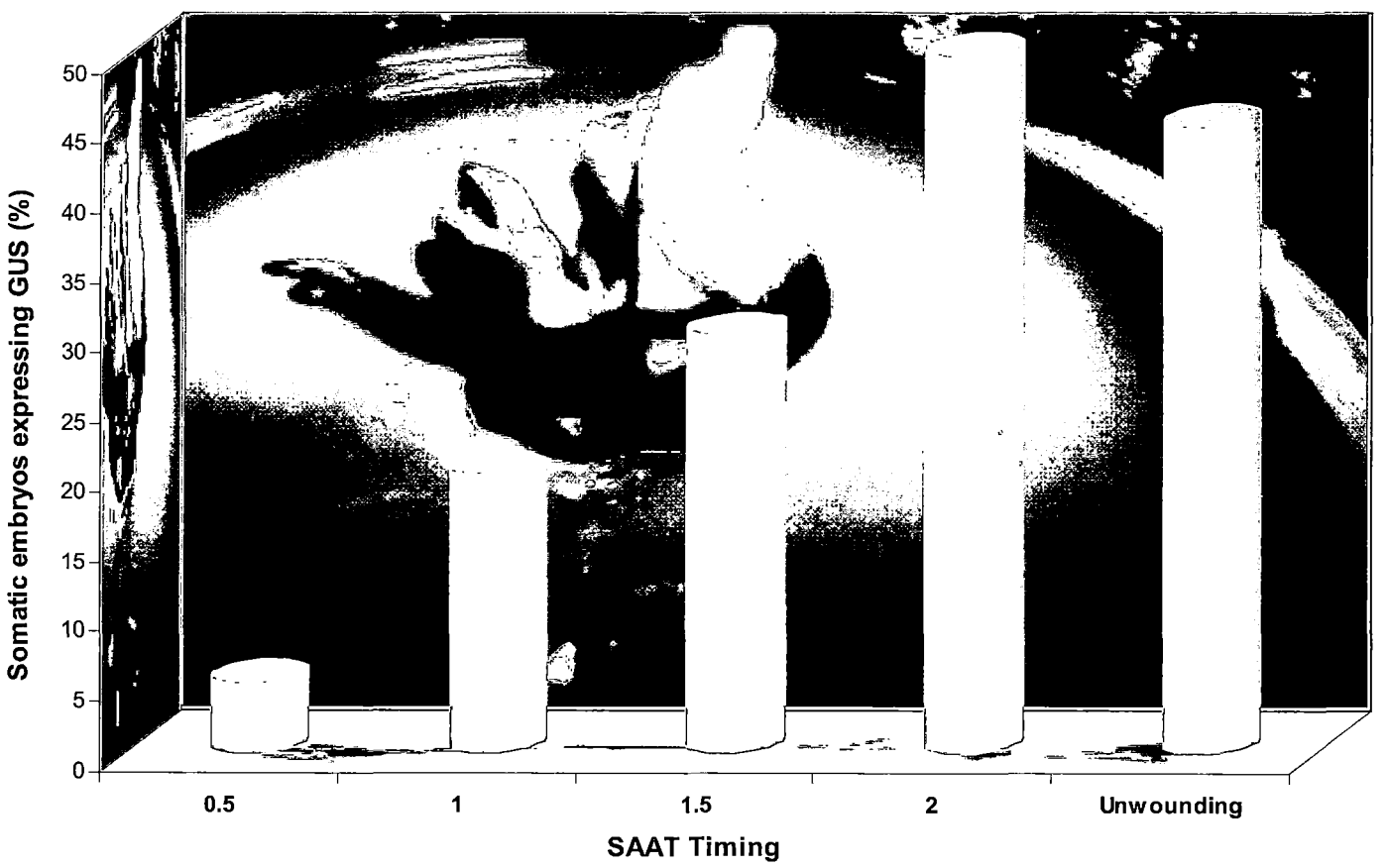
- Co-cultivation period and pH of the medium

The co-cultivation of somatic embryos with LBA4404 for 4 days resulted in high frequency transformation (Fig. 4.22c). Co-cultivation for 2-7 days is generally considered to be suitable for *Agrobacterium* mediated transformation, as reported for many plant species. More than 4 days of co-cultivation encouraged an overgrowth of bacteria with a concomitant decrease in transformation efficiency in garden pea (De Kathen and Jacobsen 1990) and flax (Dong and McHughen 1993). Co-cultivation for more than 2 days led to a decrease in transformation frequency and about 50% explant loss because of bacterial overgrowth in experiments designed to produce transgenic Datura (Ducrocq *et al.* 1994). The differential requirement of co-cultivation period largely depends upon the *Agrobacterium* strain used or the medium for bacterial culture or co-cultivation. (Mondal *et al.* 2001).

In the present study, co-cultivation medium with a pH of 5.6 yielded the highest number of transformation events (Fig. 4.22d). The influence of the pH of the co-cultivation medium on transformation efficiency is well documented in literature. Aliev *et al* (1988) observed that the virulence of *A. tumefaciens* was related to the pH of the leaf and stem cell



(a) GUS% expressed by somatic embryos under various O.D values.



