GENOMIC FINGERPRINTING OF TEA GERMPLASM AND ANALYSIS OF TRANSCRIPT ACCUMULATION OF A DEFENSE PROTEIN INVOLVED DURING INDUCED SYSTEMIC RESISTANCE

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (SCIENCE) IN BOTANY OF THE UNIVERSITY OF NORTH BENGAL

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2008

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TO WHOM IT MAY CONCERN

This is to certify that Mr. Subhas Chandra Roy has carried out his research work under my supervision. His thesis entitled "***Genomic Fingerprinting of Tea Germplasm and Analysis of Transcript Accumulation of A Defense Protein Involved During Induced Systemic Resistance***" is based on his original work and is being submitted to the University of North Bengal for the degree of Doctor of Philosophy (Science) in Botany, in accordance with rules and regulations of the University of North Bengal.

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ACKNOWLEDGEMENT

It is an immense pleasure to express my deep sense of gratitude, to my supervisor, Professor Bishwanath Chakraborty, Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal, for his noble guidance, unfailing help, advice, constant inspiration and extensive co-operation throughout the execution of this research work.

I am thankful to Professor Usha Chakraborty, Head, Department of Botany, North Bengal University and Prof. P. K. Sarkar, for rendering instrumental facilities developed under UGC-SAP (DRS-II) in this department.

Thanks are also due to Prof. A. P. Das, Dr. A. Saha, Dr. A. Sen and Mr. P. Mandal for their inspiration and constant support to finish my Ph.D. work. I acknowledge my sincere thanks to the authority of UGC for granting me two Minor Research Projects to carry out my work. Thanks are due to Mr. Babul Biswas for typing the manuscript.

Last, but not the least, I am extremely grateful to my parents for their encouragement and inspiration and immense moral support. I am really thankful to Mrs. Chhanda Roy, Mr. Aritra Roy and Mr. Sagnibha Roy for their encouragement, love and inspirations without which the completeness of this doctoral work would not have been possible.

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CONTENT

INTRODUCTION

Tea is the oldest and non-alcoholic caffeine containing beverage producing from the young shoot tips (two and a bud) of tea plant [*Camellia sinensis* (L.) O. Kuntze]. The genus *Camellia* belongs to the family Theaceae. The tea plant in its natural condition is a perennial evergreen shrub or a small tree (woody perennial) grows over a wide area from 35° S to 42° N latitude and attained the heights from 1-12 meter. Under cultivation, tea bushes are pruned to a manageable height and tender shoots are regularly plucked in tea industry. Tea is grown in more than 50 countries, mostly in plantation as a monoculture crop. It prefers a warm humid climate with well distributed rainfall and long sunshine hours. The tea leaves contain in addition to the normal constituents high levels of polyphenols (30-40%) and caffeine (2-5%). The beneficial health effects of tea are well known (Das *et al.*, 2005), and the terminal bud of tea contains the maximum caffeine, which contribute to the medical value of tea (Ramarethinam and Rajalakshmi, 2004). Tea is indigenous to India and is an area where the country can take a lot of pride. India is the world's largest tea producer followed by China. India accounted for 27.4% of world output, followed by China (24.6%), Sri Lanka (9.75%), and Kenya (9.4%). Total area under tea cultivation in India is 5.12 lakh hectares, which generates income and livelihood for nearly 20 million people in the country.

The production in 2007 was a record high of 955 million kg, up by 27 million kg compared to 2006. Darjeeling produces the world's finest quality tea on the steep hill slopes of the eastern Himalayas up to an elevation of 2000 m (Chakraborty *et al.*, 2002). It exports bio-organic tea over 40 percent of its total production to different foreign countries like France, Germany, United Kingdom and United States of America. In order to retain its 28% share of international trade and to meet the increasing domestic demand, India needs to improve the productivity of tea substantially. The northeastern state of Assam is considered the heart of India's tea industry with the state accounting for about 55% of the country's total annual tea production. So, it urgently needs of an integrated all embracing strategy so as to place the Indian tea industry on a sound and firm footing and at the same time to achieve long-term objectives for the development of elite tea clones with better quality, flavour and higher yield by doing research giving stress on genetic diversity,

germplasm conservation, breeding, crop nutrition, biological control of pests and diseases. Tea is made from the tender leaves of the different cultivars of the three species based on morphological parameters *viz. Camellia sinensis* (L.) O. Kuntze (China type), *Camellia sinensis* var. *assamica* (Masters) (Assam type) and *Camellia sinensis* sub sp. *lasiocalyx* (Planchon MS.) (Cambod or Southern type). All the three varieties of tea (*Camellia sinensis*) are highly cross-pollinated and intercrossable without any reproduction barrier, so the existing population is a mixture of three categories of tea (Wight, 1962 and Banerjee, 1992).

Assam type was developed in India and China type in China; they were subsequently introduced in to Japan. Indo-China region being a primary center of origin, India harbors a large tea biodiversity. The diversity further widens as various scientists have developed a number of region specific, high yielding cultivars in different parts of the country by introgression of some important genes from the related wild species *viz-* TV-series, UPASI-series, and other common cultivars. Unfortunately, some important traits such as abiotic stress resistant, caffeine deficiency, resistant to pathogens (fungal and bacterial) and quality-yield appear to be absent from the gene pools of tea cultivars. In order to select the right crossing combinations, it is important for plant breeders to have knowledge about relationships between genotypes that may be used in new crosses, and about total genetic diversity available in germplasm. Conventionally the tea breeders often select the parent on the basis of a few parameters *i.e.* morphology, yield and biochemical descriptors without considering any genetic basis, however these parameters are highly dependent on environmental conditions. So, plant breeders urgently need some of the unchangeable genetic markers of the varieties, which can be used for the development of new improved varieties in all respects.

Breeders can employ DNA based molecular markers because they are effective at evaluating genetic diversity among the species and cultivars and the data are easier to obtain than classical morpho-anatomical descriptors. For characterization of genome (fingerprinting): a variety of molecular marker techniques are available, which are either hybridization based or PCR based. These markers are RFLP, AFLP, RAPD, SSR, ISSR, *etc.* The advantage of molecular markers is that they are least affected by environmental factors (Varghese *et al.*, 1998) and are almost unlimited in number. They also offer a possibility to observe the genome directly, and thus

eliminate the shortcomings inherent in a phenotype observation. Molecular markers highlighted important differences in the partitioning of diversity within and among population. These include AFLP based marker analysis (Paul *et al.*, 1997), RFLP based fingerprinting (Matsumoto *et al.*, 1994; Devarumath *et al.*, 2002), RAPD profiles (Waugh and Powell, 1992; Wu *et al.*, 1994; Lee *et al.*, 1995; Wachira *et al.*, 1995, 1997; Weir *et al.*, 1997; Kaundun *et al.*, 2000; Hsiao *et al.*, 2001; Kaundun and Park, 2002; Hamik, 2003; Luo *et al.*, 2004; Roy, 2004; Li *et al.*, 2005; Roy and Chakraborty, 2007; Liang *et al.*, 2007; Cheng-Wen *et al.*, 2008), ISSR based analysis (Hsiao *et al.*, 2001; Mondal, 2002; Devarumath *et al.*, 2002, and Liang *et al.*, 2007). The CAPS markers have also been used in tea characterization (Kaundun and Matsumoto, 2003). These markers are useful for characterization, estimation of genetic relatedness and determination of genetic diversity among the tea germplasm. Comparison between RAPD, AFLP and SSR markers in different plants species has revealed that co-dominant SSRs detect the highest level of polymorphism per locus and it surveys the hyper-variable microsatellite regions of the genome, hence they have higher information content (Powell *et al.*, 1996; Maughan *et al.*, 1996). Sometimes, AFLP and RAPD despite their dominant nature can estimate relationship with a high resolution due to their multilocus approach (Powell *et al.*, 1996; Russell *et al.*, 1997; Teulat *et al.*, 2000). A number of DNA based genetic markers are now available for the effective quantification of genetic variation in plant species. The genetic diversity and phylogenetic relationship among the commercial cultigens, therefore, deserve investigation.

The introgression of pest and disease resistance genes from related wild species into modern tea cultivars over the past several decades has been done which broadened the germplasm base of the cultigens and showing resistance against targeted pathogen. But, it is not as satisfactory as it was desired. There are several major diseases of tea plant that threatens production such as red rot, blister blight, root rot, caused by several pathogens. The pathogen infects only the economically important, tender shoots which lead to enormous crop loss, estimated up to 50% depending on the severity of infection. Blister blight incited by a fungal pathogen *Exobasidium vexans* Massee is the most important leaf disease in India affecting both the quality and productivity of tea (causes 35-50% crop loss). The protections against pest and pathogens have been achieved (40-68%) by using some conventional

fungicide and pesticides but it hampered the export quality. So we need high yielding, disease resistant, quality full cultivars for more production to promote export. In order to optimize disease and pest management strategies it is important to understand the genetic capabilities of tea germplasm to defend itself against diseases. Resistant/tolerant cultivars can be screened from the available tea germplasm on the basis of some genetical parameters such as immunity of a plant against pathogen with respect to plant's own defense system. Plant has its own defense strategies to combat the pathogenic infection by producing pathogenesis-related protein/enzyme (PR-proteins).

So, elite tea clone can be identified on the basis of the chitinase gene structure- a PR-protein gene. Many attempts have already been made to analysis the crop plant germplasm on the basis of chitinase gene but not in tea plant. Plant chitinases belong to a family of pathogenesis-related (PR) proteins, which are over-expressed by plants in response to a pathogen attack (Boller *et al.*, 1983; Giazinazzi, 1987; Legrand *et al.*, 1987; Collinge *et al.*, 1993). Chitinases catalyze the hydrolysis of the β-1,4 linked N-acetylglucosamine polymers that form chitin chains, a major component of fungal cell walls and is involved in the inducible defenses of plants. Those with lysozyme activity also cleave ß-1,4 linkages between GlcNAc and *N*-acetylmuramic acid. They are present in a broad range of organisms including bacteria, fungi, plants, and animals. Recently eleven families of PR proteins (PR-1 to PR-11) were recognized and classified in different plant species (tobacco and tomato, cucumber and parsley (Van Loon *et al.*, 1985, 1994, 1997, 1999).

Plant chitinases and lysozymes are likely to have arisen from one coancestor by divergent evolution (Monzingo *et al.*, 1996). Some researchers are trying to address the question whether manipulation of defense signaling pathways, either through genetic engineering or through application of defense signal-mimicking plant protectants, will boost the plant's immunity to potential invaders or will be a burden in crop protection strategies. Researchers have tried to isolate different chitinase gene from different plant species to use it in crop improvement program in order to increase the plant's own immune system (Schlumbaum *et al.*, 1986; Metraux and John, 1989; Samac *et al.*, 1990; Roby *et al.*, 1991; Broglie *et al.*, 1991; Huynh *et al.*, 1992; Datta *et al.*,1999; Passrinho and De Vries, 2002; Sekeli *et al.*, 2003; Sharma and Kumar, 2005; Eilenberg *et al.*, 2006). Intensive studies showed that

numerous genes involved in these processes have been identified (Hammnd-Kosack and Jones, 1996). Defense response genes are induced in both incompatible and compatible plant-pathogen interactions. However, mRNA accumulation for many plant defense genes is more rapid during interactions involving a plant expressing resistance to a particular pathogen (Christensen *et al.*, 2002; Davis *et al.*, 2002). Polyphenol oxidases (PPO) are nuclear coded enzymes of almost ubiquitous distribution in plants (Mayer and Harel, 1979; Mayer, 1987). Pritsh *et al.* (2000) studied the pattern of transcripts accumulation of six-typical defense response genes, POX (peroxidase), PR-1, PR-2 (β-1,3-glucanase), PR-3 (chitinase), PR-4 & PR-5 (thaumatin like protein) in spray-inoculated panicles of both the susceptible cv. Wheatson and the resistant cv. Sumai3 of wheat. POX transcripts accumulation has been studied by Collinge and Boller (2001) in potato upon infection with *Phytophthora infestans.*

Defense proteins (PRs) related transcript accumulation have been studied in several plant species by many researchers throughout the world to characterize and interaction between host and pathogens (Cary *et al.,* 1992; Hunt *et al.,* 1993; Newmann *et al.,* 1993; Joy *et al.,* 1995; Dry and Robinson, 1994; Sherman *et al.,* 1995; Boss *et al.*, 1995; Thipyapong *et al.,* 1995; Thipyapong and Steffms, 1997; Van Gelder *et al.,* 1997; Fossdal *et al.,* 2001; McFadden *et al.,* 2001; Yu and Muehlbauer., 2001; Christensen *et al.*, 2002; Mohammadi and Kazemi, 2002; Constabel *et al.,* 1995, 2000; Li and Steffens 2002).

Chitinases are enzymes that hydrolyse ß-1,4-*N*-acetyl-D-glucosamine (GlcNAc) linkages. Those with lysozyme activity also cleave ß-1,4 linkages between GlcNAc and *N*-acetylmuramic acid. They are present in a broad range of organisms including bacteria, fungi, plants, and animals. According to their primary structure, chitinases are divided into seven classes (classes I–VII). Plant chitinases and lysozymes are likely to have arisen from one coancestor by divergent evolution (Monzingo *et al.*, 1996). The protein genealogy of chitinases shows that class I and class II chitinase genes evolved from the same ancestral gene (Shinshi *et al.*, 1990; Araki and Torikata, 1995). Moreover, a basic class II chitinase is a putative ancestor of basic class I and acidic class II chitinase genes (Ohme-Takagi *et al.*, 1998). It has also been proposed that chitinases in class IV, which are phylogenetically related to class I and II chitinases (Araki and Torikata, 1995; Hamel *et al.*, 1997; Gomez

et al., 2002), evolved from a class I chitinase gene by four deletions in the coding sequence (Araki and Torikata, 1995). Wounding of plant tissues has been shown to induce complex molecular responses many of which (caffeine) are considered plant defense response (Aneja and Gianfagna, 2001; Leon *et at.*, 2001; Cheong *et al.*, 2002). Wounding can induce formation of jasmonates and ethylene, each of which are capable of independently inducing signal transduction pathways leading to resistance against plant pests. Jasmonic acid and related compound methyl jasmonate are part of the octadecanoid pathway in plants (Strassner *et al.*, 2002; Wasternack and Hause, 2002) and in some incidences contribute to resistance to insects (McConn *et al.*, 1997; Baldwin, 1998) and pathogen (Park *et al.*, 2001). Jasmonates have been shown to function as regulators of many different plant defense genes (McConn *et al.*, 1997; Park *et al.*, 2001). Ethylene is known to induce specific subsets of pathogenesis-related proteins in many plant species (Kitajima and Sato, 1999; Thomma *et al.*, 1999). The effects of ethylene have been associated with resistance to many different plant pathogens (Abeles *et al.*, 1971; Thomma *et al.*, 1999; Park *et al.*, 2001) and insects (Abeles *et al.*, 1971; O'Donnell *et al.*, 1996; Argandona *et al.*, 2001; Bailey *et al.*, 2005).

In response to pathogens, plants synthesize two classes of chitinases with either basic or acidic isoelectric points. These two classes appear to be targeted to different parts of the cell, and there is evidence that they are differentially regulated. Acidic chitinase is found in the intercellular space of infected plants and plants treated with salicylic acid or necrotizing salt solution. Basic chitinase accumulates in the central vacuole and is systematically induced by ethylene, a stress related hormone (Broglie *et al.*, 1986; Mauch and Staehelin, 1989; Metraux and John, 1989; Samac *et al.*, 1990).

The aim of this discourse was

(a) To characterize the tea genotypes at molecular level using DNA based marker in order to elucidate the genetic diversity and phylogenetic relationships among the tea cultivars.

(b) To obtain fundamental information on callus growth and development in tea clones *in vitro* and regeneration of plantlets

(c) To study the somatic embryogenesis and regeneration of plantlets from the cotyledonary tissues in relation to improvement of genetic traits.

(d) To know the genomic constitution of tea cultivars at the chromosomal level both *in vitro* and *in vivo* conditions

(e) To generate partial sequence information of caffeine synthase gene of tea clones

(f) To determine transcript accumulation of chitinase gene specific mRNA during induced systemic resistance by abiotic elicitor

(g) To quantify chitinase specific mRNAs through cDNAs using reverse transcriptase-PCR reaction (RT-PCR)

(h) To amplify chitinase gene specific DNA fragment by PCR, T/A Cloning and sequencing of tea clone

At the onset, a brief review of literature on DNA-based molecular markers for fingerprinting the tea genome, genomic constitution at the chromosome level, tea tissue culture, partial genetic information of caffeine synthase gene and chitinase gene in conformity with the present investigation has been presented.

LITERATURE REVIEW

Taxonomic position of tea

The generic name *Camellia* was derive from Kamel, George Joseph Kamel, a German Missionary stationed in the Philippines, also wrote about plants found in Asia during the latter half of the seventeenth century. In 1753 Linnaeus had given the name of tea plant *Thea sinensis* in Vol. I of his 'Species planterum' while describing the two ornamental species as *Camellia sasanqua* and *Camellia japonica* in Vol.II. Lastly, in its session of the Botanical Congress held in Amsterdom during 1935, decided to unite the two genera *Thea* and *Camellia* into a single genus, *Camellia.* The correct scientific name of the tea plant is *Camellia sinensis* (L.) O. Kuntze since it gives recognition to the authority responsible for the union of the old name *sinensis* with the new genus *Camellia.* The internationally accepted scientific nomenclature is *Camellia sinensis* (L.) O. Kuntze. The genus *Camellia* of which tea is a member belongs to the family Theacee, tribe Gordonieae (Barua, 1989). All tea plants were placed under one species, *Camellia sinensis.* Earlier taxonomists describe the tea plant as *Camellia sinensis* (L.) for the whole tea population ignoring its varietals status. Later taxonomists (Wight, 1962; Barua, 1963 and Bezbaruah, 1971) recognized two main varieties namely China type (all narrow leaved China types of plants) and Assam type (covering the broad leaved Assam type of plants) on the basis of morphological and anatomical description of the tea plants. Two main varieties of tea were also grouped as *C. sinensis* var. *senensis* (L). and *Camellia sinensis* var. *assamica* (Masters). The Assam variety was first described by Masters (1844) as *Thea assamica.* The Assam and China varieties have different morpohological atrributes, markedly differ in their growth rate and other features, leaf size, shape, texture *etc.* Then a third type of tea plant was found which was considered as a Cambod (Cambodiensis) or southern form as described by Planchon. This plant did not differ much from the Assam type plant. Later on it was described as *Camellia assamica* sub sp. *lasiocalyx* (Planchon MS.). This third variety has been approved by different scientists (Kingdon-Ward, 1950; Sharma and Venkataramani, 1974). The present status of the tea has been considered as China type, Assam type and Cambod type as distinct variants or varieties of *Camellia sinensis* (L.) O. Kuntze, based on their distinctive morphological and morphmetric features (Bezbaruah, 1976; Wight, 1962). The morphological traits of

the three main variants of tea are therefore of considerable practical importance, not only because of their agronomical importance in providing yield and quality, but also because they provide the genetic sources for selection of better plant types in which features of agronomical significance can be incorporated.