(b) GUS%expressed by somatic embryos under various SAAT timings

Fig. 4.22 Graphical representation of somatic embryos expressing GUS% under various parameters.

sap in cotton (*Gossypium hirsutum cv.* 109F), with the best infectivity being achieved at pH 5.5. In potato leaf discs a pH of 5.6 was the optimum for inciting the highest number of tumours when five different strains of *Agrobacterium* were tested (Boudjeniba and Hunault 1989). An acidic pH of 5.5 is generally considered to be suitable as acidic pHs may induce the vir (virulence) genes (Stachel *et al.* 1986; Alt-Moerbe *et al.* 1988).

- Acetosyringone treatment

The use of the phenolic inducer, acetosyringone, did not enhance the efficiency of transformation in the present study. Similarly, in several other experiments on woody plants, particularly in plum (Mannie *et al.* 1991) and poplars (Confaloneiri *et al.* 1997), acetosyringone did not help in increasing transformation efficiency. The suppression of transformation frequency by acetosyringone has also been reported in other varieties of poplars (De Kathen and Jacobsen 1990). The inability of acetosyringone could be due to the inherent prevalence of high amounts of phenolics in woody plant tissues such as tea (Mondal *et al.* 2001).

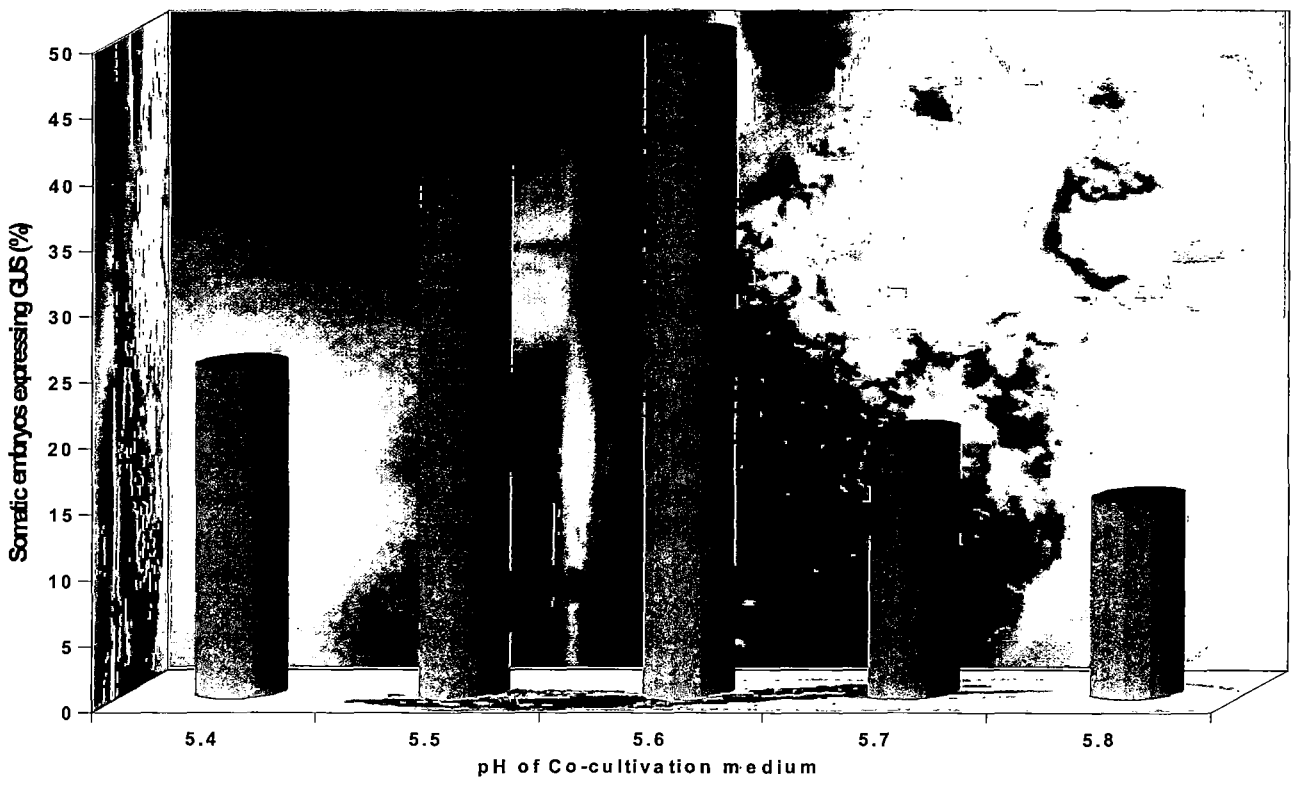
4.4.1.1. GUS activity of putative transformed somatic embryos from cotyledons and embryo-derived plants

The histochemical assay of *GUS* activity was carried with the help of the ß-Glucuronidase (*GUS*) reporter Gene Staining kit (SIGMA, product No. *GUS*-S). Transient *GUS* activity of pCAMBIA LBA4404 was detected by characteristic blue spots which appeared on the somatic embryos and leaf tissues 48h following co-cultivation. Eventually this blue colouration became uniformly distributed over the leaf surface (Fig. 4.23a). Putative transformed somatic embryos were selected, based on their growth on MS-104 medium augmented with lethal dose of Kanamycin (50mg/l) Following successful growth in MS-104 medium containing MS+0.5mg/lIBA+10mg/lBA+75mg/l kanamycin, shoot regeneration occurred (Fig. 4.23b). A very strong *GUS* signal was detected in the leaf tissues from 1 year old shoots recovered through the germination of the *GUS*-indexed kanamycin resistant somatic embryos. No endogenous *GUS* expression was detectable in somatic embryos or tissues that had not undergone an *Agrobacterium* infection.

4.4.1.2. Molecular characterization of the transformed plants

DNA obtained from several independent kanamycin resistant, *GUS*-positive lines revealed the specific predicted amplification product of 693bp with *nptII* among the transformed plants (Fig. 4.23c). This indicated the presence of marker transgene *nptII* as a single T-DNA in the transformed genome of TV-26 cultivar of tea. No amplification product was detected in DNA from untransformed shoots when subjected to PCR amplification with *nptII* specific primers (Fig. 4.23c). The failure of some kanamycin resistant transformants two samples to produce any PCR band for *nptII* specific primer may indicate false positives ('escapes') through antibiotic selection despite an increase in the kanamycin concentration from 50mg/l to 75mg/l during subsequent stages of selection. 'Escape' is a major problem in woody plant transformation (Pena *et al.* 1995). Nevertheless, it is also important to strike a balance between the require-

(c) GUS%expressed by somatic embryos under various Co-cultivation period



(d) GUS%expressed by somatic embryos under various pH of Co-cultivation medium.

Fig. 4.22 Graphical representation of somatic embryos expressing GUS% under various parameters.

ment for a high concentration of selective agent and its inhibitory effect on plant regeneration (Dong and McHughen 1993).

4.4.1.3. Establishment of transformed plants

The transformed microshoots were multipled *in vitro* on MS-104 medium containing MS+0.5mg/lIBA+10mg/lBA and rooting was established by transferring the *in vitro* grown microshoots onto semi-liquid 1/2MS medium containing 0.5mg/l IBA and also subjected to 1μM TDZ for multiplication of roots as it enhanced the number of roots production in *in vitro* grown whole tea plantlets (Fig.4.23d).

4.4.1.4. Transfer of transformed plants to Soil

Plantlets were transplanted to pots containing a mixture of vermiculite and soil (Fig. 4.23e). They were maintained for 2 or 3 weeks under plastic covers in order to avoid their dessication in the growth chamber or in the laboratory. It is possible to maintain plantlets in flasks for several months in a cold chamber under light before transplanting them. Four weeks after transplantation, pots were transferred to the greenhouse. Plantlets derived from cotyledons exhibit healthy growth and their growth is the same as that of seedlings under natural conditions.

4.4.2. Particle Bombardment- Mediated Transformation of tea

The ultimate goal of plant genetic engineering is to produce novel transgenic plants with improved agronomic traits. The production of transgenic plants depends among other factors on the stable introduction of foreign gene into the plant genome. A large number of gene transfer methods are currently available. However, the method of gene transfer using the particle Delivery System (PDS/1000He, Biorad, Inc., USA) is the most versatile (Christou, 1996) and has proven successful in introducing foreign genes not only to the nucleus but also to the chloroplasts in plants (Svab & Maliga, 1993). In the present study the method is used by standardizing a number of parameters involving transformation through particle bombardment in leaf tissue of *in vitro* grown plantlet of tea and callus of tea derived from *in vitro* grown epidermal layers, intact stem segments (nodal and internodal) and without epidermal layers (stripped segments) (Table 4.8). The construct used was pZP200KC engineered for nuclear expression from the 35S promoter. The most important part of the experiment was sterilization of the discs, stopping screens, gun parts, gun vaccum chamber components etc. Several pieces of *in vitro* grown leaf tissue and 15-20 days old embryogenic callus formed on MS+Kin.0.2mg/ml+NAA2mg/ml were used as samples to be shot in present experiment. Preparation of samples for 6 shots was taken over with utmost care keeping in mind the preference for addition of the components in order like 50μl tungsten suspension+ 10μl DNA in TE buffer (1μg/μl) + 50μl 2.5M $CaCl_2$ + 20μl 0.1M Spermidine (free base). For shooting the callus BioRad shooting kit was used containing one 1100p.s.i. rupture disc (the small dark brown discs), one flying disc (the large orange discs), and one stopping screen (the wire mesh screens) for each shot as per the user's instructions.