Tea growing regions of India

South-East Asia is the original birth place of tea and China is the first country to use tea as a beverage. From this region, tea spread to different countries of the world such as China, Japan, Taiwan, Bangladesh, Indonesia, Srilanka, U.S.S.R., Kenya, Turkey, Iran, Africa, India and others. In India tea plantation is mainly concentrated into two traditional regions, one in north-east and other in South-India. In addition, tea is grown in a very limited area in Kangra (Himachal Pradesg) and Dehra Dun (Uttar Pradesh) valleys of North-West India (Figure 1 and Table 1).

Table 1. Tea growing regions of India.

Regions	Tea areas
1. North-East India (N.E.India)	Assam valley, Barak valley, Cachar, Dooars, Terai, Darjeeling, Sikkim, Orissa, Bihar.
2.North-West India	Kangra Valley (H.P), Dehra Dun (UP)
3.South India (Kamataka,Kerala & Tamil Nadu)	Annamalai (Cinchona), Travancore (Vandiperiyar), High Ranges (Munnar), Nilgiris (Coonoor), Wyannad (Mepppadi).

Genetic resources and natural variability

Tea plant belongs to the genus *Camellia,* family theaceae. The other ten genera under this family are *Stuartia, Schima, Franklinia, Yunnanea, Gordonia, Tutcheria, Laplacea Pyrenaria, Eurya, Ternstroiemia* and *Adenanthera* (Chang, 1981). Chang (1981) reported over 200 species under the genus *Camillia,* which is the most important of all the genera.

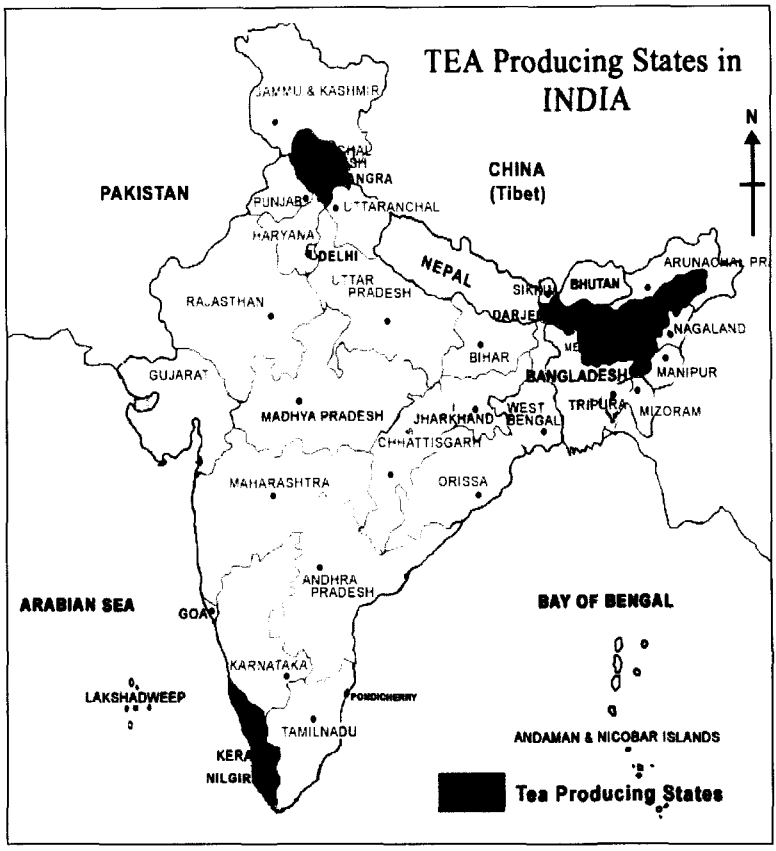


Figure 1. Map of India showing the three main tea producing regions.

Tea population of today are highly heterozygous because of free cross-pollination for many years among all the taxa owing to their cross compatible and inter- fertile nature. Species hybrids, at least involving a number of related species exist in cultivated tea population (Barua, 1965 and Bezbaruah, 1974). Quality trait of tea particularly Darjeeling tea might be due to incorporation of a number of genes from other species including non-tea or wild species growing in the same habitat. Barua (1965) reported that the complex hybrids of the three cultivated forms of tea one or more of the wild species of *Camellia* occur in the tea population particularly in the Darjeeling district of north-east India, which is considered as "Champenge of tea" for its distinctive quality and flavour. Tea was hybridized with non-tea species *Camellia irrawadiensis, C. taliensis, C. lutescens* to develop caffeine deficient tea but industrially was not viable. The above three non-tea species are devoid of caffeine.

The cultivated population can broadly be classified on the basis of morphological, anatomical and biochemical proximity to the main taxa into three types-Assam type (*Camellia assamica* Masters), China type (*Camellia sinensis* L.)

and Cambod type or Southern form (*Camellia assamica* ssp. *lasiocalyx* Planchon. MS). Since, China is centre of origin of *Camellia*, where maximum genetic diversity and most of the species of China type and others are found in the Yunnan province of South-west China. The north-east India mainly Assam region bordering Burma is the origin of Assam tea plant where maximum diversity will respect to genetically and morphological characters are found. Other *Camellia* species prevailed in this region (north-east) are *Camellia caudata, C. caduca, C. irrawadiensis, C. taliensis, C. kissi, C. drupifera* and related genera like *Eurya, Pyrenaria, Schima and Gordonia.*

So, genetic resources of tea composed of cultivated varieties, non-cultivated or wild species and genera, land races, improved clones, breeding hybrids and seed stocks. In the hybrids, considerable species introgression has also taken place. For commercial use, tea clones must carry the desirable characters for cultivation like cup quality, disease, pest and drought resistance *etc.* Quality differs from one kind of tea to another. The China kind of tea has a quality different from that of the Assam kind. Darjeeling tea is made from Chinery bushes. Different kinds of tea are made in different parts of the world from more or less morphologically distinct populations.

Tea Germplasm in India

Unfortunately no central pool of tea germplasm exists anywhere in the world. India can claim to be one of the pioneers in scientific approach to tea cultivation in the world. Collection of tea germplasm was started in India at Tocklai Experimental Station (TES), Jorhat, Assam in 1900, soon after the discovery of the indigenous tea plant in north-east India. It is the largest single collection of *Camellia* germplasm anywhere in the world (Singh and Bera, 1994). The collection is enriched by 14 species with 2532 accessions maintained at three main centres *viz.* Jorhat, Nagrakata and Darjeeling (table 2 and 3). Maximum diversity is found in *Camellia assamica* followed by *C. assamica* ssp. *lasiocalyx*, mainly collected from the North-East areas of India.

The collection of tea germplasm virtually started with the discovery of wild tea in Assam by Robert Bruce in 1823 and the collection was extended to Vietnam, Cambodia, Japan and China by various scientists. It is observed that over 60% of the world tea acreage received basic planting materials directly or indirectly from Indian tea germplasm. The geographical distribution of more important *Camellia* species (Table 2) suggests the possibilities of existence of wild varieties in the region

bordering Assam-Burma and China. There is a wide genetical base for wild tea as expressed in its phenotypic variation.

Indeed, most existing tea cultivars had their origins linked to one or the other of these genetic materials and they still offer opportunities for selecting a wide range of superior cultivars with greater genetic diversity. The tea germplasm is important not only because it supplies genes to modify and improve cultivars and hybrids but also because in it the mutant genes that originated naturally have accumulated over thousands of years. This natural resource must therefore be saved in order to delve deeper into the molecular level to develop molecular blueprints for tea cultivars endowed with potentialities for high yield and quality. The desirable genetic characters can be introduced by inter-specific hybridization only on the basis of knowledge of the evolutionary organization of the tea gene pool differentiation. So, the characterization of germplasm and its preservation can only ensure the availability of the wide range of gene pool in tea for future use in tea improvement.

Table 2: Collection of tea germplasm in North-East India. A total of 2532 accessions have been collected and preserved in the field Gene bank at the three main locations.

Sl. No	Location	No. of Accessions
1	Tocklai Experimental Station, Jorhat, Assam	**1724**
	i) Primitive seed sources, natural variants	1279
	ii) Improved seed/clonal cultivars	196
	iii) Polyploids	174
	iv) Wild and related *Camellia* species.	75
2	Nagrakata Sub-station, West Bengal	**555**
	i) Natural variants and breeding stocks	490
	ii) Improved seed/clonal cultivars	65
3	Clonal Proving Station, Ging Tea Estate, Darjeeling	**253**
	i) Natural variants and breeding stocks	208
	ii) Improved seed/clonal cultivars	45
		Total: 2532

Table: 3: *Camellia* germplasm available in the Tocklai Experimental Station, Jorhat, Assam, North-East India.

Species	Source of Collection	No. of Accessions
Camellia assamica	Assam, Manipur, Sri Lanka, South India.	2337
Camellia sinensis	China, Darjeeling Hills	35
C. assamica ssp *lasiocalyx*	Indo China, Mayanmar, Assam	60
C. kissi (drupifera)	Meghalaya	50
C. caudata	Assam	-
Eurya japonica	North-East India	7
E. acuminata	North-East India	2
Gordonia excelsa	North-East India	2
G. imbricata	Sri Lanka	2
C. japonica	USA, Japan	-
C. sasanqua	USA, Japan	-
C. irrawadiensis	Upper Mayanmar	2
C. japonica var. *kyoniski*	Japan	1
C. rosiflora	Sri Lanka	1
Total		2507

Source: Singh and Bera (1994).

Tea gene pool

It has been suggested that the tea might have arisen from the same basic genome because most of the species of the genus *Camellia* maintain a comparable chromosomal structure and numbers (table 4). The cultivated varieties contain diploid chromosome number 2n=30 but except a seed population in Vietnam showed triploid Chromosome constitution 2n=3x=45 (Bezbaruah, 1971). The wild species *Camellia*

caudata, *C. kissi* and *C. irrawadiensis* have diploid chromosome numbers as like the cultivated varieties, 2n=30. The *C. sasanqua* is a hexaploid having 2n=6x= 90.

Table 4. Chromosome numbers in *Camellia* species.

Species	Chromosome number (2n)
Camellia sinensis	
var. *assamica*	30, 45
var. *sinensis*	30
C. kissi	30
C. caudata	30
C. irrawadiensis	30
C. japonica	30,45
C. rosaeflora	45
C. sasanqua	90
Camellia spp.	30, 45

Source: Bezbaruah, 1971.

Genetic variability utilized

Existing wide natural genetic variability present in the tea populations are due to free hybridization among the tea species and varieties (Singh and Bezbaruah, 1988; Singh and Bera, 1994; Barua, 1965). Elite tea clones are selected or created using the available existing genetic variability in tea germplasm. There is no scope for further genetic evolution to occur in the clones (asexual reproduction). Seeds can be used for the creation of evolution naturally through sexual reproduction. It is this feature of the seed which provides variability for the selection of clones. The inherited characters such as quality and cup strength are positively associated with some morphological features like pubescence (Venkataramani and Sharma, 1974) phloem index and vascular index. However, these factors are subjected to large phenotypic or environmental variations. The selection of right bushes and the development of improved tea clones is a laborious method and take 7-10 years to release a commercial planting clone (Bezbaruah, 1974, 1984; Singh, 1996; Barua, 1965). The clones are selected for good yield and cup quality in addition tolerance to pests, diseases, water stresses (drought, water logging) and rooting behaviour. Many clonal cultivars over 165 have been developed by Tocklai Experimental Station, Jorhat for commercial cultivation in North-East India (Singh, 1992, 1989) and 40 clones in

South India (Sharma and Satyanarayana, 1987, 1989) and two in Himachal Pradesh (table 5).

Table 5. Release of clonal cultivars in India.

Region	No. of Clones selected from		Total
	Natural variability	Created variability	
N.E India			
Plains	125	8	136
Darjeeling Hills	27	2	29
Total	155	10	165
South India	40	-	40
Himachal Pradesh	2	-	2
Grand Total	197	10	207

After Singh, 1994.

Natural as well as created genetic variability has been exploited for the development of these clones. Singh and Handique (1991) was selected a clone tolerant to drought and water logging from old seed grown regions. Usually it takes 10-15 years to select a clone, which is quite long; it needs to be reduced by developing more efficient selection criteria. The modern techniques of molecular biology and biotechnology may be used for early screening of selected clones. Genetic variability was created through recombination, mutation, polyploidy and tissue culture to some extend, and thus more efforts can be given to create genetic variability all over the world by tea breeders. Selection of seedlings for yield and other attributes holds a great potential and should be exploited for genetic improvement of tea. Genetic markers for identifying superior clones in the population may be introduced. The local Assam types crossed freely with the plants brought from China and Indo-China, giving India an edge in terms of great variability in germplasm collection, which has been a breeders' delight. Enterprising tea planters made initial selections of desirable seed material for raising plantations suited to their tea estates. Exploitation of a wide genetic variability in the early years by mass selection gave a number of distinct seed varieties or jats. Considering the present state

of tea plantation in the world and current tea improvements, total displacement of seed plants by vegetative clones can possibly be ruled out. It is more likely and desirable that development of seed varieties and selection of clones should proceed simultaneously. Indian tea plantations, the tea improvement scheme is commonly referred to as the "clone-seed cycle". Clonal plantings are gaining more popularity in major tea growing countries, where old seedling teas are being uprooted and replaced with a few popular clones, whose cultivation is a dead end to evolution. It is leading to narrowing of genetic base of tea plantations. It needs to be arrested by preserving interesting bushes in the old-seed grown sections besides planting diverse clones. Development of polyclonal (similar to composites in corn) or biclonal (double cross hybrids) have the advantage of genetic adaptability and uniformity within a narrow plant type. So, conservation of fast depleting, valuable germplasm present in the existing old plantations is strongly advocated. It assumes greater significance in view of a narrowing genetic base by depending upon a few clonal varieties in breeding programs, which is fraught with danger of genetic vulnerability.

Thus the first clone was selected and released as cultivar code No. 19/29/13- later named as TV1 (Tocklai vegetative 1) in the year 1949. Since then clones have been released with the last clone TV30 in 1993. The clone TV_1 is a hybrid of Assam and China type tea, with good rooting efficiency and drought resistant capability. The bio-chemical characteristics are summarized in table 6.

There are several major diseases of tea plant that threatens production such as red rot, blister blight, root rot, caused by several pathogens. The pathogen infects only the economically important, tender shoots which lead to enormous crop loss, estimated up to 50% depending on the severity of infection (Table 7). Blister blight incited by a fungal pathogen *Exobasidium vexans* Massee is the most important leaf disease in India affecting both the quality and productivity of tea (35-50%). The protections against pest and pathogens have been achieved (40-68%) by using some conventional fungicide and pesticides but it hampered the export quality. So we need high yielding, disease resistant, quality full cultivars for more production to promote export. There are some strategies have been developed by the various researchers to enhance the defense mechanism of the plant through the manipulation of genes that are related to stresses (abiotic and biotic) tolerance (Table 7).

Table 6. Bio-chemical characteristics of TV1.

Green Leaf		**Made Tea**	
Moisture %:	79.68	Theaflavins %:	1.77
PPO activity (QO_2 µl/mg):	15.82	Thearubigins %:	14.04
Total Oxygen uptake (µl/mg) :	18.03	Crude fibre % :	9.80
Total polyphenolics %:	26.10	Total polyphenolics %:	16.23
Tannin %:	20.20	Tannin %:	14.25
Water soluble solids %:	42.85	Caffeine %:	3.49
Caffeine %:	3.52	Chlorophyll a (mg/g):	0.051
Chlorophyll a (mg/g):	0.361	Chlorophyll b(mg/g):	0.004
Chlorophyll b(mg/g):	0.185	Ash %:	6.41
Total Carotenoids (mg/g):	0.89	Total colour%:	6.06

Source: Special Bulletin, Tocklai Vegetative Clones, 1994, TRA, Calcutta.

The transgenic tobacco plant was developed by inserting the glutathione-S-transferase gene of cotton and showed enhanced resistance to methyl viologen (Yu *et al.*, 2003). A novel superoxide dismutase gene was overexpressed in Alfalfa plant which showed increased level of winter survival (abiotic stress) without a detectable increase in photosynthetic oxidative stress (McKersie *et al.*, 2000). In order to optimize disease and pest management strategies it is important to understand the genetic capabilities of tea germplasm to defend itself against diseases.

Table 7. Crop loss due to diseases and pests in tea plantation and their genetic improvement.

Stresses	Causal organism	% yield loss
Weeds	*Mimosa pudica* *Amaranthus spinosus* *Solanum khasianum* *Ageratum conyzoides*	9
Insect pests	Lepidopterans and Hemipterans	6-14
Diseases Leaf diseases	*Exobasidium vexans* *Corticium theae* *Pestalotia theae* *Colletotrichum theae-sinensis*	35-50
Stem diseases Root disease	*Tunstallia aculeate* *Poria hypobrunnea* *Phomopsis theae* *Hypoxylon sepens* *Ustulina zonata* *Hypoxylon asarcodes* *Armillaria melloa* *Xylaria* species	35
Abiotic stresses	Oxidative stress, salinity, drought, cold.	10

DNA-based Molecular Markers: During past two decades, a series of DNA based markers techniques have been developed and used for germplasm characterization. Fundamental differences among the different categories of DNA-based markers are summarized below. These markers can be primarily classified in two major categories:

I) *Hybridization based*-especially primed markers from known and characterized regions of the genome.