4.4.2.1. GUS activity of putative transformed embryogenic callus and embryo-

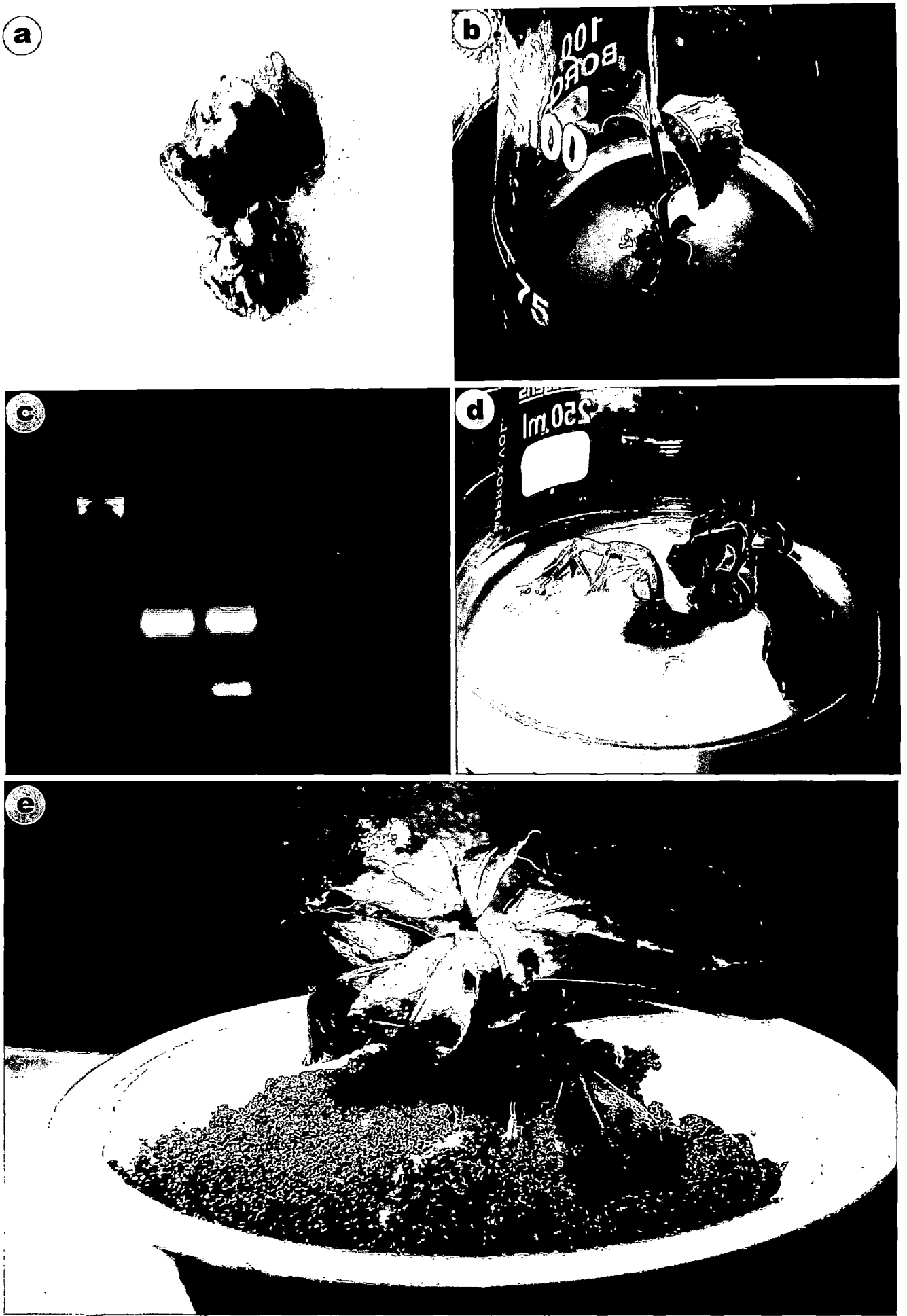


Fig. 4.23 Transgene integration in somatic embryos from cotyledon and their regeneration stages. **(a)** GUS assay showing blue staining of transformed somatic embryos. **(b)** Shooting of transformed somatic embryos from cotyledon of TV-26 cultivar. **(c)** PCR amplification for confirmation of transgene (*NptII gene*) into the tea (TV-26) plant. Lane M=λ-DNA/*HindIII* digest, Lane 1: (+)ve control (plasmid DNA LBA4404), Lane 2: Transformed plant DNA and Lane 3: (-)ve control (non-transformed plant DNA) **(d)** Rooting from transformed *in vitro* grown shoots of TV-26 cultivar. **(e)** Pot transfer and hardening of transformed *in vitro* grown plantlet of TV-26 cultivar.

derived plants

Transient expression of the construct (pZP200KC and pCAMBIA) was monitored in the callus and leaf tissue, 24-48 hours after shooting and co-cultivation, by staining the shot callus and leaf tissue for *GUS* activity (Bansal *et al.*, 1992). The histochemical assay of *GUS* activity was carried with the help of the ß-Glucuronidase (*GUS*) reporter Gene Staining kit (SIGMA, product No. *GUS*-S). Transient *GUS* activity was detected by characteristic blue spots which appeared on the callus and leaf tissue (70%), 48h following co-cultivation. Eventually this blue colouration became uniformly distributed over the entire callus and leaf tissue (Fig. 4.24a). Putative transformed embryogenic calli were selected, based on their growth on MS-104 medium augmented with lethal dose of Kanamycin (50mg/l). Following successful growth of these embryogenic calli in MS-104 medium containing MS+0.5mg/lIBA+10mg/lBA+75mg/l kanamycin shoot regeneration occurred (Fig. 4.24b). A very strong *GUS* signal was detected in the leaf tissues from 1 year old shoots recovered through the differentiation of the *GUS*-indexed kanamycin resistant embryogenic calli. No endogenous *GUS* expression was detectable in embryogenic calli that had not undergone particle bombardment.

4.4.2.2. Molecular characterization of the transformed plants

DNA obtained from several independent kanamycin resistant, *GUS*-positive lines revealed the specific predicted amplification product of 693bp with *nptII* in the transformed plant (Fig. 4.24c). This indicated the presence of marker transgene *nptII* as a single T-DNA in the transformed genome. No amplification product was detected in DNA from untransformed shoots when subjected to PCR amplification with *nptII* specific primers (Fig. 4.24c). The failure of some kanamycin resistant transformants in one of the transformed plant to produce any PCR band for *nptII* specific primer may indicate false positives ('escapes') through antibiotic selection despite an increase in the kanamycin concentration from 50mg/l to 75mg/l during subsequent stages of selection. 'Escape' is a major problem in woody plant transformation (Pena *et al.* 1995). Nevertheless, it is also important to strike a balance between the requirement for a high concentration of selective agent and its inhibitory effect on plant regeneration (Dong and McHughen 1993).

4.4.2.3. Establishment of transformed plants

The transgenic microshoots were multipled *in vitro* on MS-104 medium containing MS+0.5mg/lIBA+10mg/lBA and rooting was established by transferring the *in vitro* grown microshoots onto semi-liquid 1/2MS medium containing 0.5mg/l IBA and also subjected to 1µM TDZ for multiplication of roots as it enhanced the number of roots production in *in vitro* grown whole tea plantlets (Fig. 4.24d).

4.4.2.4. Transfer of transformed plants to Soil

Plantlets were transplanted to pots containing a mixture of vermiculite and soil (Fig. 4.24e). They were maintained for 2 or 3 weeks under plastic covers in order to avoid their dessication in the growth chamber or in the laboratory. It is possible to maintain plantlets in flasks for several months in a cold chamber under light before transplanting them. Four