RFLP (Restriction Fragment Length Polymorphism),

The development of this marker requires extensive cloning and sequencing of the target sequences to design a specific probe. RFLP is a co-dominant single locus marker and therefore highly expensive during application also. This marker could be developed from coding as well as from non-coding regions of the genome.

II) PCR-based-arbitrarily primed anonymous markers: Some are dominant markers others are co-dominant markers. The examples of dominant markers are-

RAPD (Randomly Amplified Polymorphic DNA),
ISSR (Inter Simple Sequence Repeats),
AFLP (Amplified Fragment Length Polymorphisms) markers,
The examples of co-dominant markers are-
CAPS (Cleaved Amplified Polymorphic Sites),
STMs (Sequenced Tagged Microsatellite Sites), and
SCAR (Sequence Characterized Amplified Regions) markers.

These markers are very commonly used because no prior sequence information is required prior to their use. In addition, these markers are multilocus markers and they provide information from several loci per assay, which reduces the cost per locus.

These markers may direct single locus, oligolocus, or multiple locus differences, and the markers detected may be inherited in a presence/absence, dominant, or co-dominant manner. Brief descriptions of each of the DNA markers are available from the information contained in the Plant Genome website (http//www.nal.usda.gov/pgdic/tutorial/lesson4.htm) and recent reviews of molecular markers useful in mapping plant genomes (Karp *et al.*, 1997).

Botstein *et al.* (1980) heralded the ear of DNA based molecular genetic markers by proposing the Restriction Fragment Length Polymorphisms (RFLP) technique which provides the basis of a new type of genetic diagnostic tool that permits direct identification of a genotype in any tissue and developmental stage, in an environment-independent manner. RFLP-based linkage maps have since been developed in a number of plant and animal species. This genetic diagnostic tool can provide great benefits to plant breeders (Tanksley *et al.*, 1989) through which one can linkup the trait of interest to the DNA markers. Quantitative traits, particular interest to breeders, could be resolved into Mendelian factors and diagnosed with linked RFLP markers in a breeding population (Paterson *et al.*, 1988).

DNA fingerprinting is a term that was first coined by Jeffreys *et al.*(1985) to describe a multilocus RFLP pattern for the identification of individual on the basis of

polymorphisms of minisatellite sequences which are called as variable number of tandem repeats (VNTRs, Nakamura *et al.*, 1987). Reviews of DNA fingerprinting with these methods have been provided by Nybom (1991) and by Weising *et al.* (1989). The report of RFLP loci in humans with as many as 80 different alleles suggested the possibility of greatly enhanced informativeness per locus. Such VNTR loci consist of sets of tandemly repeated oligonucleotide core sequences that were termed "minisatellites" by Jeffreys *et al.* (1985). The core sequences vary in length from 11 to 60 base pairs and the repetitive region is flanked by conserved endonuclease restriction sites. Thus, the length of the restriction fragment produced by this type of genetic locus is proportional to the number of oligonucleotide core units it contains. The DNA fingerprinting in plants was first referred by Ryskov *et al.* (1988) who reported the use of the M13 repeat phage probe. Human minisatellite probes and synthetic simple repeat oligonucleotide probes have also been utilized.

Variable Number of Tandem Repeats (VNTRs): All eukaryotic genomes contain some regions of highly repetitive DNA, some of them are tandemly repeated and varies in repeat number so, named as variable number tandem repeat (VNTR) which have higher mutation rates than that of the single copy DNA. These repetitive regions have been categorized into three classes-

i) Satellite: The first class of repeated DNA sequence elements was discovered by density gradient centrifugation of sheared total genomic DNA from various eukaryotic organisms. This revealed small 'satellite' bands above and/or below the main mass of DNA. This satellite DNA was found to be composed of long tandem repeats of 100 to 5000 bp core sequences clustered at chromosome ends (telomeres) and centromeres.

ii) Minisatellite: Jeffreys *et al.* (1985) described this class of repetitive DNA as 'minisatellites', with 11-60 bp core repeats occurring in tandem arrays of up to 1000 units, distributed throughout the genome. Variation in the number of tandem repeats of minisatellite DNA (VNTRs) have been used as molecular markers to detect high levels of polymorphism, even between closely related individuals and populations of a single species. VNTRs are generally assayed by a multilocus RFLP approach that involves digesting genomic DNA with a restriction endonuclease, blotting to a membrane, and hybridizing with a labeled probe. The probe can be a copy of any minisatellite repeat unit, but a set of

probes developed by Jeffreys *et al* gave good results across widely divergent groups of species. The multiple, 'anonymous' fragments generated with a VNTR probe are very useful for determining parentage or identity (genetic fingerprinting), but do not allow for the identification of alleles or the determination of genotypes. Thus these markers are difficult to use for measurements of relatedness among individuals or for phylogenetic analyses.

iii) Microsatellite: An even more ubiquitous class of repetitive DNA, which is composed of 1-6 bp core units that are tandemly repeated throughout the genome in arrays of few to many thousands of copies. These are less polymorphic than minisatellites, but occur more frequently in the genome. The abundance of this type of marker was suggested by the occurrence of a dinucleotide repeat such as $(CA)_{n>10}/(GT)_{n>10}$ as many 50,000 times in the human genome. This type of reiterated sequence has been termed as a Short Tandem Repeat (STR; Edwards *et al.*, 1991), a microsatellite (Litt and Luty, 1989) or a Simple Sequence Repeat (SSR; Weber and May, 1989). As is generally the case with VNTR loci, the DNA sequence flanking SSRs are conserved, allowing the selection of PCR primers that will amplify the intervening SSR in all genotypes of the target species. The major source of variation is replication slippage resulting in different array length. Microsatellites currently gain tremendous importance as markers for genetic mapping and fingerprinting. Microsatellite oligonucleotides are excellent DNA fingerprinting probes. Differences in length at an SSR locus are detected by DNA amplification *via* PCR, using a pair of oligonucleotide primers that complement unique sequences flanking the microsatellite (Weber and May, 1989). The sizes of the amplified products are precisely determined by acrylamide gel electrophoresis. This type of assay generates single-locus data that provide alleles and genotypes. Evolutionary distances can be calculated between the various alleles at a locus based on the assumption of a stepwise mutation process that results in the alleles closest in size being most closely related by descent from common progenitor. They showed Co-dominant Mendelian inheriantance. The primary drawback of SSR-based markers is the effort and cost required to develop specific primer pairs for each polymorphic locus. This involves the cloning and sequencing of large numbers of genomic DNA fragments containing SSRs. However, much progress is occurring in this area, so

costs associated with marker isolation and characterization is likely to diminish. Another problem is that microsatellites with four base-repeats might not be randomly distributed in a genome but clustered at the centromeres or telomeres. This limits the usefulness of such mutilocus probes in genetic linkage analysis and population studies.

Inter Simple Sequence Repeats (ISSR): Inter Simple Sequence Repeats (ISSR) marker was developed by Zietkiewicz *et al.*, (1994). Instead of SSR, the ISSR markers can be used to circumvent the problems associated with the SSR markers. ISSR is a technique in which primers that anneal to microsatellites during PCR lead to amplification of the piece of DNA between two primers. For this system two primers are constructed to anneal to microsatellites [(CA)n or (GT)n]with one extra nucleotide in the 3′-end. The extra nucleotide will permit amplification only if the primer binds to a 5′-end of a microsatellite with a suitable first nucleotide in the flanking sequence. Such extra nucleotides are termed anchors and they assure that amplification will always start from the 5′-end of the microsatellite. Amplification will only take place when two suitable microsatellites with suitable first flanking nucleotides are in a distance suitable for PCR amplification. Because of the many microsatellites in most genomes of higher plants, ISSR will often amplify 25 to 50 ISSR products in one reaction. Polymorphisms are mostly of the dominant type because of changes in the anchoring nucleotides. There is no need of cloning and sequencing of any DNA part of the genome under investigation.

RFLP is the most reliable polymorphism which can be used for accurate scoring of genotypes. RFLP are co-dominant and can identify unique locus. When cDNAs with known gene function are used as markers, the chromosomal position of the specific gene or genes can be identified on the chromosome. Using RFLP markers, genetic maps have been developed in many plant species. The polymorphism detected by RFLP markers is very reliable as it involves the recognition by specific restriction enzymes and hybridization with a specific probe (according to Southern, 1975). RFLP markers facilitate the selection of progenies with desirable genotypes in a shorter span of time. However, RFLP analysis is labour intensive and time consuming. One of the major problems in using RFLP marker technology in breeding programs is the expense involved. The expense includes not only the material and supplies but also less definable costs such as quality technical

support and radioisotope permits (Tanksley *et al.*, 1989) and it requires relatively large amount of DNA (5-10μg) and is refractory to automation. While variation in length or sequence of diagnostic DNA is used to measure degree of relatedness between individual organisms or inheritance in progeny populations, the identification of these molecular markers requires prior knowledge of DNA sequence, cloned and characterized probes, and considerable experimental manipulation.

To circumvent these limitations in the RFLP technique, a novel DNA amplification-based strategy was conceived. Single, arbitrary primer-based DNA amplification techniques (DAF, RAPD and AP-PCR) were developed (Williams *et al.*, 1990; Welsh and McClelland, 1990; Caetano-Anolles *et al.*, 1991a, 1991b, 1992a), extending the utility of Polymerase Chain Reaction (Mullis, 1986) to general genome analysis. Because of a plethora of terms, a general acronym was proposed as MAAP (Multiple Arbitrary Amplicon Profiling, Caetano-Anolles *et al.*, 1992b, 1993). The discrete amplifiable DNA regions, also termed amplicons in the PCR are determined by primer annealing to partially or perfectly complementary sites on each DNA strand. Strategies like random amplified polymorphic DNA (RAPD) analysis (Williams *et al.*, 1990), arbitrarily primed PCR (AP-PCR; Welsh and McClelland, 1990), and DNA amplification fingerprinting (DAF; Caetano-Anolles *et al.*, 1991a) target multiple annealing sites without the requirement of prior knowledge of template sequence were developed independently and apparently concurrently in the three laboratories. They have been widely used for the construction of genetic maps (Reiter *et al.*, 1991), for simplified identification of markers linked to traits of interest (Michelmore *et al.*, 1991), for genetic fingerprinting (Welsh *et al.*, 1991), for genetic diagnostics (Horn *et al.*, 1989; Horn and Rafalski, 1992), and in population genetics (Arnold *et al.*, 1991).

I. **AP-PCR** (Arbitrarily Primed-PCR) developed by Welsh and McClelland (1990), which uses PCR-primers of length 18 to 32 nt of arbitrary sequence to amplify target DNA under low stringency (*i.e.*, low annealing temperature) conditions for two amplification cycles followed by a series of cycles of high stringency amplification. Amplification product was resolved by polyacrylamide gel electrophoresis and was detected by autoradiography.

II. **RAPD** (random amplified polymorphic DNA) procedure was invented by Williams *et al.*, (1990), in which an arbitrary primer of either 9 or 10 nt in length was used to produce PCR products. RAPD products are routinely resolved on agarose gel electrophoresis and visualized by ethidium bromide staining on UV light. RAPD is widely used because of its simplicity and low-cost instrumentation.

III. **DAF** (DNA Amplification Fingerprinting) developed by Caetano-Anolles *et al.* (1991a, b). Of all MAAP procedures, DAF utilizes the shortest primer, down to 5 nt in length. The optimal length was found to be 8 nt a length which does not produce efficient amplification with RAPD. DAF products are routinely separated by polyacrylamide gel electrophoresis, and stained with an improved silver staining method for visualization of bands.

There are several examples of genetic diversity and relationship studies on different plant species by different workers (Joshi and Nguyen, 1992, 1993; Tanaka *et al.*, 1996; Tsumura *et al.*, 1996; Wachira *et al.*, 1995 and 1997; Paul *et al.*, 1997; Wolfe *et al* ., 1998; Lai *et al.*, 2001; Wendel and Liu, 2001; Vijayan and Chatterjee, 2003; Roy, 2004; Vijayan *et al.*, 2004; Nagaraju *et al.*, 2002, 2004; Matsumoto *et al.*, 2004; Eapen *et al.*, 2004; Roy and Chakraborty, 2007).

RFLPs were the first DNA based co-dominant markers used for genetic diversity studies. Matsumoto *et al.*, (1994) cloned the phenylalanine ammonia-lyase (PAL) cDNA from tea, and used it as RFLP probe. They were able to distinguish Assam hybrids and Japanese tea varieties with low and high levels of catechins. The PAL gene was shown to be single copy and multiallelic. The major disadvantage of RFLPs is that they are highly expensive and time consuming both during development and application. The advent of PCR (Polymerase Chain Reaction) led to development of another co-dominant marker, known as CAPS (Cleaved Amplified Polymorphic Sites) or PCR-RFLP. Kaundun and Matsumoto (2003) analysed the genetic diversity of *sinensis* and *assamica* cultivars of tea using CAPS markers for three genes, namely PAL, CHS_2 (Chalconesynthase) and DFR (Dihydroflavonol 4-reductase), which are involved in phenylpropanoid pathway leading to biosynthesis of catechin and tannin. The study showed that polymorphisms in PAL and DFR genes was due to indels in the introns of both genes as well as due to point mutations as revealed by restriction

digestion of amplicons. Although, they did not find any particular CAPS profile specific to *seninsis* or *assamica*, the combined data set from all the CAPS markers could group the cultivars according to their respective varietals status.

A greater level of variation was observed in CHS_2 gene, which is expected due to fact that CHS is a multigene family comprising of three non-allelic genes with high sequence similarity (Takeuchi *et al.*, 1994). As more and more genes from tea are being cloned and sequenced, there is great future potential for CAPS markers. These expressed sequence tags (EST) projects in tea have produced a large number of sequence data (Park *et al.*, 2004). Presently, there are over 2000 ESTs for tea available in NCBI data Bank. CAPS markers based on these ESTs can also be used for linkage analysis and marker-assisted selection in tea because these are from the expressed regions of the genome.

Although majority of these are uncharacterized sequences, these can still be used for development of CAPS markers in future. Several important genes controlling quality traits in tea such as beta-primeverosidase (Mizutani *et al.*, 2002), caffeine synthase (Kato *et al.*, 2000) and polyphenol oxidase (GenBank Accession no. AF 269192) have been already cloned and sequenced. Identification of polymorphisms in these genes can help in developing varieties with improved quality traits through marker-aided selection. STMs markers are another type of co-dominant markers, which are the most desirable markers for genetic studies due to inherently high levels of allele density (Tripathi and Negi, 2006). These markers are based on flanking sequences of microsatellites, which are tandem repeats of 2-5 base pairs. Development of STMs markers is highly expensive because it requires isolation, cloning and sequencing of portions of genome containing these repeats. Till date, only 15 microsatellite sequences from *C. sinensis* have been reported (Freeman *et al.*, 2004). Few microsatellites sequences from *C. japonica* have also been reported earlier (Ueno *et al.*, 1999). These markers are non-transferable between species except very closely related ones and therefore need to be developed for each species separately. Kaundun and Matsumoto (2002) attempted to transfer four nuclear microsatellites from *C. japonica* and seven chloroplast microsatellites from *Nicotiana tabacum* into *C. sinensis*. The CAPS markers have also been used in tea characterization (Kaundun and Matsumoto, 2003).

Molecular markers highlighted important differences in the partitioning of diversity within and among population. The potential use of different molecular markers such as RFLP (Matsumoto *et al.*, 1994), RAPD (Lee *et al.*, 1995; Wachira *et al.*, 1995; Kaundun *et al.*, 2000; Kaundun and Park, 2002) and AFLP (Paul *et al.*, 1997) have been reported in tea. These markers are useful for characterization, estimation of genetic relatedness and determination of genetic diversity among the tea germplasm. Comparison between RAPD, AFLP and SSR markers in different plants species has revealed that co-dominant SSRs detect the highest level of polymorphism per locus and it surveys the hyper-variable microsatellite regions of the genome, hence they have higher information content (Powell *et al.*, 1996; Maughan *et al.*, 1996). Sometimes, AFLP and RAPD despite their dominant nature can estimate relationship with a high resolution due to their multilocus approach (Powell *et al.*, 1996; Russell *et al.*, 1997; Teulat *et al.*, 2000).

Mishra and Sen-Mandi (2004) has developed a DNA marker, which is associated with drought tolerance in tea. The usefulness of molecular techniques, including RAPD, in the characterization and identification of plant germplasm has great advantage over the phenotypic characterization because of their independence from environmental variations (Varghese *et al.*, 1998). This RAPD marker has been utilized extensively for the characterization of tea genetic resources and estimation of genetic diversity were reported earlier by many workers (Bera and Saikia, 1999; Wachira *et al.*, 1995, 1997; Paul *et al.*, 1997; Raina and Marimuthu, 1999; Tanaka *et al.*, 1996). The RAPD methodology has provided informative data consistent with other markers, especially at the intraspecific level (Dos Santos *et al.*, 1994; Lerceteau *et al.*, 1997) and it is cost effective for large-scale population genetic analysis.

The PCR based anonymous markers have also been used in genetic diversity studies on tea. The markers are as such: RAPD, ISSR and AFLP (Wachira *et al.*, 1995; Paul *et al.*, 1997; Lai *et al.*, 2001; Balasaravanan *et al.*, 2004). The PCR based markers has been used to create a genetic linkage map in tea by Hackett *et al.* (2000). Using RAPD, Kaundun *et al.* (2000) observed higher level of genetic diversity in Korean cultivated accessions of tea as compared to accessions from Japan and Taiwan. However, the intra-population genetic diversity of six Korean tea populations was found to be relatively lower than the expected diversity of natural populations (Kaundun and Park, 2002). This was attributed to narrow genetic base of the tea

samples introduced from China and considerable reduction in population size following mass destruction of tea populations during fourteenth century.