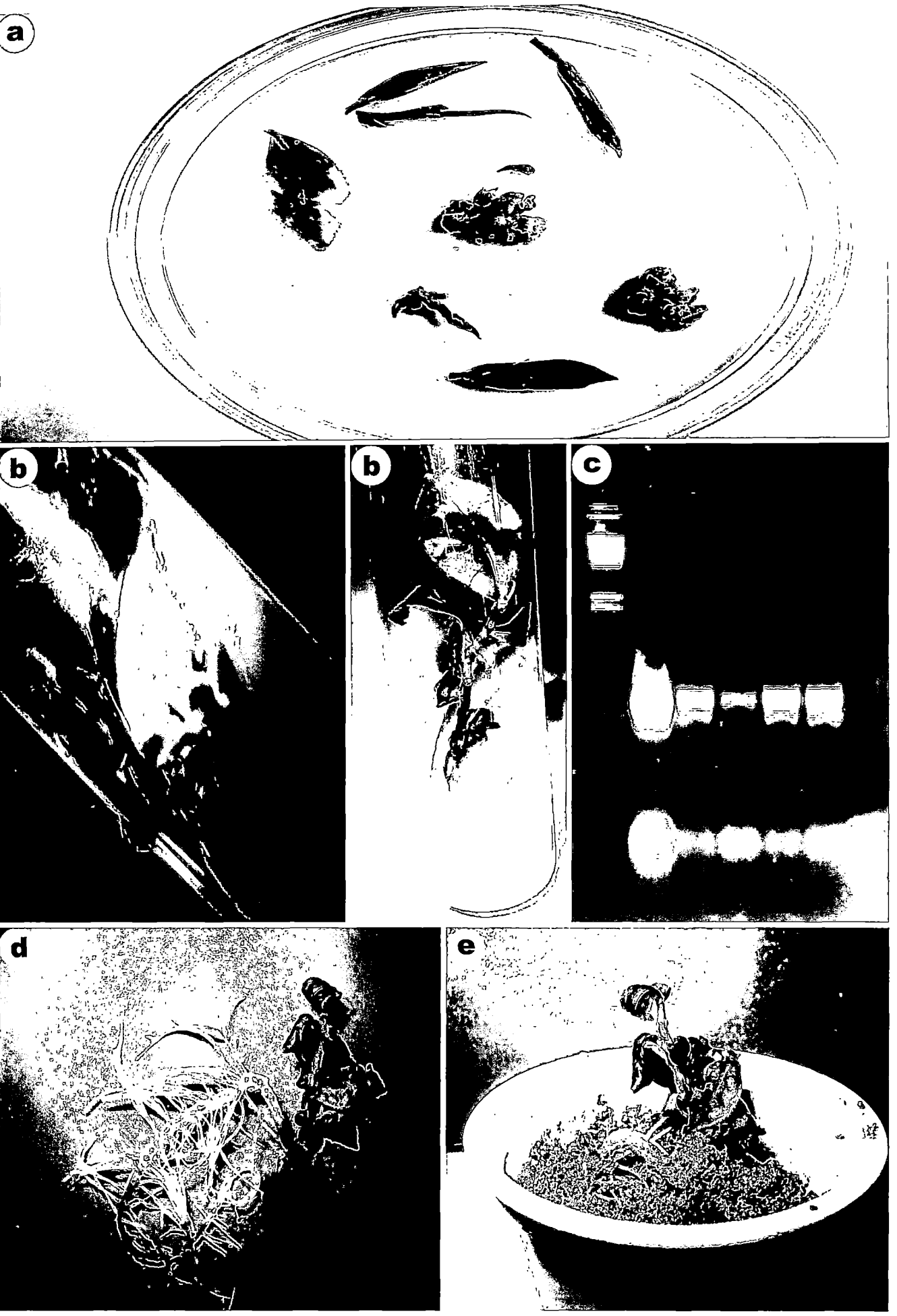


Fig. 4.24 Transgene integration in callus and leaf from stem segment and their regeneration stages. **(a)** GUS assay showing blue staining of transformed callus and leaf. **(b)** Shooting of transformed callus from stem segment of TV-26 cultivar. **(c)** PCR amplification for confirmation of transgene (*NptII* gene) in to the tea plant. Molecular marker in Lane M=λ-DNA/*HindIII* digest, Lane 1-(+)ve control (plasmid DNA pZP200KC), Lane2-5- Transformed plant DNA and Lane 6 (-)ve control (non-transformed plant DNA). **(d)** Rooting from transformed *in vitro* grown shoots of TV-26 cultivar. **(e)** Pot transfer and hardening of transformed *in vitro* growth plantlet of TV-26 cultivar.

weeks after transplantation, pots were transferred to the Greenhouse in Sikkim Council of Science and Technology to provide them a cooler atmosphere as per their requirements. Plantlets derived from Epidermal and stem segments exhibit healthy growth and their growth is the same as that of seedlings under natural conditions.

4.5. *Agrobacterium* mediated transformation of tea vs. Particle mediated bombardment of tea

The stability of transgene expression during development and its germline transmission by both the transformation methods remains to be elucidated as it will take several years for these plants to flower and set fruit. Nevertheless, the protocol for producing transgenic tea plants by both the methods namely *Agrobacterium* mediated transformation of tea and Particle mediated bombardment of tea developed in the present study should serve as an useful experimental basis for desirable gene introgression aiming at the genetic improvement of the tea in future.

Both the methods of transformation were found to be effective in the present study. In *Agrobacterium* mediated transformation the *Agrobacterium* contamination chances are higher as it is very difficult to remove and there are no *chances* of *Agrobacterium* contamination in particle mediated bombardment of tea. Also there is no need of applying any antibiotic to the transformed tissue which may have adverse effect on its growth. Therefore, the growth of the tissue in case of particle mediated bombardment of tea is higher in comparison to *Agrobacterium* mediated transformation and the contamination rate is also low in case of particle mediated bombardment of tea than *Agrobacterium* mediated transformation. The GUS activity was also seen to be higher (70%) in case of particle mediated transformation of tea in comparison of 50% as seen in *Agrobacterium* mediated transformation of tea. Thus, particle bombardment method can also be a method of choice in near future for tea transgenics. Further attempts can be carried out for transformation of tea by applying this method for transferring the fungal or drought resistance genes in tea genome.

Chapter 5
Conclusion

In Conclusion the following points are highlighted:

- Various tea cultivars available in North Bengal region were collected and maintained.
- Genomic DNA isolation from fresh & tender leaf samples of various cultivars was done.
- Detection of genetic variability and the Phylogenetic relationship among the tea cultivars were established using various PCR based fingerprinting methods like Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP) and Microsatellite markers.
- Sequencing was done for ten cultivars of tea chosen on the basis of PCR-RFLP method revealing maximum polymorphism.
- Induction of somatic embryos and regeneration of whole plantlet from cotyledon and embryogenic callus of tea was done.
- Histological studies of embryogenic stages of tea was done.
- Different gene transfer methods like *Agrobacterium* mediated transformation and Particle bombardment using gene gun were standardized for transformation of tea.
- Induction and multiplication of callus tissue from the genetically transformed explants was achieved on the antibiotic selective medium.
- Differentiation was done of genetically transformed tissue by subjecting it various hormone combination &concentrations.
- Confirmation for the integration of transgene into tea nuclear genome using GUS assay and by PCR analysis using *nptII* specific primer was done successfully.

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Appendix

Appendix I

• **L.B. medium: (Liquid)**

Bactopeptone-10g/lt. (Hi-media, Cat#RM001)

NaCl-10g/lt. (Hi-media, Cat#RM1150)

Yeast Extract-5g/lt (Hi-media, Cat#RM027)

The above mentioned components were dissolved in 1 litre of double distilled water and then autoclaved.

• **L.B. medium: (Solid)**

Bactopeptone-10g/lt. (Hi-media, Cat#RM001)

NaCl-10g/lt. (Hi-media, Cat#RM1150)

Yeast Extract-5g/lt. (Hi-media, Cat#RM027)

Agar-1.2% (Hi-media Cat# RM201)

The above mentioned components were dissolved in 1 litre of double distilled water and then autoclaved.

• **MS-104 medium: Liquid** (Hi-media Cat# PT0018)

Macroelements	**Amount required**
KH_2PO_4	170.0mg/l
KNO_3	1900.00mg/l
$MgSO_4$	180.54mg/l
NH_4NO_3	1650.00mg/l
Microelements	**Amount required**
$CoCl_2.6H_2O$	0.025mg/l
$CuSO_4.5H_2O$	0.025mg/l
FeNaEDTA	36.70mg/l
H_3B0_3	6.20mg/l
KI	0.83mg/l
$MnSO_4.H_2O$	16.90mg/l
$Na_2Mo0_4.2H_2O$	0.25mg/l
$ZnSO_4.7H_2O$	8.60mg/l

Vitamins	Amount required
Glycine	2.00mg/l
Myoinositol	100.0mg/l
Nicotinic acid	0.50mg/l
Pyridoxine HCl	0.50mg/l
Thiamine HCl	0.10mg/l

To it was added 30gm/l sucrose (Hi-media Cat# RM1158), 0.332mg/l $CaCl_2$ (Hi-media Cat# MB034), 500mg/l MES buffer (Hi-media Cat# RM1128). pH was adjusted to 5.6 and the volume was made up to 1000ml with double distilled water. It was then autoclaved for 15 minutes at 15psi and cooled to room temperature and hormones were added.

- **MS-104 medium: Solid** (Hi-media Cat# PT0018)

Macroelements	Amount required
KH_2PO_4	170.0mg/l
KNO_3	1900.00mg/l
$MgSO_4$	180.54mg/l
NH_4NO_3	1650.00mg/l
Microelements	**Amount required**
$CoCl_2.6H_2O$	0.025mg/l
$CuSO_4.5H_2O$	0.025mg/l
FeNaEDTA	36.70mg/l
H_3B0_3	6.20mg/l
KI	0.83mg/l
$MnSO_4.H_2O$	16.90mg/l
$Na_2Mo0_4.2H_2O$	0.25mg/l
$ZnSO_4.7H_2O$	8.60mg/l
Vitamins	**Amount required**
Glycine	2.00mg/l
Myoinositol	100.0mg/l
Nicotinic acid	0.50mg/l
Pyridoxine HCl	0.50mg/l
Thiamine HCl	0.10mg/l

To it was added 30gm/l sucrose, 0.332mg/l $CaCl_2$, 500mg/l MES buffer and 1% agar. pH was adjusted to 5.6 and the volume was made up to 1000ml with double distilled water. It was then autoclaved for 15 minutes at 15psi, cooled to room temperature and hormones were added.

• **MSO medium: Liquid** (Hi-media Cat# PT0018)

Macroelements	**Amount required**
KH_2PO_4	170.0mg/l
KNO_3	1900.00mg/l
$MgSO_4$	180.54mg/l
NH_4NO_3	1650.00mg/l
Microelements	**Amount required**
$CoCl_2.6H_2O$	0.025mg/l
$CuSO_4.5H_2O$	0.025mg/l
FeNaEDTA	36.70mg/l
H_3B0_3	6.20mg/l
KI	0.83mg/l
$MnSO_4.H_2O$	16.90mg/l
$Na_2Mo0_4.2H_2O$	0.25mg/l
$ZnSO_4.7H_2O$	8.60mg/l
Vitamins	**Amount required**
Glycine	2.00mg/l
Myoinositol	100.0mg/l
Nicotinic acid	0.50mg/l
Pyridoxine HCl	0.50mg/l
Thiamine HCl	0.10mg/l

To it was added 30gm/l sucrose, 0.332mg/l $CaCl_2$, 500mg/l MES buffer. pH was adjusted to 5.6 and the volume was made up to 1000ml with double distilled water. It was then autoclaved for 15 minutes at 15psi and cooled to room temperature. No hormones were added.

• **MSO medium: Solid** (Hi-media Cat# PT0018)

Macroelements	**Amount required**
KH_2PO_4	170.0mg/l
KNO_3	1900.00mg/l
$MgSO_4$	180.54mg/l
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Microelements	**Amount required**
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$CuSO_4.5H_2O$	0.025mg/l
FeNaEDTA	36.70mg/l
H_3B0_3	6.20mg/l
KI	0.83mg/l
$MnSO_4.H_2O$	16.90mg/l
$Na_2Mo0_4.2H_2O$	0.25mg/l
$ZnSO_4.7H_2O$	8.60mg/l
Vitamins	**Amount required**
Glycine	2.00mg/l
Myoinositol	100.0mg/l
Nicotinic acid	0.50mg/l
Pyridoxine HCl	0.50mg/l
Thiamine HCl	0.10mg/l

To it was added 30gm/l sucrose, 0.332mg/l $CaCl_2$, 500mg/l MES buffer and 1% agar. pH was adjusted to 5.6 and the volume was made up to 1000ml with double distilled water. It was then autoclaved for 15 minutes at 15psi, cooled to room temperature and no hormones were added.