Wachira *et al.* (1995) used RAPD to estimate genetic diversity and taxonomic relationships in 38 clones belonging to the three tea varieties, China, Assam and Cambod type. Extensive genetic variability was detected in these clones and showed that 30% of the total diversity resided among population while 70% diversity resided within populations based on Shannon's diversity index. Cluster analysis using RAPD data could separate the three populations in a manner consistent with both the present taxonomy of tea and with the known pedigree of some clones. The study also showed the usefulness of RAPD in discriminating the commercial clones, even those that cannot be distinguished on the basis of morphological and phenotypic traits. Wachira *et al.*, (1997) studied the genetic relationship of 20 species of the genus *Camellia* using RAPD and organelle-specific PCR. The result was generally consistent with the classification based on morphology and the relationship revealed from comparative photochemical data.

This implied that the results of RAPD and ISSR studies were highly congruent. However, ISSRs seemed to be more discriminative in cultivar identification then RAPDs because cultivars that could not be distinguished by RAPDs markers were separable based on ISSR markers. This finding suggests that the evolution rate of ISSRs might be faster than that of RAPDs in the tea samples studied. ISSR markers are, therefore, suitable for use in the study of genetic diversity and determination of genetic relationships of closely related tea cultivars. Other studies also indicated that ISSR markers exhibit higher levels of polymorphism than RAPD markers (Yang Quiros, 1993; Nagaoka and Ogihara, 1997; Parsons *et al.*, 1997; Esselman *et al.*, 1999). The observed difference between ISSR and RAPD might be explained in terms of functional constraints since some RAPD bands may be associated with functionally important loci (Penner, 1996).

The fast evolutionary rate and the hyper variability of ISSR may suggest that ISSR bands represent neutral markers (Esselman *et al.*, 1999). Results showed that Assam tea had the smallest within group diversity while native wild tea had the largest diversity for both RAPD and ISSR markers. Three clones of Assam tea were included in the present study. These clones represent only a small portion of the genetic variation of Assam tea. This might account for the small within group

diversity of Assam tea observed. Six samples of native wild teas were studied, and the diversity within native wild tea was larger than China tea, which consisted of 21 cultivars. The result indicated that the native wild tea is highly variable and the germplasms of native wild tea might be useful in breeding programs.

Linkage maps of canola have been constructed using isozymes, RAPDs, RFLPs, (Thormann *et al.*, 1994) and to a limited extent using microsatellites (Kresovich *et al.*, 1995). The RFLP markers generally represent single and low copy sequences. SSRs are widespread in the genome and the average heterozygosity index of these markers has been reported to be higher than any other single locus approach (Roder *et al.*, 1995; Powell *et al.*, 1996). AFLP approach has the ability to expedite the construction of a saturated linkage map as it detects a high level of polymorphisms in DNA in a single lane. The segregating loci mostly map on the independent locations. AFLP maps are currently being made in a number of crops.

The AFLP technology developed by Vos *et al.* (1995) is a more reliable and robust technique. This marker technology has received great attention in genetic diversity studies during recent years. The first paper on genetic diversity of tea using AFLP was by Paul *et al.* (1997), who studied diversity of 32 tea clones comprising of Indian and Kenyan origin. A total of 73 bands generated with 5 AFLP primer combinations revealed that the intramorphotype genetic diversity was higher in Chinary types than in Assam or Cambod types.

Shannon's index of diversity was used to partition the total variation, and they found that most of the diversity was detected within populations, with 79% of the variation being within and 21% being between populations of India and Kenyan tea. A dendrogram constructed on the basis of band sharing separated the tea samples into China, Assam and Cambod types. The Pearson's correlation coefficient between similarity matrices based on RAPD and ISSR was 0.811. The Mantel test revealed that the correlation was highly significant ($P<0.001$). The clustering obtained in this study was consistent with the morph-types of the accessions used in this study. The principal component analysis revealed that the Assam clones from India and Kenya were highly related which supports the history of migration of tea from India to Kenya (Singh and Bera, 1994).

Balasaravanan *et al.* (2003) studied the genetic diversity of 49 tea accessions from UPASI comprising of Assam, China and Cambod types. A total of 1555 bands obtained with 3 primers combinations supported the findings of Paul *et al.* (1997) revealing an overall higher genetic diversity with China type accessions than Assam types. The study also revealed a very low diversity within South Indian cultivars, which may be due to selection from a narrow gene pool.

Towards the ultimate goal of genetic improvement of tea, efforts are required to construct a high-density linkage map using molecular markers and integration of economically important traits on this linkage map. A beginning has been made by Hackett *et al.* (2000), who used RAPD and AFLP markers for construction of a linkage map of *C. sinensis*. As tea is highly cross-pollinated, conventional mapping populations such as F_2 selfed or BC_1 cannot be developed. Alternative approaches such as pseudo test cross that have been used in other tree species can also be used in tea (Grattapaglia *et al.*, 1996; Hackett *et al.*, 2000). The biclonal seed stock populations, which are readily available in various tea germplasm institutes, can be used for this purpose. Clonal seed orchards or seed baris can be established using genetically diverse accessions identified based on genetic diversity and morphological data. This will generate excellent raw material for future selection of clones with desirable combination of traits and for further use in research such as QTL (Quantitative Trait Loci) mapping and marker-assisted breeding.

It is relatively easy to turn the RAPD assay into a secondary PCR assay through DNA sequencing of the RAPD band of interest and conversion to allele-specific PCR, allele-specific ligation (Landergren *et al.*, 1988) or a sequence characterized amplified region (SCAR) assay (Paran and Michelmore, 1993). Additional sequence polymorphisms may be detected in RAPD bands using restriction enzyme divergence, single strand conformational polymorphism assay (SSCP; Orita *et al.*, 1989), or denaturing gradient gel electrophoresis (DGGE; Myers *et al.*, 1986) may be applied to individual RAPD bands.

The advent of polymerase chain reaction (PCR) favored the development of different molecular techniques such as random amplified of polymorphic DNA (RAPD), simple sequence repeats (SSR or microsatellite), sequence tagged sites (STS), random amplified microsatellite polymorphism (RAMP) and inter simple sequence repeat polymorphism (ISSR) and so on (Saiki *et al.*, 1988; Welsh and

McCleland 1990; Williams *et al.*, 1990; Akkaya *et al.*, 1992; Tragoonrung *et al.*, 1992; Zietkiewicz *et al.*, 1994; Wu *et al.*, 1994a; Nagaoka and ogihara, 1997).

Molecular markers successfully developed during the last two decades have largely overcome the problems that are associated with phenotype-based classification. Initially, isozymes (Chengyin *et al.*, 1992) and Restriction Fragment Length Polymorphisms (RFLPs) (Botstein *et al.*, 1980; Beckmann and Soller 1986; Tanksley *et al.*, 1989; Miller and Tanksley, 1990; Wang *et al.*, 1992) served as reliable markers for genetic analyses in plants. But PCR based techniques developed in recent years such as Random Amplified Polymorphic DNA (RAPDs) Williams *et al.*, 1990; Welsh and McClelland 1990), Inter Simple Sequence Repeats (ISSR) (Zietkiewicz *et al.*, 1994), Amplified Fragment Length Polymorphisms (AFLPs) (Vos *et al.*, 1995), and Simple Sequence Repeats (SSRs) (Weber and May, 1989), also called microsatellites, sequence tagged sites (STS), random amplified microsatellite polymorphism (RAMP) provide DNA markers that are dispersed throughout plant genomes (Saiki *et al.*, 1988; Tragoonrung *et al.*, 1992; Wu and Tanksley, 1993; Wu *et al.*, 1994) and are easier to reproduce and analyze.

High levels of polymorphism and their co-dominant nature have made SSRs ideal markers for studying genetic diversity in plants (Akkaya *et al.*, 1992; Morgante and Olivieri, 1993; Plaschke *et al.*, 1995). However, the time and cost of identifying SSR motifs and designing primers for regions flanking SSRs have restricted the widespread use of microsatellites in plants (Beckmann and Soller, 1990; Roder *et al.*, 1995). ISSR markers, which show dominant inheritance, use SSR repeat-anchored primers and are being used as an alternate tool in diversity studies. ISSR markers are useful in detecting genetic polymorphisms among accessions by generating a large number of markers that target multiple microsatellite loci distributed across the genome.

Further, they are simpler to use than the SSR technique as prior knowledge of the target sequences flanking the repeat regions is not required (Zietkiewicz *et al.*, 1994; Tsumura *et al.*, 1996; Nagaoka and Ogihara, 1997; Nagaraju *et al.*, 2002; Bornet *et al.*, 2002). It has been shown that different markers might reveal different classes of variation (Powell *et al.*, 1996; Russell *et al.*, 1997). It is correlated with the genome fraction surveyed by each kind of marker, their distribution throughout the genome and the extent of the DNA target which is analyzed by each specific assay

(Davila *et al.*, 1999). Of these techniques, RAPD has several advantages, such as simplicity of use, low cost, and the use of small amount of plant material. ISSR markers, which involve PCR amplifications of DNA using a primer, composed of a microsatellite sequence anchored at 3' or 5' end by 2-4 arbitrary, could be used to assess genetic diversity (Qian *et al.*, 2001). ISSRs have been used for cultivar identification in maize (Kantety *et al.*, 1995; Pejic *et al.*, 1998), potatoes (Prevost and Wilkinson, 1999), wheat (Nagaoka and Ogihara, 1997), cotton (Dongre and Parkhi, 2005) and barley (Fernandez *et al.*, 2002; Tanyolac, 2003).

Restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (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). The above two techniques are slow and expensive and are not amenable for assessment of genetic variation in large number of genetic analysis. More recently, PCR-based, random amplified polymorphic DNA (RAPD) marker, developed by Williams *et al.* (1990) have been employed widely in cultivars identification and characterization (Halward *et al.*, 1992; Sharma *et al.*, 1995; Lai and Hsiao, 1997; De Bustos *et al.*, 1998; Mandolino *et al.*, 1999). Inter-simple sequence repeat polymorphism (ISSR) markers developed by Zietkiewicz *et al.*, (1994) can also be employed for the study of genetic variation among the cultivated tea species (Wolff *et al.*, 1995, Tsumura *et al.*, 1996; Fang and Roose, 1997; Chen *et al.*, 1998).

Several types of molecular markers have been employed previously to quantify the genetic diversity within tea germplasm collections in different countries (Tanaka *et al.*, 1996). Wachira *et al.* (1995, 1997) have used this molecular marker system to determine genetic diversity and differentiation within and between cultivated tea and related *Camellia* species. Paul *et al.* (1997) studied the diversity and genetic differentiation of Indian and Kenyan tea using AFLP markers. Lai *et al.* (2001) studied the genetic relationships in cultivated tea clones and native wild tea in Taiwan using RAPD and ISSR markers. A few Tocklai variety (TV clones) has also been characterized on the basis of RAPD analyses by Bera and Saikia (1999). Matsumoto *et al.* (1994) characterized the genetic diversity in tea cultivars on the basis of PAL (phenylalanine ammonia lyase) cDNA probe analysis. Devarumath *et al.* (2002) evaluated the genetic integrity of micropropagated diploid and triploid elite tea clones on the bases of RAPD, ISSR and RFLP fingerprinting. Kaundun *et al.* (2000)

also studied the genetic diversity among elite tea accessions using RAPD markers. Six Korean tea populations were analysed on the basis of RAPD-PCR to reveal their genetic diversity among the tea populations by Kaundun and Park (2002). The difference in the generation of bands per primer was probably due to the differences in the primer sequence and primer-template DNA interaction (Weir *et al.*, 1997). According to Rafalski *et al.* (1991) variation in the number of bands in RAPD profiles is independent of the complexity of the genome. ISSR markers, which involve PCR amplifications of DNA using a primer composed of a microsatellite sequence anchored at 3′ or 5′ end by 2-4 arbitrary, could be used to assess genetic diversity (Qian *et al.*, 2001). ISSRs have been used for cultivar identification in maize (Kantety *et al.*, 1995; Pejic *et al.*, 1998), in potatoes (Prevost and wilkinsom, 1999), in barley (Fernandez *et al.*, 2002, Tanyolac, 2003).

So far only a few attempts have been made to characterize the genetic diversity in tea by using molecular markers. These include AFLP based marker analysis (Paul *et al.*, 1997), RFLP (Wachira *et al.*, 1995; 1997; Devarumath *et al.*, 2002; Matsumoto *et al.*, 2004), RAPD profiles (Kaundun *et al.*, 2000; Lai *et al.*, 2001; Park *et al.*, 2002; Luo *et al.*, 2004; Roy, 2004; Li *et al.*, 2005; Chen *et al.*, 2005; Roy and Chakraborty, 2007; Cheng-Wen *et al.*, 2008), ISSR based analysis (Lai *et al.*, 2001; Mondal and Chand, 2002 and Devarumath *et al.*, 2002; Liang *et al.*, 2007). In the current study we report the use of RAPD and ISSR markers for assessing the genetic diversity and relationships among 21 cultivated clonal genotypes from three tea varieties.

Tissue Culture for Genetic Improvement

The tea plant is generally propagated vegetatively by rooting of the single stem node cutting. Although, vegetative propagation of elite clones has been used for large scale multiplication of plants with desirable attributes in respect to yield and quality. But it has some drawbacks of such clonal plantations, noted over the years in field, is yield variability under adverse/stress conditions. Vegetative clonal plantations are also relatively prone to pathogens in comparison to seed grown plantations (Barua, 1989). Quite often, the regenerated cuttings gradually lose their vigour in the long run and if the source plant itself is infected, the cuttings also carry the disease.

Like may other woody species, it has traditionally been propagated vegetatively because it is highly heterozygous and seedlings are too variable for commercial use (Banerjee *et al.*, 1992). The ever-increasing demand for higher productivity and better quality of tea has resulted in the need for mass multiplication of elite clones. Tea is propagated largely through seeds or cuttings, but a major concern associated with seed raised populations has been the occurrence of high genetic variability. Tissue culture technique can be employed to eliminate the problem. Tissue culture technology and mass clonal propagation with advancements in the field of genetic engineering are likely to make a major impact on the tea industry. *In vitro* tissue culture techniques include rapid mass clonal propagation of selected clones, production of pure breeding lines, germplasm storage and exchange, interspecific and intergeneric hybridization, development of haploids, polyploids and somaclonal variants, somatic embryogenesis leading to the overall improvement in yield and quality of tea.

Plantlets were regenerated *in vitro* from callus by Wu and Li (1976). A number of reports appeared on *in vitro* culture of tea using almost all the plant parts *viz.* leaf, node, internode, cotyledon, embryo, root, anther, and pollen. Tissue culture provides a method of rapid multiplication of selected genotypes (Nakamura, 1989; Kato, 1986, 1996; Arulpragasam and Latiff, 1990; Rajkumar and Ayyappan , 1992; Vieitez and Borciela, 1992; Sood *et al.*, 1993; Akula and Dodd, 1998; Mondal *et al.*, 2004). The application of tissue and organ culture for multiplication of elite tea clones has been advocated. Tissue and cell culture technology along with advancements in the field of genetic engineering are likely to make a major impact on the industry in the areas of rapid and mass clonal propagation of selected clones, in disease elimination and development of stress (biotic and abiotic) tolerant clones, production of pure breeding lines through haploid culture technology, germplasm storage and exchange, mutant selection and development and for the overall improvement in quality and yield of tea (Jain and Newton, 1990).

The plantlets were transferred to rooting medium for root initiation, after which they were hardened or acclimatized. However, only a few hardened tissue culture derived plants were transferred to field. The use of modern technology for mass multiplication of tea for commercial plantings is limited largely due to problems of rooting, hardening and survival after field transfer of tissue culture raised plants.

Phukan and Mitra (1984) obtained shoot buds from the nodal segments without an intervening callus phase. Banerjee and Agarwal (1990) studied rooting in vitro in *Camellia sinensis*. Micropropagation of tea was first reported by Kato (1985) followed by several others (Arulpragasam and Latiff, 1986; Akula and Dodd, 1998; Saratchandra *et al.*, 1988; Jha *et al.*, 1992). Micropropagation has not yet been commercially adopted as a means of propagating tea due to the fact that tea is easily propagated vegetatively by single leaf cuttings. The tissue culture method is not season bound, and is suitable for production of large number of plantlets and can be profitably used in the development of gene banks and for multiplication of novel elite plants obtained through other biotechnological approaches. Moreover, meristem cultures can be used for regeneration of disease free explants which can be transported from one place to another without quarantine problems. While plant tissue culture is essentially used as a method of clonal (true-to-type) propagation, tissue culture induced variation also provides an additional tool for enhancing genetic variability, including the incorporation of desirable traits like high yield. Variation in cultured cells and plants regenerated from these is referred as somaclonal variation (Larkin and Scowcroft, 1981; Evans *et al.*, 1981).

There have been several studies aimed at the production of haploid tea plants using anther culture (Raina and Iyer, 1983). Haploid plants thus obtained can be diplodized to obtain homozygous diploids for use in breeding programmes and for developing inbred lines. If homozygosity achieved at all loci, which would greatly improve the process of selection and helps in screening mutants. Hence, haploid technology has great potential for initiating investigations on genetic and genetic engineering for the improvement of tea plant. Haploids obtained by anther and pollen culture can be depolarized to get homozygous diploids for use in the breeding program to get inbreeds lines.

The first observation of *in vitro* somatic embryogenesis was made in *Daucus carota* (Reinert, 1958, 1959; Steward *et al.*, 1958). Somatic embryo formations in *Camellia* sp. were investigated by many workers (Vieitez and Borciela, 1990; Jha *et al.*, 1992; Kato, 1996; Wachira and Ogunda, 1995; Ponsamuel *et al.*, 1996).