• **YEM medium: (Liquid)**

Yeast extract-0.4g/lt (Hi-media, Cat#RM027)

Mannitol-10.0g/lt (Hi-media, Cat#RM570)

NaCl -0.1g/lt (Hi-media, Cat#RM1150)

$MgSO_4.7H_2O$-0.2g/lt (Hi-media, Cat#RM1281)

K_2HPO_4-0.5g/lt (E Merck India, Cat#17885)

- **YEM medium: (Solid)**

Yeast extract-0.4g/lt (Hi-media, Cat#RM027)

Mannitol-10.0g/lt (Hi-media, Cat#RM570)

NaCl -0.1g/lt (Hi-media, Cat#RM1150)

$MgSO_4.7H_2O$-0.2g/lt (Hi-media, Cat#RM1281)

K_2HPO_4-0.5g/lt (E Merck India, Cat#17885)

Agar-1.2% (Hi-media Cat# RM201)

Appendix II

Composition of Buffers:

- **C-TAB Extraction buffer**

2% (W/V) C-TAB (Hi-media Cat# RM164)

1.4M NaCl (Hi-media, Cat#RM1150)

20mm EDTA (pH-8.0) (Hi-media Cat# RM1197)

100mM Trizma base (pH-8.0) (SIGMA Cat# T1503)

0.2% Mercaptoethanol (Hi-media, Cat#RM2895) just before use

54g of molecular biology grade Trizma base (Sigma, USA Cat#T-1503, Tris (hydroxymethyl) aminomethane, $C_4H_{11}NO_3$ FW-121.1) was dissolved in 800ml of sterile distilled water, pH was adjusted to 8.0. It was divided into two parts; to one part 7.44g EDTA was added. In other part 81.82g NaCl, 20g CTAB (Hexadecyl trimethyl ammonium bromide, $C_{19}H_{42}NBr$) was added. Both the parts were then mixed and to it 0.2% Mercaptoethanol was added.

- **DNA Loading buffer (6X Concentration)** (Fermentas Cat# R0611)

(TypeIII, Sambrook *et al.*, 2001)

0.25% Bromophenol blue

0.25% Xylene cyanol FF

30% Glycerol in DD H_2O

Two and a half grams of Bromophenol blue and Xylene cyanol was dissolved in 1000ml of 30% Glycerol.

- **dNTP mix** (Finnzymes Cat#F560L)

10mM dATP, 2'-Deoxyadenosine 5'-Triphosphate, minimal diphosphate, sodium salt.

$C_{10}H_{12}N_5O_{12}P_3Na_4$, F.W-579.2

10mM dGTP, 2'-Deoxyguanosine 5'-Triphosphate, minimal diphosphate, sodium salt

$C_{10}H_{12}N_5O_{13}P_3Na_4$, F.W-595.1

10mM dCTP, 2' Deoxycytidine 5'-Triphosphate, minimal diphosphate, sodium salt

$C_9H_{12}N_3O_{13}P_3Na_4$, F.W-555.1

10mM dTTP, 2' Deoxythymidine 5'-Triphosphate, minimal diphosphate, sodium salt

$C_{10}H_{13}N_2O_{14}P_3Na_4$, F.W-570.1

• **GUS staining solution** (SIGMA USA, Cat# GUS-S)

Reagent A, Product No. R6147

200mM sodium phosphate, pH 7.0 with 4mM EDTA-2.5ml

Reagent B, Product No. R6272

100mM potassium ferricyanide-10µl

Reagent C, Product No. R6397

100mM potassium ferrocyanide-10µl

Deionized water-5.5ml

Methanol-2.0ml

5-Bromo-4-Chloro-3-Indolyl-ß-D-Glucuronide (X-GlcA), Cyclohexylammonium Salt, Product No. ß 0522-20µl

The staining solution may be stored at 2-8°C in the dark for one month.

• **Taq buffer (10X) (Supplied with Taq polymerase, Finnzymes Cat#F501L)**

10mm Tris-HCl (pH-8.8)

1.5mM $MgCl_2$

50mM KCl

0.1% Triton X-100

• **TE-Tris EDTA Buffer (pH-8)**

10mM Tris (pH-8.0)

10mM EDTA (pH-8.0)

1.21g molecular biology grade Trizma base (Sigma, USA Cat#T-1503, Tris (hydroxymethyl) aminomethane, $C_4H_{11}NO_3$,FW-121.1) was dissolved in 400ml of double distilled water and the pH was adjusted with Conc. HCl (Hi-media, Cat#RM5955) to 8.0 and sterilized by autoclaving. Similarly 0.372g Di-Sodium EDTA was dissolved in 400ml of double distilled water. The solution was stirred properly and the pH was adjusted with NaOH (Hi-media, Cat#RM1183) pellets and sterilized by autoclaving. Both the solutions were then mixed and the volume was made upto 1 litre sterilized double distilled water.

- **TBE- Tris-Borate EDTA Buffer (5X Concentration)**

0.045M Tris-Borate

0.001M EDTA (Hi-media Cat# RM1197)

Preparation of 5X stock:

54g of molecular biology grade Trizma base (Sigma,USA Cat#T-1503, Tris (hydroxymethyl) aminomethane, $C_4H_{11}NO_3$, FW-121.1) and 27.5g Boric acid (Hi-media, Cat#MB007) were dissolved in 800ml of sterile double distilled water. To it 20ml of 0.5 EDTA (pH-8.0) was added.

TBE was used in a final concentration of 1X, so the 5X stock was diluted to 1X.

- **Washing solution**

70% Ethanol (E Merck Cat# 101076HBD)

10mM Ammonium acetate (Hi-media, Cat#MB033)