Tea (*Camellia sinensis*) plants are perennial, and highly heterozygous, requiring many years of selection to incorporate favourable traits. Nakamura (1988) has induced adventitious embryo formation on the tea cotyledon of *Camellia sinensis*

and other *Camellia* sp. The adventitious bud formation was investigated in tea stem culture by Nakamura (1988). The somatic embryogenesis in tea (*Camellia sinensis*) has been investigated by Akula and Akula (1999). A novel plant growth regulator, betaine was used in tea tissue culture for rapid induction of somatic embryogenesis (Akula *et al.*, 2000). Somatic embryogenesis from immature leaves has been investigated by Kato (1996). Mondal *et al.* (2001b) have shown the *in vivo* somatic embryogenesis in tea (*Camellia sinensis*) from cotyledons. Transgenic tea plant has been developed by *Agrobacterium*-mediated transformation of somatic embryos in tea (*C. sinensis*) (Mondal *et al.*, 2001). The somatic embryo has been obtained from embryonic tissues of *Camellia japonica* L. (Vieitez and Barciela, 1990).

The RAPD marker has been used appropriately for a number of crop species to detect genetic integrity among micropropagated plants (Isabel *et al.*, 1993; Rani *et al.*, 1995; Rani and Raina, 2000; Mondal and Chand, 2002). Genetic variation may occur in the tissue culture raised tea plant as a result of mutation during micropropagation (Mondal and Chand, 2002). The other markers RAPD, ISSR and RFLP were used to examine the genetic integrity of micropropagated tea plants, UPASI-26, UPASI-3 and UPASI-27 (Devarumath *et al.*, 2002).

The micropropagated plants of both the UPASI-3 and UPASI-27 (Assam type) revealed complete stability but the regenerated clone UPASI-26 (China type) showing lack of complete genetic integrity. That means China type clone UPASI-26 is prone to genetic changes during propagation. Two Assam type clones UPASI-3 and UPASI-27 exhibited complete stability during *in vitro* propagation. It is indicating that the genetic changes in tea clones are genotype dependent rather than culture condition dependent (Devarumath *et al.*, 2002). Somaclonal variation has been detected in micropropagated plants of a number of species for commercial importance as well as genetic integrity was maintained in the tissue culture derived plants (Isabel *et al.*, 1993; Rani and Raina, 2000; Derarumath *et al.*, 2002). Hashmi *et al.* (1997) has detected somaclonal variants in each regenerated plants from tissue culture using RAPD markers, that reveals that genetic changes occur during *in vitro* culture in peach.

Genomic Constitution as Revealed by Chromosome Karyotype

The taxonomic status of the present day cultivated tea is a subject of much debate and controversy. Cultivated tea has been classified into three types i) China type, ii) Assam type and iii) Cambod type on the basis of morphology *i.e. C. sinensis* var. *sinensis*, *C. sinensis* var. *assamica* and *C. assamica* sub sp. *lasiocalyx* (Cambod type). All the three types of tea are almost self-sterile and outbreeding in nature coupled with frequent spontaneous hybridizations that take place between different types of tea, resulting in highly heterozygous plants ranging from China type to Assam type, has further complicated the taxonomic status of tea. The cultivated tea is cytogenetically very poorly understood. The investigations are largely restricted to mere determination of zygotic numbers. Although preliminary meiotic analysis have been conducted in few cases but the available information is not sufficient to draw any conclusion or sometimes conflicting (Bezbaruah, 1971).

Except for the few natural triploids and polyploids reported by Bezbaburah (1971), the cultivated tea plant is a diploid with a chromosome number of 2n = 30. The chromosome number in tea was determined by many investigators (Bezbaruah, 1971). Root-tip method was generally used for mitotic studies which necessitated digging up of roots. To avoid this, Bezbaruah adopted the shoot-tip method for examination of mitotic chromosomes in tea and other related species after necessary modification and standardization. Bezbaruah (1971) made detailed karyotype analysis of 30 tea clones belonging to the three races of tea, ten from each race, as well as of plants of a few allied species. The somatic chromosome number of 30 was common to all the tea clones and plants of the allied species. The chromosome of tea, as of other *Camellias*, is short but there is a gradation of size from the longest to the shortest. The chromosomes have median to sub-median primary constrictions. In a few clones, one or two pairs of chromosomes may have secondary constrictions. Clones belonging to the three races of tea do not reveal any major differences in their karyotype, although some minor differences exist. However, none of the minor differences could be correlated with any morphological feature of the plants. No irregularities are observed in the meiosis of any of the clones. In many genera and species, the process of evolution is accompanied by different types of karyotypic changes whereas in others morphological difference and divergence occur without any visible change in chromosome morphology.

This phenomenon is attributed to cryptic gene mutation. Close morphological similarity of the tea chromosomes suggests that the observed differences in growth and form of plants of the different races of tea are the results of mutative changes of the genes as found in *Pinus* and *Quercus*, where the karyotype is closely similar although they are different species. Karyotype analyses of different cultivars of tea showed a smooth decrease in size of chromosome from the longest to the shortest. Naturally evolved polyploids in tea are very rare. Only a few natural triploids (2n=45) have so far been reported from Japan, South India (Venkataramani and Sharma, 1974). Ackerman (1977), and Bezbaruah (1971) mentioned that the ploidy level in the genus *Camellia* ranged up to hexaploid with 2n=90 chromosomes. Ackerman (1977) proposed that segmental allopolyploid might have originated during the course of evolution in diploid level and continued in the polyploids through inter-crossing among them. Bezbaruah (1971) reported that the chromosomes of tea plant were primitive but other classified them as of advanced status depending on the symmetry and asymmetry of chromosomes respectively. The length of tea chromosomes ranged from 1.24μm to 4.20μm. The 'r' value (ratio of long arm to short arm) for all the 15 pairs of chromosomes ranged from 1.00 to 1.91. The chromosomes were classified morphologically on the basis of relative lengths, position of the centromere and presence or absence of secondary constriction. The meiotic division in Assam and hybrid tea were regular with 15 bivalents at diakinesis and telophase I and the segregation was regular (Bezbaruah 1971). Bezbaburah (1971, 1976) observed two satellite pairs of chromosomes in the complement of *C. assamica*. Lack of predominant satellites indicated tea as being inherent in low activity of the nucleolus organizer, which restricts the chromosomes to produce secondary constrictions.

In tea, preliminary studies on the detailed karyotypic analysis and nucleolar organizer carried out at Tea Research Foundation, Kenya, revealed that the nucleolar numbers correspond to multiples of the somatic chromosome number (2n=30). Nucleolar number per cell was shown to reflect ploidy. Some NORs and therefore, ribosomal gene clusters were observed to be located in telomeric regions of some chromosomes. Banding technique and differential staining of chromosomes to study the karyotypic differences between the species are of immense importance in the future strategy of cytological studies in tea. The predominant occurrence of trivalent in the triploid UPASI-3 indicates either autotriploid or segmental allotriploid nature of

polyploid. Based on the meiotic analysis of diploid tea clones, it might be considered to be segmental allotriploid in origin. More studies are, however, needed to establish some conclusion regarding the origin of three different types of tea varieties.

The cytology of cultivated tea is very poorly understood. The clones manifested a chromosomal homology in chromosomal pairing during meiosis, between the genomes of tea clones, which are known to be "highly heterozygous". It appears that the genomes of the involved taxa are not sufficiently differentiated but exhibit cryptic hybridity, which is sufficient to differentiate clones in their morphology as well as their agronomic traits. The occurrence of trivalent in the only triploid cultivated clone UPASI-3 (2n =3x =45) indicates that it might have originated as segmental allo-triploid.

Most of the teas under cultivation are diploid with two sets of basic chromosomes, though natural triploids and pentaploids have also been reported (Bezbaruah, 1971, 1976; Sharma and Venkataramani, 1971). Bezbaruah (1971) observed that the quality of the natural triploid and tetraploids are inferior to the diploids, because of the hardiness of the triploids and tetraploids. Natural polyploids have more diverse genetic variation in terms of desirable traits which may be incorporated by hybridizing them with better quality diploids (Bezbabaruah, 1976). Tea polyploids (triploids and tetraploids) have clearly demonstrated the superiority of over domesticated diploids in morphological and agronomical characteristics.

They showed increased plant vigour and winter hardiness compare to diploids. The triploids generally do not set fruits though they bear flowers, so the energy required for the development of fruit, is diverted towards the development of vegetative parts. Chromosome number is the basic criterion for differentiations of a species. Most of the chromosomal investigation carried out on tea (Sharma and Raina, 2006) till now of different species and varieties (Somatic and meiotic) which showed a remarkably constant number 2n = 30 in all cases except for a few natural triploids (Bezbaruah, 1971). Karyotype study revealed that the clones belonging to the three main races of tea (diploid) did not show any major differences in their gross morphology and karyotype, although some minor differences existed (Bezbaruah, 1971). The knowledge of the genomic constitution and genetic markers are pre-requisites for development of the elite improved tea varieties. Almost self-sterile and out-breeding nature of the tea plant, coupled with frequent and spontaneous

hybridization of different species and genera, resulted in highly heterozygous plants ranging from very small leaf China type to very large leaf Assam type. The hybridization is so extreme that sometimes molecular markers could not resolve correct identification of cultivated clones.

The tea varieties are essentially out breeders and a free exchange of gene pools accounts for an endless series of continuous morphological variability in many taxa. In spite of what little data accumulated on cytology of different members of tea, certain aspects of the cytology of some of the important cultivated varieties are yet to be investigated. In recent years it has been repeatedly discussed that the role of gene mutation in the evolution of species was over emphasized in the past. Consequently, due emphasis was not laid on structural alternation of chromosomes in the origin of species. Refined techniques, worked out lately by several workers have been adapted to trace karyotype differences between species. The importance of karyotype study in establishing phyletic relationship and evolutionary trends is well recognized. With the aid of improved chromosome techniques it has been possible to work out the chromosomal basis of intervarietal and even inter-strain differences (Datta, 1974). The chromosomes derived their prominence as a tool in taxonomy from their direct relation to the genetic system of which they are an integral part. The comparative organization of chromosomes may indicate the extent of relationship and a variety of ways in which the related individuals have evolved, besides commenting upon the adequacy of correct taxonomic placements.

In view of the scanty work on tea species and the importance of karyotype analysis in relation to phylogenetic relatedness, the present investigation was undertaken which includes detailed chromosome analysis in commercially important ten tea clonal varieties. Further, for a proper understanding of cytological situation in any plant material under *in vitro* conditions, it is imperative to consider the *in vivo* cytological constitution of the taxon used for induction and growth of the callus. It is also expected that the cytological situation in an explants tissue would be reflected in that of callus derived from it. For a proper assessment of *in vitro* cytological observations it seems to be justified to undertake a detailed cytological investigation on the experimental materials under *in vivo* conditions. The significance of *in vitro* cytology of explants source for a meaningful interpretation of its corresponding *in vivo* cytology. The varieties included in the present scheme of work are TV23, TV25,

T78, T383, TV30, HV39, TeenAli17, TV29, TV26, and UPASI-26 all of which are used to produce commercial tea in the tea industries.

Transgenic Tea through Genetic Engineering

Plant protection is a major challenge to agriculture world wild. One of the effective strategies for disease resistance in plants has been the incorporation of disease resistant genes into commercially acceptable cultivars. The most attractive initial candidates for manipulation of the single gene defense mechanism approach. The genes encoding for chitinase and β1,3- glucanase can be used because these two enzymes hydrolyze chitin and β-1,3-glucans, which are structural components of the cell walls of several fungi. Major emphasis has been given to the plant hydrolases such as β1,3-glucanase (PR-2) and chitinase (PR-3), as they are capable of clearing fungal cell walls resulting in pathogen growth inhibition (Neuhaus,1999) and moreover, the products of the hydrolysis can act as elicitors of further defense responses (Boller *et al.*, 1998). Both the β 1,3-glucanase and chitinase are highly abundant proteins in plant involved in diverse physiological and developmental processes .They can act either alone or in combination strengthening their antifungal activity. Their accumulation is not restricted only to resistant plants but is often observed in compatible plant-pathogen interactions or even non-pathogenic combination. Constitutive expression of individual PRs in transgenic plants can lead to reduced pathogen growth and symptom expression, consistent with a role of PRs in the expression of acquired resistance (Ryals *et al.*, 1994).

There is strong evidence that experience of β-1,3 glucanase transgene alone or in combination with chitinase transgene regulated by the strong CaMV 35S RNA promoter can reduce the susceptibility of plants to infection by certain fungi. Transgenic tobacco plants expressing a soybean β-1,3-glucanse can elicitor releasing β-1,3-glucanase or the tobacco class II β-1,3 glucanse PR -2b, that showed reduced symptoms when injected with *Alternaria alternata* or the oomycetes *Phytophthora parasitica* var. *nicotianae* and *Perenospora tabacina*. Wubben *et al.* (1996) reported on the tissue-specific expression of genes encoding the hydrolytic enzymes, β-1,3-glucanse and chitinase, as studied by means of *in situ* hybridization. No preferential accumulation of gene transcripts in tissue near penetrating hyphae was observed in compatible or incompatible interaction. Activation of natural weapons before

infection, called systemic acquired resistance (SAR) is initiated by pathogens, or pathogen-derived elicitors, as well as a number of chemical compounds. Among the main defense genes, which are switched on in response to pathogen infection, belong to those encoding PRs.

The association of PRs with SAR, but of with ISR, has led to the hypothesis that accumulation of PRs is not a pre-requisite for the induction of resistance, but that PRs contribute to the protective state (Van Loon and Van Strien ,1999). SAR is dependent on the accumulation of SA, but not JA or ethylene. It appears that only when increases in the levels of any of these signals occur, PRs become detectable in the infected plants. Spraying plants with solution of salicylic acid (SA) mainly induces the acidic PR-1 and acidic β-1,3-glucanase genes (Bol *et al.*, 1990). Salicylic acid is a naturally occurring compound in tobacco plants and the levels of endogenous salicylic acid rise after TMV infection, not only in the infected leaves but also in the uninfected parts of the plant, which show inducted resistance. Resistance can be local and restricted to the treated tissue or be induced systemically. The induction of resistance usually coincides with the accumulation of pathogenesis related proteins and their transcripts in resistant tissue (Kuc, 1982).

Despite major advances in the field of biotechnological approaches in case of tea improvement, the molecular markers, *in vitro* tissue culture as well as chromosomal studies and chitinase gene accumulation in tea remain elusive. In the years ahead, new genomic and proteomic technologies will assist in the identification and characterization of tea germplasm and transgenic gene expression for the creation of disease resistance improved tea. In view of the many limitations to horizontal improvement, transgenic technology is the most popular method for vertical crop improvement (Galun and Breiman, 1997). An ever moving goal, that the scientists of each of the tea regions try to achieve these goal through conventional/non-conventional breeding of tea plants that are not only high yielding with good cup characters but are also more tolerant to biological and abiological stresses (Bhattacharya *et al.*, 2004).

The conventional breeding programme can not fully achieve the target goal due to some inherent problems in tea (Mondal *et al.*, 2004). The tea characteristics like tolerance to stresses, yield and quality are multigenic characters and can not be dealt in a time effective manner with the conventional breeding technique. With the

consideration above all, transgenic tea development can be a more effective technology for pyramiding desirable genes into tea for better adaptation, quality and yield (Bhattacharya and Ahuja, 2001; Bhattacharya *et al.*, 2006). Production of transgenic plants requires cells or tissues that are not only competent for gene transfer and integration but also ones that would regenerate successfully into *in vitro* plants (Cahteau *et al.*, 2001; Gelvin, 2000).

Generally, preference has been given for the development of somatic embryogenesis or a proliferating callus from which adventitious shoot buds or somatic embryos can be regenerated in recurrent cycles. Preterminal meristemic tissues like the apical or the axillary buds are generally not preferred especially in the *Agrobacterium*-mediated gene transfer system in order to avoid the formation of chimeras or mosaic plants (Riva *et al.*, 1998). Since somatic embryogenesis is one of the most worked out regeneration system in tea (Jain and Newton, 1990) was employed in the gene transfer system through *Agrobacterium* harboring a binary vector pBIN19, containing the plasmid p35SGUSINT and developed first transgenic tea (Mondal *et al.*, 1999). Later Mondal *et al.* (2001a) optimized certain parameters to express the GUS gene (β-glucoronidase) in transformed somatic embryo and regeneration of plantlets by somatic germination. Micro-shoots of the transgenic tea plants was excised and further multiplied *in vitro* and later micro-grafted on the seedling derived root stocks of the same cultivar. Some other parameters for efficient transformation of tea explants have been investigated by many workers (Mondal *et al.*, 2004).

Agrobacterium tumefaciens mediated gene transfer technique has been used by several workers using different explants for genetic transformation in tea (Matsumoto and Fukui, 1998, 1999; Biao *et al.*, 1998; Luo and Liang, 2000). The putative transformed leaf callus with stable gene integration had been confirmed after molecular characterization through PCR and southern hybridization by these workers (Matsumoto and Fukui, 1989, 1999; Biao *et al.*, 1998). It is common to occur multiple insertion of transgene into the genome, rearrangement of the genes and/or deletions of the integrated transgene in the regenerated plants (Svitashev *et al.*, 2000). Biolistic mediated genetic transformation had also been initiated to transform tea callus by many workers (Akula and Akula, 1999; Prakash *et al.*, 1999).

The natural infection of tea plants with *Agrobacterium* has been reported *in* woody plants and attempted has taken in tea by several workers (Biao *et al.*, 1998; Matsumoto and Fukui, 1998, 1999; Luo and Liang 2000; Mondal *et al.*, 1999, 2001a, b) using different explants systems such as *in vitro* grown leaf, and somatic embryos. In view of all this, transgenic appear to be the most time effective technology for stacking desirable genes into tea for better adaptation, quality and yield (Bhattacharya and Ahuja, 2001) irrespective of the limitations of a specific region of a particular product quality.

Although the first successful report on the regeneration of tea plantlets from the cotyledon derived calli was published by Wu and his team (Wu *et al.*, 1981). Kato (1986) and Yan and Ping (1983) have reported the induction of somatic embryos when mature cotyledon sections were cultured on MS medium supplemented with BAP and IBA. Then the higher rates of somatic embryogenesis and secondary somatic embryogenesis have been reported by several workers using immature cotyledons (Nakamura 1988; Akula and Dodd, 1998; Mondal *et al.*, 1999; Palni *et al.*, 2006). The artificial seeds have been produced successfully from the somatic embryos in cultivated tea by Mondal *et al.* (2000) and Akula *et al.* (2000). The inter-specific hybrid embryos were rescued from immature somatic embryos before abortion (Nadamitsu *et al.*, 1986). The plantlets are obtained either through the somatic embryogenesis route or by induction of adventitious shoots followed by rooting. Akula and Dodd (1998) used mixture of sand, peat and vermiculite (1:2:1) in nursery pots that were kept under moist conditions in a green house for successful acclimatization.

Caffeine is synthesized from the nucleoside xanthosine in four sequential reactions. The first step in the pathway is the methylation of the nucleoside xanthosine by S-adenosylmethionine, which is catalyzed by the enzyme xanthosine-7-methyltransferase (XMT). The product, 7-methlyxanthosine is hydrolyzed to 7-methylxanthine, and undergoes further methylations to theobromine and caffeine. Although there are some *Camellia* species (Tea) that produce low level of caffeine, but these are not readily available for commercial use. To achieve this some researchers have utilized the antisense RNA technology for the production of decaffeinated tea (Kato *et al.*, 2000; Stiles *et al., 2000*), for the tea drinkers those who prefer caffeine less tea. Because high caffeine consumption has some negative health

impacts, which include palpitation, gastrointestinal disturbances, increased blood pressure, insomnia, anxiety and tremor.

Many attempts have already been made to analysis the crop plant germplasm on the basis of chitinase gene but not in tea plant. Plant chitinases belong to a family of pathogenesis-related (PR) proteins, which are over-expressed by plants in response to a pathogen attack (Giazinazzi, 1987; Boller, 1983; Collinge *et al.*, 1993; Legrand *et al.*, 1987). Some researchers are trying to address the question whether manipulation of defense signaling pathways, either through genetic engineering or through application of defense signal-mimicking plant protectants, will boost the plant's immunity to potential invaders or will be a burden in crop protection strategies. Researchers have tried to isolate different chitinase gene from different plant species to use it in crop improvement program in order to increase the plant's own immune system (Sekeli *et al.*, 2003; Eilenberg *et al.*,2006; Metraux and John,1989; Roby *et al.*,1991; Samac *et al.*,1990; Datta *et al.*, 1999; Broglie *et al.*, 1991; Sharma and Kumar., 2005; Schlumbaum *et al.*, 1986; Huynh *et al.*, 1992; Wu *et al.*, 1994; Passrinho and De Vries 2002).

Pathogenesis related Proteins and respective genes for Plant's Own Defense system

Since their discovery in tobacco leaves hypersensitively reacting to TMV by two independently working groups (Van Loon and Van Kammen, 1970; Gianinazzi, 1982; Van Loon, 1985, 1997), pathogenesis-related proteins (initially named "b" proteins) have focused an increasing research interest in view of their possible involvement in plant resistance to pathogens. This assumption flowed from initial findings that these proteins are commonly induced in resistant plants, expressing a hypersensitive necrotic response (HR) to pathogens of viral, fungal and bacterial origin. Later, however, it turned out that b-proteins are induced not only in resistant, but also in susceptible plant-pathogen interactions, as well as in plants, subjected to abiotic stress factors (Van Loon *et al.*, 1994).

The term "pathogenesis-related proteins" (PRs) was coined by Antoniw *et al.*, (1980), which have been defined as "proteins encoded by the host plant but induced only in pathological or related situations", the latter implying situations of non-pathogenic origin. To be included among the PRs, a protein has to be newly expressed

upon infection but not necessarily in all pathological conditions. Pathological situations refer to all types of infected states, not just to resistant, hypersensitive responses in which PRs are most common; they also include parasitic attack by nematodes, insects and herbivores. Induction only by abiotic stress conditions is not a sufficient criterion for inclusion as PRs. These considerations imply that the characteristics of the induction of PRs take priority over other identifying features, such as chemical properties or cellular localization (Van Loon *et al.*, 1994; Van Loon, 1999). Originally, five main groups of PRs (PR-1 to PR-5) were characterized by both molecular and molecular-genetic techniques in tobacco, numbered in order of decreasing electrophoretic mobility.

Each group consists of several members with similar properties (Bol *et al.*, 1990). Odjakova and Hadjiivanova (2001) have reviewed in details the defense system in plants. The present status of the Pr-protein and its role in plant defense system has been reviewed by Edreva (2005). PR-1 is the most abundant, reaching up to 1-2 % of total leaf proteins. PRs of group 5 share significant amino acid sequence homology with the sweet tasting protein in the fruits of the tropical plant *Thaumatococcus daniellii*, and have been named thaumatin-like (TL) proteins (Cornelissen *et al.*, 1986). By then seventeen families (PR-1 to PR-17, table 8) were recognized and classified for tobacco and tomato, with the families PR-8 and PR-10 being also present in cucumber and parsley, respectively (Christensen *et al.*, 2002).

PRs have dual cellular localization-vacuolar and apoplastic, the apoplast being the main site of their accumulation (Van Loon, 1999). Apart from being present in the primary and secondary cell walls of infected plants, PRs are also found in cell wall appositions (papillae) deposited at the inner side of cell wall in response to fungal attack (Jeun, 2000). Interestingly, they are detected in the cell walls of invading fungal pathogens and in the space formed between cell walls and invaginated plasma membrane of fungi (Jeun, 2000; Jeun and Buchenauer, 2001). Acidic and basic PRs are identified, each of these counterparts having both apoplastic and vacuolar localisation (Buchel and Linthorst, 1999). Earlier data show that acidic tobacco PR-1 is localized in the apoplast, whereas basic tobacco PR-1 accumulates in the vacuole (Bol *et al.*, 1990). This may be valid for one PR family (PR-1) in a host plant, such as tobacco, but cannot be generalized as a differential localization feature of acidic and basic proteins in plants.

Table 8. Recognized families of pathogenesis-related proteins

Families	Type member	Properties
PR-1	Tobacco PR-1a	antifungal
PR-2	Tobacco PR-2	β-1,3-glucanase
PR-3	Tobacco P, Q	chitinase type I,II, IV,V,VI,VII
PR-4	Tobacco 'R'	chitinase type I,II
PR-5	Tobacco S	thaumatin-like
PR-6	Tomato Inhibitor I	proteinase-inhibitor
PR-7	Tomato P_{69}	endoproteinase
PR-8	Cucumber chitinase	chitinase type III
PR-9	Tobacco 'lignin-forming peroxidase'	peroxidase
PR-10	Parsley 'PR1'	'ribonuclease-like'
PR-11	Tobacco 'class V' chitinase	chitinase, type I
PR-12	Radish Rs-AFP3	defensin
PR-13	Arabidopsis THI2.1	thionin
PR-14	Barley LTP4	lipid-transfer protein
PR-15	Barley OxOa (germin)	oxalate oxidase
PR-16	Barley OxOLP	'oxalate oxidase-like'
PR-17	Tobacco PRp27	unknown

In the leaves PRs are present in mesophyll and epidermal tissues. They are also localized in the abscission zone of leaves and inflorescence, abscission zone at the stem-petiole junction, and vascular tissue of stems and petioles. PRs in plants are coded by a small multigene family. Since their discovery, regulation of PRs has been a highly active research area. Putative plasma membrane localized receptors of PRs inducers are suggested, and secondary signals of PRs induction, such as salicylic acid (SA), jasmonic acid and ethylene, are established. Many of these secondary signals are well-known inducers of PRs expression (Durner *et al.*, 1997; Poupard *et al.*, 2003).

Cross-talks are common between signaling pathways mediated by these secondary messengers. Thus, SA-independent/jasmonate dependent, and *vice-versa* pathways of PRs induction have been demonstrated. It has been proven that PRs synthesis is regulated at transcriptional level; the exact mechanisms of transcriptional regulation have been ones of the most active fields of PR gene studies. Several *cis-*

regulatory elements in PR-promoters mediating PR gene expression have been identified. These include Wbox, GCC box, G box, MRE-like sequence, SA-responsive element (SARE) (Zhou, 1999). New mutants are developed providing clues into the better understanding of the regulation of PRs (Delaney, 2000). PRs are synthesized following a long lag period (no less than 8 h) the synthesis proceeds *in situ*, *i.e.* PRs are not translocated from the site of their induction to other plant parts, as proven by elegant grafting experiments (Gianinazzi *et al.*, 1982). By using PRs patterns the origin of *N. tabacum* from the wild progenitors *N. sylvestris* and *N. tomentosiformis* was confirmed (Ahl *et al.*, 1982).

The amino acid sequence GHYTQVVW is a particularly well-conserved region in the two groups of proteins, suggestive of the functional role of this domain (Van Loon and Van Strien, 1999). Peter Tiffin (2004) has studied the comparative evolutionary histories of chitinase genes in the genus *Zea* and family Poaceae. Plants are exposed to very different attackers, including microbial pathogens and herbivorous insects by Van Loon (2001). To protect themselves, plants have evolved defensive strategies to counteract potential invaders. Recent advances in plant defense signaling research have revealed that plants are capable of differentially activating inducible, broad-spectrum defense mechanisms, depending on the type of invader encountered. The plant hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are major players in the network of defense signaling pathways. Cross-talk among SA, JA and ET dependent signaling pathways is thought to be involved in fine-tuning the defense reaction, eventually leading to the activation of an optimal mix of defense responses to resist the intruder. Genetic engineering of the biosynthetic pathways of these signaling compounds and the development of protective chemicals mimicking their mode of action provide useful tools for the development of new strategies for crop protection. However, there is evidence for antagonism between SA-dependent resistance to microbial pathogens and JA-dependent resistance to herbivorous insects: once a plant is conditioned to express resistance against microbial pathogens it may become more susceptible to attack by herbivores, and *vice versa*. Yet, the evidence for tradeoffs between pathogen and insect resistance is contradictory.

It has been focused on recent experimental evidence on the relationship between SA, JA and ET dependent induced resistance to microbial pathogens and

herbivorous insects. In addition, some researchers are trying to address the question whether manipulation of defense signaling pathways, either through genetic engineering or through application of defense signal-mimicking plant protectants, will boost the plant's immunity to potential invaders or will be a burden in crop protection strategies. Sekeli *et al.* (2003) have isolated and cloned chitinase-I gene from winged bean seed and characterized its structure in relation to defense system. Chitinase catalyses the hydrolysis of β-1,4-N-acetyl-D-glucosamine linkages of the fungal cell wall polymer chitin and is involved in the inducible defenses of plants. Chitinase gene fragments were isolated from a winged bean seed cDNA library using two sets of degenerate primers corresponding to the conserved regions of chitinase class I and IV proteins.

The poly-A mRNA was reversed transcribed and further amplified using RT-PCR to measure its expression. They isolated and cloned 1.1 Kb fragment of the chitinase gene. A nucleotide sequence comparison identified the fragment as a Class I basic chitinase cDNA; this fragment was subsequently used as a probe to screen for a full-length transcript from the cDNA library. Library screening resulted in the isolation of a 1324 bp clone, which is designated as CHRZP; encoding a polypeptide of 289 amino acids. Isolation and characterization of chitinase genes from pitchers of the carnivorous plant *Nepenthes khasiana* has been conducted by Eilenberg *et al.* (2006). Metraux and John (1989) have studied in details the chitinase gene isolation and cloning. Roby *et al.* (1991) studied the regulation of chitinase *in vitro* in protoplast culture inducing the defense system with ethylene and other elicitors. Samac *et al.* (1990) have critically demonstrated the isolation and cloning of chitinase gene in model plant *Arabidopsis thaliana*. Datta *et al.* (1999) have shown that transgenic rice plant over expressing PR-5 gene can be more resistance against sheath blight disease pathogen *Rhizoctonia solani*.

Chitinases are enzymes that hydrolyse ß-1,4-*N*-acetyl-D-glucosamine (GlcNAc) linkages. Those with lysozyme activity also cleave ß-1,4 linkages between GlcNAc and *N*-acetylmuramic acid. They are present in a broad range of organisms including bacteria, fungi, plants, and animals. According to their primary structure, chitinases are divided into seven classes (classes I–VII). Plant chitinases and lysozymes are likely to have arisen from one coancestor by divergent evolution (Monzingo *et al.*, 1996). The protein genealogy of chitinases shows that class I and

class II chitinase genes evolved from the same ancestral gene (Shinshi *et al.*, 1990; Araki and Torikata, 1995). Moreover, a basic class II chitinase is a putative ancestor of basic class I and acidic class II chitinase genes (Ohme-Takagi *et al.*, 1998). It has also been proposed that chitinases in class IV, which are phylogenetically related to class I and II chitinases (Araki and Torikata, 1995; Hamel *et al.*, 1997; Gomez *et al.*, 2002), evolved from a class I chitinase gene by four deletions in the coding sequence (Araki and Torikata, 1995).

Among many other functions, chitinases can stimulate embryo development (van Hengel *et al.*, 1998) and seed development (Van Damme *et al.*, 1999). Besides chitin, the primary substrate for chitinase, which is not present in plants, chitinases can also hydrolyse arabinogalactan proteins (AGPs) (van Hengel *et al.*, 2001), rhizobial Nod factors (Staehelin *et al.*, 1994) and other lipo-chito-oligosaccharides (LCOs) (Dyachok *et al.*, 2002). Since the enriched pattern of proteins found in cell suspensions includes seed-specific proteins, it has been assumed that some somatic cells show endosperm properties (Kragh *et al.*, 1993). It has been hypothesized that endosperm and embryo interact during their development (Berger, 1999). The evidence supporting endosperm–embryo interaction is derived from the carrot system. The EP3 chitinase that is expressed in the endosperm of carrot, rescue somatic embryos of carrot *ts11* variant (van Hengel *et al.*, 1998).

Wounding of plant tissues has been shown to induce complex molecular responses many of which are considered plant defense response (Aneja and Gianfagna, 2001; Leon *et at.*, 2001; Cheong *et al.*, 2002). Wounding can induce formation of jasmonates and ethylene, each of which are capable of independently inducing signal transduction pathways leading to resistance against plant pests. Jasmonic acid and related compound methyl jasmonate are part of the octadecanoid pathway in plants (Strassner *et al.*, 2002; Wasternack and Hause, 2002) and in some incidences contribute to resistance to insects (McConn *et al.*, 1997; Baldwin, 1998) and pathogen (Park *et al.*, 2001). Jasmonates have been shown to function as regulators of many different plant defense genes (McConn *et al.*, 1997; Park *et al.*, 2001). Ethylene is known to induce specific subsets of pathogenesis-related proteins in many plant species (Kitajima and Sato, 1999; Thomma *et al.*, 1999). The effects of ethylene have been associated with resistance to many different plant pathogens

(Abeles *et al.*, 1992; Thomma *et al.*, 1999; Park *et al.*, 2001) and insects (Abeles *et al.*, 1992; O'Donnell *et al.*, 1996; Argandona *et al.*, 2001; Bailey *et al.*, 2005).

Plant chitinases belong to a family of pathogenesis-related (PR) proteins, which are over-expressed by plants in response to a pathogen attack (Giazinazzi, 1987; Legrand *et al*, 1987; Boller, 1989; Collinge *et al.*, 1993). Chitinases catalyze the hydrolysis of the β-1,4 linked N-acetylglucosamine polymers that form chitin chains, a major component of fungal cell walls. Chitinases have been divided based on their structure into at least four classes (classes i to iv; Hamel, *et al.*, 1997). Even though chitinases have been shown to inhibit the hyphal tip growth of many fungi *in vitro.* A plant's natural defense mechanisms are often insufficient to prevent an invasion by the pathogen (Neuhaus *et al.*, 1999). The consequences of plant disease caused by fungal pathogens can be significant losses in crop quality and yields. Plants over-expressing chitinases under the control of a strong constitutive promoter have been engineered and have shown improved resistance against fungal pathogens under laboratory conditions (Broglie *et al.*, 1991).

Further, plants constitutively over-expressing a hybrid endochitinase exhibited improved tolerance to fungal diseases in field tests (Grison *et al.*, 1996). The expression of chitinases in plants is therefore useful to enhance resistance in plants against fungal pathogens. Chitinases expressed in plants are also reported to have anti-insect activity. Additionally, chitinases are useful in industrial processes aimed at the bioconversion of shellfish chitin waste. Although expression of chitinases can be useful to reduce infection by fungal pathogens and other pests, constitutive overexpression of foreign proteins in crop plants has a potentially yield-reducing metabolic cost. Moreover, it is commonly found that particular chitinases only have antifungal activity against a narrow range of fungal pathogens. Roby *et al.* (1991) have been studied the regulation of a chitinase gene promoter by ethylene and elicitors in bean protoplasts to improve the crop protection mechanism. Recent studies suggest that the production of enzymes capable of degrading the cell walls of invading phytopathogenic fungi may be an important component of the defense response of plants. The production of transgenic plants with enhanced resistance to attack by the fungal pathogen *Rhizoctonia solani* has been developed (Broglie *et al.*, 1993). Payne *et al.*, (1990) isolated the cDNA of chitinase gene from tobacco.

Jeyaramraja *et al.* (2005) have studied the role played by certain factors, physical barriers and chitinase enzyme, associated with blister blight resistance in tea. Sharma and Kumar (2005) studied the differential display-mediated identification of three drought-responsive expressed sequence tags in tea [*Camellia sinensis* (L.) O. Kuntze] and have shown the PR-5 like protein (pathogenesis related protein) induction in drought conditions. Kirubakaran and Sakthivel, (2007) had cloned and over expressed the barley chitinase gene in *E. coli*, which was believed to be involved in plant defense responses to pathogen infection. The molecular weight of chitinase is 35 kDa that was isolated and purified. Purified chitinase exerted broad-spectrum antifungal activity against *Botrytis cinerea* (blight of tobacco), *Pestalotia theae* (leaf spot of tea), *Bipolaris oryzae* (brown spot of rice), *Alternaria sp.* (grain discoloration of rice), *Curvularia lunata* (leaf spot of clover) and *Rhizoctonia solani* (sheath blight of rice). Due to the potential of broad-spectrum antifungal activity barley chitinase gene can be used to enhance fungal-resistance in crop plants such as rice, tobacco, tea and clover. Genetic transformation of pigeon pea with rice chitinase gene had been investigated by Kumar *et al.* (2004) for enhanced resistance activity against pathogenic attack.

Plants are endowed with various defense related genes and they express a variety of defense products in response to pathogen attack as a consequence of transcriptional activation. Accumulation of defense gene transcripts generally commences within minutes to hours around the infection sites, and several hours or days later at distant sites over the whole plant. During the local and systemic responses of plants, a large group of defense enzymes, PR-proteins and signal molecules are synthesized in high amounts to display a broad spectrum of antimicrobial activity. Though intensive studies, numerous genes involved in these processes have been identified (Hammnd-Kosack and Jones, 1996). Defense response genes are induced in both incompatible and compatible plant-pathogen interactions. However, mRNA accumulation for many plant defense genes is more rapid during interactions involving a plant expressing resistance to a particular pathogen (Davis *et al.*, 1993; Christensen *et al.*, 2002; Davis *et al.*, 2002). Polyphenol oxidases (PPO) are nuclear coded enzymes of almost ubiquitous distribution in plants (Mayer and Harel, 1979; Mayer, 1987).

However, there are very few reports of transcripts accumulation studies with regard to the role of defense enzyme like glucanase, chitinase, phenylalanine

ammonia lyase (PAL), peroxidase, chalcone synthase, PPO (Demeke and Morris, 2002) and LOX involved in blister blight infection. Therefore other examples of defense enzymes and PR proteins transcripts accumulation during induction of resistance with various other inducers and also during host-pathogen interactions is briefly presented here. The research progress on the molecular characterization of the defense enzyme PPO is also briefly outlined.

Pritsh *et al.* (2000) studied the pattern of transcripts accumulation of six-typical defense response genes, POX (peroxidase), PR-1, PR-2 (β-1, 3-glucanase), PR-3 (Chitinase), PR-4 & PR-5 (thaumatin like protein) in spray-inoculated panicles of both the susceptible cv. Wheatson and the resistant cv. Sumai3 of wheat. POX transcripts accumulation has been studied by Collinge and Boller (2001) in potato upon infection with *Phytophthora infestans*. They isolated two genes, a putative peroxidase. Fossdal *et al.* (2001) isolated cDNA encoding the putative defense related and basic plant peroxidase SPI2 (Spruce pathogen induced-2) with an estimated molecular mass of 34 kDa from roots of Norway spruce (*Picea abies*) seedlings. McFadden *et al.*, (2001) reported the expression of PRs- cadinene synthase, acidic chitinase, basic chitinase, β-1, 3-glucanase, phenylalanine ammonia lyase (PAL) and PR10 transcripts in cotton infected by *Verticillum dahlia*. Transcripts of defense response genes PR-1, PR-2 (β-glucanase), PR-3 (Chitinase), PR-4 & 5 (thaumatin like) and PR-9 (Peroxidase) transcripts accumulated in wheat spikes upon infection with *Fusarium graminareum* and treated with BTH (Yu and Muehlbauer, 2001). Christensen *et al.* (2002) reported the isolation of two cDNA clones from barley after inoculation with *Blumeria graminis f.sp.hordei*. The encoded protein of these cDNA clones belong to a new family of plant pathogenesis proteins designated as PR-17. Mohammadi and Kazemi (2002) showed the involvement of PPO in resistance against *Fusarium graminareum* during induced resistance. PPO catalyzes the oxygen dependent oxidation of phenols to quinines. Because of their conspicuous reaction products and their wound and pathogen inducibilities PPOs have frequently been suggested to participate in plant defense against pests and pathogens (Constabel *et al.*, 1995; Thipyapong *et al.*, 1995; Thipyapong and Steffins, 1997). However, the role of the enzymes in plant defense against pathogens has not been studied much.

There are only a few reports of showing the involvement of PPO during defense against plant pathogens. Overexpression of polyphenol oxidase in transgenic

tomato plants resulted in enhanced resistance to *Pseudomonas syringae* (Li and Steffens, 2002). PPOs appear to be nearly ubiquitous among plants (Sherman *et al.*, 1995), but the many possible roles they play have not been totally resolved. PPO activity is often thought of as a defense mechanism based on the appearance of PPO reaction products upon wounding or pathogenesis and the inducible nature of PPO upon wounding (Mayer and Harel, 1979; Constable *et al.*, 1995) showed that antisense down-regulation of constitutive and inducible PPO activity results in a hyper susceptibility to pathogens in tomato (*Lycopersicon esculentum*). Overexpression of PPO in tomato results in enhanced resistance to bacterial disease (Li and Steffens, 2002). These results strengthen the assertion of a defense role for the enzyme. Although there is speculation as to the mode of action involved in such defensive roles, the actual mechanisms have not been elucidated.

Various studies have reported the cloning and characterization of the multigene families encoding PPOs from different plant species, including faba bean (Cary *et al.*, 1992), potato (Hunt *et al.*, 1993), tomato (Newmann *et al.*, 1993) and Virginian pokewood (Joy *et al.*, 1995). In 1994, Dry and Robinson described the molecular cloning and characterization of grape berry PPO. Southern analysis suggested the presence of only one gene in the grapevines. Moreover, high level of gene expression was found in young, developing berries, whereas expression in mature tissues was low. More recently Boss *et al.* (1995) isolated a full length cDNA clone encoding apple PPO and described it as a multigene family. These studies have revealed a high degree of sequence conservation among the investigated PPOs. Leaf PPO sequences can be roughly divided into three domains, with a central domain containing the copper-binding sites (Van Gelder *et al.*, 1997).

Reverse-transcriptase-polymerase chain reaction (RT-PCR) has become an important tool to examine levels of mRNA transcripts because of its high sensitivity. The powerful amplification potential of PCR has assured its use in the detection of low-abundance mRNA in cells and tissues. This technique is particularly valuable in cases where the amount of an RNA species present is so minute that traditional RNA analysis methodologies such as Northern blot or RNase protection assay may not be adequate. It has been estimated that RT-PCR is thousands of times more sensitive than Northern blot analysis. The technique consists of two parts: synthesis of cDNA from RNA by reverse transcription, and amplification of a specific cDNA by

polymerase chain reaction (PCR). The method requires very little RNA and differs from Northern blotting because it is somewhat tolerant of degraded RNA, as long as the RNA is intact within the region of interest.

Because PCR targets only DNA sequences, mRNA is first converted by reverse transcriptase (RT) to single-stranded complementary DNA (cDNA), which can be amplified by PCR reaction. Avian myeloblastosis virus (AMV) or Moloney murine leukemia virus (MMLV) reverse transcriptases are commonly used for cDNA synthesis. For reverse transcription to occur, mRNA must be primed at the 3' end. This can be easily achieved by using primers of random sequences that will hybridize to their respective complementary RNA sequences along the RNA molecule. Thus, this random-primed method can be used for any RNA species, including ribosomal and messenger RNA. For mRNA, most of which ends in 3' polyadenlyation, specific 3' priming can be effected by annealing with oligo(dT). If the 3' sequence of a target RNA is known, a 3' primer can be used to specifically prime the RNA, resulting in a cleaner PCR product profile. Priming with any of these three methods, followed by extension with reverse transcription will result in a cDNA template for the 5' sense primer in the PCR reaction, allowing the PCR to proceed.

As long as an RNA molecule can be reverse-transcribed to render a target sequence for an ensuing PCR reaction, even partially degraded RNA can be amplified and be detected. The quantitative measurement of specific mRNA species is of major importance for studies on gene expression. Northern blotting is a relatively insensitive method requiring microgram amounts of RNA. It is time consuming and semi-quantitative at best. Because of the limitations of Northern blotting, various strategies have been developed for quantitation of cDNA by polymerase chain reaction (PCR)-based methods (Schweitzer and Horikoshi, 1992), *i.e.* RT-PCR technique.

So, there is no report on chitinase gene isolation and cloning from tea plants and no initiative has been taken for the improvement of Indian tea crop by manipulating the defense protein like chitinase. The present investigation is aimed to partially clone the chitinase gene of tea [*Camellia sinensis*] for its characterization at the molecular genetic level using bioinformatics algorithm.

MATERIAL AND METHODS

3.1. Plant material

Tea, *Camellia senensis* (L.), belongs to the family Theaceae. The cultivated taxa comprise of three main natural hybrids, which are: *Camellia sinensis* (L.) O. Kuntze (China type), *Camellia sinensis* var. *assamica* (Masters) (Assam type) and *Camellia sinensis* sub sp. *lasiocalyx* (Planchon MS.) (Cambod or Southern type). Tea plant is an evergreen, perennial tree (Plate 1, fig. A) but for commercial use it is pruned in regular intervals (Plate 1, fig. C). Leaf is the main criterion by which three types of tea are classified, briefly, Assam type has biggest leaves, China type has smallest leaves and Cambod type leaves size are in between of Assam and China type.

3.1.1. Collection

Twenty-one tea genotypes from three different varieties (*Camellia sinensis* var. *sinensis*; *Camellia sinensis* var. *assamica* and *Camellia sinensis* sub species *lasiocalyx*) of tea were used for the present study (Table 9). These genotypes were collected from the different tea germplasm centre of India (Darjeeling Tea Research Centre (DTRC), Kurseong, West Bengal; The United Planter's Association of Southern India (UPASI), Valparai, Tamil Nadu and Tocklai Experimental Station (TES), Jorhat, Assam and are presently being maintained under similar cultural practices at Tea Germplasm Bank, Department of Botany, North Bengal University, West Bengal, India (Plate 1, fig. B) by propagating through single stem node cuttings to maintain genetic identity according to Banerjee (1993).

3.1.2. Clonal propagation

Tea plants are mostly vegetatively propagated through single-node cutting. Fresh clonal cuttings of the tea varieties were propagated in sleeves containing sandy soil (3:1 of sand and soil) with a pH ranging from 4.5- 4.8 and were maintained in a green Agro-net house. In the early stage, limit of light tolerance was at 25% and after rooting progressed 50% light tolerance was maintained according to Banerjee (1993).

3.1.3. Maintenance in glasshouse

Clonal propagated tea plants (T383) of 6 months old were transferred to 6 cm and 12 cm diameter plastic pots as well as 24 cm earthen pots containing soil supplemented with 20% green manure and were maintained in the glasshouse for

experimental purposes in optimum day light (11 h), relative humidity (55-75%) and temperature (30-35°C), at which the photosynthetic rate was maximum, were provided to these plants according to Banerjee (1993).

Table 9. Tea genotypes from three different tea varieties.

Varieties	Tea genotypes
i)*Camellia sinensis* var. *sinensis*	**China type**
Sl-1	T383
Sl-2	T78
Sl-3	T135
Sl-4	HV39
Sl-5	TeenAli17
Sl-6	AV2
Sl-7	BS/7A/76
ii)*Camellia sinensis* var. *assamica*	**Assam type**
Sl-8	UPASI-3
Sl-9	UPASI-9
Sl-10	UPASI-26
Sl-11	TV20
Sl-12	TV21
Sl-13	TV27
Sl-14	TV28
iii)*Camellia sinensis* sub spp. *lasiocalyx*	**Cambod type**
Sl-15	TV18
Sl-16	TV19
Sl-17	TV22
Sl-18	TV23
Sl-19	TV25
Sl-20	TV26
Sl-21	TV30

Sl: Serial no.

3.2. Genomic fingerprinting by RAPD and ISSR markers

For characterization of genome (fingerprinting): A variety of molecular marker techniques based on hybridization or PCR are available for characterization of genome (fingerprinting). In the present investigation for the fingerprinting of twenty one tea clones PCR-based marker development method *i.e.* Randomly Amplified Polymorphic DNAs (RAPD) and Inter Simple Sequence Repeats (ISSR) were used.

Plate 1 [Fig. A-C]: (A) Tea plant grown without pruning; (B) Tea germplasm maintained by pruning and (C) Commercial tea plantation in plain lands of Northern Region of West Bengal, India .

3.2.1. Preparation of genomic DNA extraction buffer

The following buffers for DNA extraction were prepared by mixing appropriate amount of desired chemicals with distilled water and adjusted the desired pH using 1N HCl or 1N NaOH solution in all the cases.

CTAB buffer
2% CTAB (Cetyltrimethylammonium bromide)
1.5% PVP K-30 (polyvinylpyrrolidone)
1.4M NaCl
20mM EDTA
100mM Tris-HCl (pH 8.0)
0.1% β-mercaptoethanol (add before use)

10x Tris EDTA (TE buffer)
100mM Tris-HCl (pH 8.0)
10mM EDTA (pH 8.0)

10x TAE electrophoresis buffer

Tris base	48.5g
Glacial acetic acid	11.42ml
0.5M EDTA (pH 8.0)	20ml
H_2O to make fmal vol.	1000ml

6x Gel loading buffer (DNA)
0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF
40% (w/v) sucrose in H_2O

Sodium acetate (3M, pH 5.2)
40.83g Sodium acetate 100ml
Adjust pH with glacial acetic acid

Ethidium bromide (10mg/ml)
1g EtBr 100ml H_2O

1% Agarose gel solution
250 mg agarose dissolved in 25ml 1x TAE buffer

Proteinase K stock (20 mg/ml) 10ml

Proteinase K	200mg
50mM Tris (pH 8.0)	
1.5mM Calcium acetate	
Make final volume	10ml
Use 100µg/ml in reaction	

Proteinase K reaction buffer
0.01M Tris-Cl (pH 7.8)
0.005 M EDTA
0.5% SDS
50µg/ml Proteinase K

RNase A stock solution (10mg/ml)

RNase A	100mg
0.01 M Sodium acetate	
(pH 5.2) added to make	
Final volume	10ml
Heated to 100°C	15 min
Stored at	-20°C

RNase A treatment buffer
10mM Tris-Cl (pH 8.0)
0.1 M EDTA (pH 8.0)
0.5% (w/v) SDS
Added RNase A stock solution
20µg/ml

3.2.2. Genomic DNA isolation

Tender unfolded leaf samples (from twenty one tea genotypes) were collected from the clonally propagated plants and stored immediately at -80 °C for DNA extraction. The total genomic DNA was extracted from the stored leaf samples using

the modified CTAB method of Murray and Thompson (1980). Fresh unfolded tender leaf tissue (1g) was ground in a precooled mortar and pestle using liquid nitrogen. The pulverized material thus obtained, was transferred to 15ml polypropylene tube containing 4ml of preheated CTAB extraction buffer (65°C) with 20 µl Proteinase K stock solution. Then it was incubated at 65 °C in a water bath for 30 minutes with occasional gentle shaking after adding 1 ml of 10% w/v SDS. Equal volume of phenol: chloroform: isoamylalcohol (25:24:1) mixture was added, and the mixture was shaken vigorously. The mixture was centrifuged at 10,000 rpm for 10 minutes and supernatant was transferred to a new centrifuge tube. Again added equal volume of a mixture of chloroform: isoamylalcohol (24:1) and centrifuged at 10,000 rpm for 8 minutes and the supernatant was transferred to a new tube. DNA was precipitated by adding double volume of chilled (-20°C) absolute ethanol followed by addition of $1/10^{th}$ volume of 3M Sodium acetate (pH 5.5) and kept at -20°C for overnight for total DNA precipitation.

3.2.3. Genomic DNA purification

DNA purification was done according to the method of Sambrook and Russell (2001). The precipitated DNA was spooled out and washed 2-3 times in 70% ethanol and dissolved in 500µl RNase A treatment buffer containing 20µg/ml RNase A solution for purification in a 1.5 ml eppendorf tube. It was incubated at 37 °C for 1h to remove the RNA contamination. Then extracted with equal volume of chloroform: isoamylalcohol (24:1) and centrifuged at 10,000 rpm for 8 minutes and the supernatant was transferred to a new tube. DNA was precipitated by adding double volume of chilled (-20°C) absolute ethanol followed by addition of $1/10^{th}$ volume of 3M Sodium acetate (pH 5.5) and kept at -20°C for overnight for total DNA precipitation. The purified DNA pellet was collected by centrifuging at 5000 rpm for 5 minutes in a microfuge. The excess ethanol was air dried and purified DNA was finally dissolved in 250µl TE buffer (pH 8.0) for further use in PCR amplification.

3.2.4. Measure DNA concentration using Spectrophotometer

For quantitating the amount of DNA, readings were taken at wavelengths of 260 nm and 280 nm in a UV-*vis* Spectrophotometer (Shimadzu-160). The reading at 260 nm allows calculation of the concentration of DNA in the sample. An OD of 1 corresponds to ~ 50 µg/ml for double stranded DNA. The ratio between the readings

at 260 nm and 280 nm (OD_{260}: OD_{280}) provides an estimate of the purity of the DNA. A pure preparation of DNA has OD_{260}: OD_{280} value of 1.8. Concentration of the purified genomic DNA in each case was adjusted to 10 ng/µl in a different aliquots and stored at -20°C for further use.

3.2.5. Agarose gel electrophoresis to check DNA quality

1% (w/v) agarose gel was cast by melting 250 mg agarose in the presence of 25 ml 1xTAE buffer in a microoven until a clear, transparent solution was achieved. The melted solution was then poured into a mold after adding ethidium bromide solution to a final concentration 0.5 µg/ml, set appropriate comb and allowed to harden. Upon hardening the gel, it was placed in the Mini-submarine gel electrophoresis tank (Bio-Rad, Sub-Cell GT) and added the 1x TAE buffer sufficient to cover the gel to a depth of ~1 mm (according to Sambrok and Russell, 2001).

3.2.6. Preparation of DNA samples for electrophoresis

Taken 20 µl purified DNA from each samples and mixed with 4 µl of 6x DNA gel loading buffer. The DNA sample mixed gently and slowly loaded 25 µl sample into the slots of the submerged gel using a micropipette. Low range DNA ruler (Bangalore Genei) also loaded into the slot on either of the sides as a DNA size marker.

3.2.7. Run gel electrophoresis for DNA fractionation

The electrical lead of the gel tank was attached firmly and applied electric supply at constant current 90 mA and voltage 75 Volt (Bio-Rad Power Pac-300) at least for 90 minutes. The DNA was migrated from cathode to anode (positive anode). Run was continued until the bromophenol blue and xylene cyanol FF have migrated an appropriate distance through the gel. Then turn off the electric current and removed the gel from the tank and examined the gel on UV-transilluminator and photographed for analysis.

3.2.8. Preparation of buffers for genomic fingerprinting

The following buffers for PCR amplification were prepared by mixing appropriate amount of desired chemicals with distilled water and adjusted the desired pH using 1N HCl or 1N NaOH solution in all the cases.

10x PCR Amplification buffer
500mM KCl
100mM Tris-HCl (pH 8.0)
15mM $MgCl_2$
0.1% Gelatin

Taq storage and dilution buffer
20mM Tris-HCl (pH 8.0)
0.1 mM EDTA
1mM dithiothreitol (DTT)
100mM KCl
0.5% Tween-20
0.5% Igepal
50% Glycerol (v/v)

PCR reaction mixture (Final volume 25µl)

10x Amplification buffer	2.5µl
10mM dNTPs (pH 8.0)	2.0µl
20µM Random primer	5.0µl
Taq polymerase 1U	1.0µl
Template DNA (30-35ng)	10.0µl
Sterile H_2O	4.5µl

3.2.9. PCR amplification for RAPD fingerprinting

Twelve RAPD primers (L5, OPA-1, OPA-4, OPA-9, OPA-11, OPA-12, OPA-13, OPA-16, OPA-18, OPA-19, OPB-10 and OPB-20) (Table 10) were used for PCR amplification of the genomic DNA of twenty one tea clones according to Williams *et al.*(1990). RAPD reactions were performed in a 25µl reaction mixture containing 10 µl template DNA, 2.5 µl PCR buffer, 2 µl dNTPs mixture, 5 µl of single 10-mer random primer, 1µl of 1 Unit Taq DNA polymerase (all chemicals and primers are procured from Bangalore Genei, Bangalore, India) and final volume was adjusted by adding sterile distilled water. A negative control with no DNA was included in each PCR run. The PCR amplification reactions were performed in a Gene Amp PCR System 2400 (Perkin-Elmer) programmed for an initial denaturation step of 94 °C for 4 minutes followed by 35 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C. After the last cycle, a final step of 7 min at 72 °C was added to allow complete extension of all amplified fragments and reaction was ended at 4 °C forever.

Table 10. RAPD primers used for the detection of polymorphism in *Camellia sinensis.*

Sl. Nos.	Primer code	Sequences (5' to 3')
1.	L5	acgcaggcac
2.	OPA-1	caggcccttc
3.	OPA-4	aatcgggctg
4.	OPA-9	gggtaacgcc
5.	OPA-11	caatcgccgt
6.	OPA-12	tcggcgatag
7.	OPA-13	cagcacccac
8.	OPA-16	agccagcgaa
9.	OPA-18	aggtgaccgt
10.	OPA-19	caaacgtcgg
11.	OPB-10	ctgctgggac
12.	OPB-20	ggacccttac

3.2.10. PCR amplification for ISSR fingerprinting

Seven ISSR primers (ISSR13, ISSR14, ISSR17, ISSR18, ISSR814.1, UBC830 and UBC825) were used in this profiling. The PCR amplification of the total genomic DNA from 21 tea genotypes (Table 9) was done according to the method of Tsumura *et al.*(1996). Amplification was carried out in 25 µl of reaction mixture same as RAPD amplification containing 10 µl template DNA, 2.5 µl PCR buffer, 2 µl of dNTPs mixture, 5 µl of single ISSR primer, 1µl of 1 Unit Taq DNA polymerase. PCR reactions were performed using an Perkin-Elmer's Gene Amplification Thermal Cycler programmed for an initial denaturation step at 94 °C for 4 minutes followed by 35 cycles of 1 min at 94 °C for denaturation, 1 min at 50 °C for primer annealing and 2 min at 72 °C for primer extension. A final step of 7 min at 72 °C was carried out for polishing the ends of PCR products and reaction was ended at 4 °C forever.

3.2.11. Fractionation of PCR products in 1% agarose gel

Both the PCR amplified products (RAPD and ISSR) were size fractionated in 1% agarose gel and electrophoresed in 1X TAE buffer (0.04 M Tris-acetate, 1 mM EDTA, pH=8.0) at 75 V, 90 mA current for 90 minutes (Power Pac-300, Bio-Rad) in a Sub-Cell-GT electrophoresis system (Bio-Rad). Ethidium bromide (0.5µg/ml final

concentration) was added into the agarose gel during its solidification to stain the DNA. The banding patterns were visualized with a UV-transilluminator (UV~312nm) and documented by a Canon digital camera. A low range DNA ruler was used as a molecular size marker. The amplification was repeated twice and only reproducible clear bands were scored for genetic analysis.

3.3. PCR amplification for chitinase gene

Chitinase gene specific DNA fragment of 201 bp was PCR amplified using chitinase gene specific primer pair. Genomic DNA sample was taken from tea clone T383.

3.3.1. Preparation of gene amplification buffer solution

Gene specific PCR amplification mixture buffer was prepared as given below and the desired pH was adjusted using 1N HCl or 1N NaOH solution in all the cases.

Gene specific PCR reaction mixture (Final volume 25µl)

10x Amplification buffer	2.5µl
10mM dNTPs (pH 8.0)	2.0µl
20µM forward primer	2.5µl
20µM reverse primer	2.5µl
Taq polymerase 1U	1.0µl
Template DNA (30-35ng)	10µl
Sterile H_2O	4.5µl

3.3.2. DNA isolation

The tender unfolded leaves of T383 cultivar was used to extract and isolate the genomic DNA according to the CTAB method of Murray and Thompson (1980) as already described in section 3.2.2.

3.3.3. DNA purification

The same methodology was used for DNA purification which has already been described in section 3.2.3.

3.3.4. Measure DNA concentration using Spectrophotometer

The protocol as stated in section 3.2.4. was followed to quantify the genomic DNA concentration.

3.3.5. Chitinase gene specific PCR amplification

The chitinase gene specific 20-mer primer pair was used to amplify the genomic DNA. Total volume of 25 µl PCR reaction mixture contains the following chemical ingredient: 30-35ng genomic DNA of tea clone T383, 2.5 µl of 10x PCR buffer, 20 µM of each primer pair (forward and reverse primer), 2 µl of dNTPs and 1U Taq DNA polymerase and final volume 25 µl was adjusted with sterile water. PCR conditions for gene specific amplification was as follows- 35 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C, at last a final extension at 72°C for 7 min was given for preparing same length of PCR products.

3.3.6. Fractionation of chitinase gene product

Chitinase gene specific PCR products were fractionated on 1% agarose gel electrophoresis and visualized on UV-transilluminator and photographed, the procedure was already described in section 3.2.11.

3.3.7. Purification of Chitinase gene fragment

The PCR amplified DNA fragment of 201bp was purified and cloned into TA cloning (pGEM-T *Easy*) vector (Promega, Madison, WI) before sequencing. PCR amplified product was purified using phenol extraction procedure. Pool the PCR products from the replicates about 150 µl and added equal volume of Tris-HCl saturated phenol and mixed gently. Centrifuge the mixture at 8000 rpm for 5 min and collected the upper aqueous phase containing DNA in a fresh eppendorf tube (1.5ml). The equal volume of chloroform: isoamylalcohol (24:1, v/v) was added and mixed thoroughly. After centrifugation at 10,000 rpm for 5min, the supernatant was taken in a fresh tube and added $1/10^{th}$ volume of 3M sodium acetate (pH 5.2). DNA was precipitated by adding double volume of chilled (-20° C) absolute ethanol and incubated for 2 h at -20°C for total precipitation of the DNA fragments. DNA samples were centrifuged at 10,000 rpm for 7 min to remove supernatant and washed the DNA pellet with 70% ethanol twice. Carefully removed ethanol and allowed the DNA pellet to air dry in the hood to remove remaining ethanol. DNA pellet was resuspended in 30µl TE buffer, pH 8.0 (10 mM Tris-HCl, pH8.0, 1 mM EDTA, pH 8.0) and measured the concentration by Spectrophotometry. Made different aliquots in 0.2 ml tube at a concentration of 10 ng/µl and stored at 4°C for cloning in pGEM-T *Easy* vector.

3.4. T/A Cloning of chitinase gene fragment

T/A cloning vector pGEM-T *Easy* of Promega was utilized in the present PCR cloning system because it is most suitable for PCR product cloning due to presence of a single overhanging 3′ deoxythymidine residue (T) (Plate 7, fig. A & C). This 3′-T overhanging easily makes complementary base pair with the 3′-A overhang of the PCR product. Since *Taq* DNA polymerase has a non-template dependent terminal transferase activity, which adds a single 3′ deoxyadenosine (A-residue) to the ends of PCR products, which allows for increased ligation efficiency with the vector. It is always prefer to use new PCR product during cloning otherwise the terminal 3′ deoxyadenosine residue has a tendency to fall off over time (from 3-4 days old PCR product). The cloning efficiency ranging between 60-80 clones per plate. The optimal ratio of PCR/vector is 3:1. The pGEM-T *Easy* vector is 3015bp in size.

3.4.1. Preparation of buffer for T/A cloning

The following buffers for T/A cloning and transformation were prepared by mixing appropriate amount of desired chemicals with distilled water and adjusted the desired pH using 1N HCl or 1N NaOH solution in all the cases.

10x Vector ligation buffer

0.66M Tris-HCL (pH 7.6)
50mM $MgCl_2$
50mM DTT
10mM ATP

Vector ligation mixture (Total 12µl)

10x ligation buffer	1.2µl
pGEM-T Easy vector	1.0µl
T4 DNA ligase	1.0µl
PCR product (50ng DNA)	8.0µl
Sterile H_2O	0.8µl

LB medium (Luria-Bertani medium)

Tryptone	10g
Yeast ectract	5.0g
NaCl	10g
Make final volume with H_2O	1000ml

Sterilized by autoclaving at 15 psi 20 min
For agar plate add 15g/l Bacto agar

SOC medium composition

Tryptone	10g
Yeast ectract	5.0g
NaCl	10g
KCl (250mM)	10ml
$MgCl_2$ (2M)	5.0ml
Glucose (1M)	20ml

H_2O to make final vol. 1000ml

IPTG (20% w/v, 0.8M) solution

IPTG	2g

H_2O to make final volume 10 ml
Filter through 0.22µm disposable filter

X-gal solution (2% w/v)

X-gal	2g
H_2O	final volume 100 ml

3.4.2. Ligation into pGEM-T *Easy* vector

Chitinase gene fragment of 201 bp was ligated into the linear pGEM-T *Easy* vector before transformation into the *E. coli* host cell strain JM109 for cloning the recombinant vector carrying the foreign gene fragment, according to the manufacturer's protocol (Promega). For the recombinant vector preparation, two tube of 0.2 ml was prepared, first tube containing 8 µl purified chitinase gene specific PCR product (50-80 ng DNA of 201bp) and second tube was prepared without PCR product but water 8µl (a control that will indicate the background of vector self-ligation). Added to each tube 1 µl pGEM-T *Easy* vector (50 ng), 1.2 µl 10X ligation buffer, 1 µl T4 DNA ligase (0.25unit/µg of DNA for cohesive end ligation), and 0.8 µl H_2O to make total volume 12 µl. The ligation reaction mixture was incubated overnight at 16°C to ligate the chitinase gene fragment with the linear pGEM-T *Easy* vector to make them circularized. Then the ligated products were used for standard transformation assay. Transformation of chemically competent *E. coli* host cell strain JM109 was done with the PCR fragment ligated pGEM-T *Easy* vector.

3.4.3. Transformation of *E. coli* host JM109

The following media were prepared for the transformation purposes- LB medium, LB agar medium and LB agar plus 50 µg/ml ampicillin plates. The day of transformation, puts LB (Luria-Bertani) agar (plus 50 µg/ml ampicillin) plates in 37°C to dry for an hour. Then added 40 µl of 20 mg/ml chromogenic substrate, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) + 8 µl of 200 mg/ml IPTG (isopropyl β-D-thiogalactoside) with a sterile spreader under the laminar hood to absorb the reagent into medium for blue-white colour screening. Put back at 37°C till needed. Thaw 50 µl host cells and added 2 µl of ligation mixture on ice while cells are thawing, pipette very gently to mix, and incubated on ice 30 minutes then proceed to one shot chemical transformation. Host cells were given heat shock in 42°C water bath for 1 minute. Immediately transferred the tube to ice for 2 minutes. Added 250 µl SOC medium, capped the tube tightly and shaked horizontally (200 rpm) at 37°C for 1 h prior to plating. Spreaded 50-100 µl of the transformed cells (SOC medium) on prewarmed selective LB agar plate (LB agar containing ampicillin + Xgal + IPTG) and incubated the plate at 37°C overnight (12-19 h) in inverted position. Removed the plates from the incubator and stored it at 4°C for 6 h to develop blue colour to its full

extent. Then the colonies carrying recombinant plasmids were identified through blue-white colour screening.

3.4.4. Screening of recombinant positive colonies

Positive colonies carrying recombinant plasmid were identified by α-complementation test (Sambrook and Russell, 2001). The *E. coli* host strain JM109 synthesizes ω-fragment of β-galactosidase and complement with the α-fragment of the β-galactosidase, which encoded by the empty (non-recombinant vector) plasmid vector. Colonies that carry intact plasmid (non-recombinant) produced active β-galactosidase and were blue in colour in the IPTG/X-gal selective medium. Colonies that carry recombinant pGEM-T *Easy* vector (with 201 bp chitinase gene fragment) could not produce active β-galactosidase and were white in colour in the selective medium. White recombinant colonies were picked with autoclaved toothpicks and inoculated into 1.5 ml of LB/ampicillin medium in an eppendorf tube for multiplication by keeping it for overnight at 37°C with 200 rpm agitation.

3.4.5. Isolation of plasmid DNA for sequencing

The plasmid vectors (recombinant pGEM-T *Easy* vector) containing PCR insert of 201 bp was isolated from the transformed cells according to Sambrook and Russell (2001). The bacterial culture in eppendorf tube was centrifuged at 5000 rpm for 5 min to collect the bacterial cells. Resuspend the bacterial pellet in 100 µl of ice-cold alkaline lysis solution I (50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA) and shaked vigorously. Added 200 µl alkali lysis solution II (0.2 N NaOH and 1% (w/v) SDS), then added 50 µl alkali lysis solution III (60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml H_2O to made final volume 100 ml). Closed the tube tightly and disperse the lysis solution through inverting the tube several times then kept on ice for 5 minutes. Bacterial lysate was centrifuged at 10,000 rpm for 5 minutes at 4° C in a microfuge. Transferred the supernatant in a fresh tube and added equal volume of phenol: chloroform (1:1, v/v). Centrifuged at 10,000 rpm for 5 minutes and taken the aqueous upper layer to a fresh tube. Precipitated the plasmid DNA by adding 2 volumes of chilled (-20° C) ethanol and kept at 4° C for 1 h. Then the precipitated plasmid DNA was collected by centrifugation at 5000 rpm at 4° C in a microfuge. DNA pellet was washed twice with 70% ethanol then recovered the DNA by centrifugation at 5000 rpm for 5 minutes and

air dried the plasmid DNA pellet by evaporating the ethanol (10-15 minutes) at room temperature. Dissolved the DNA pellet in 50 µl TE (pH 8.0) containing 20 µg/ml RNase A (DNase-free) and kept at 37° C for 1 h to made it RNA contamination free. The DNA solution was vortex gently for a few seconds and stores the DNA solution at -20° C for future use. The amplified product was analyzed on 1% agarose gel to check the quality according to the protocol already described in section 3.2.11.

3.4.6. DNA sequencing

The above purified recombinant plasmid DNA was used for sequencing purposes. DNA sequencing was done bi-directionally using universal primer by ABI-PRISMS Big-Dye terminator cycle sequencing method and separated on Applied-BioSystem (ABI-3700-96) DNA sequencer to get sequence information of the 201 bp chitinase gene fragment (By Bangalore Genei, Bangalore, India).

3.4.7. DNA sequence analysis

DNA sequence information of the above 201 bp was analyzed using the alignment software of BLASTS algorithm (http://ingene2.upm.edu.my/Blast, Altschul *et al.*, 1997) for the different characteristics of DNA sequences. The other software program such as GENSCAN was also run to analyze the DNA sequences for the identification of the putative chitinase gene sequence with respect to peptide information.

3.4.8. Submission to GenBank (Accession no. EF673751)

Chitinase gene specific sequence information of 201 base pair was deposited to NCBI GenBank through BankIt procedure and approved as a sequence of chitinase gene after complete annotation and given the accession no. EF673751.

3.5. Caffeine synthase gene amplification by PCR reaction

Caffeine synthase gene specific genomic DNA sequence was amplified using gene specific primer in polymerase chain reaction. DNA samples from tea clones HV39 and T383 were used in this gene specific PCR amplification.

3.5.1. Preparation of DNA isolation buffer

As described in section 3.2.1.

3.5.2. Genomic DNA isolation

As described in section 3.2.2.

3.5.3. Purification of genomic DNA

As described in section 3.2.3.

3.5.4. Preparation of PCR amplification buffer

As described in section 3.3.1.

3.5.5. Caffeine synthase gene specific PCR amplification

The forward (TCS1, Tea caffeine synthase 1) and reverse (TCS2) primer (oligonucleotide) pair of caffeine synthase gene was used for partial amplification of the caffeine synthase gene from genomic DNA of two tea clones (T383 and HV39). The sequence of the caffeine synthase gene specific primer was 5′ - tcttcaaaggcctgtcgtct-3′ for forward and 5′- tccccttgtttaatgccaag-3′, for reverse primer. The primer pair was so designed (from GenBank Accession no-AB031280 of caffeine synthase gene of 1438bp cDNA) as to give amplification product of the internal sequences of the caffeine synthase gene of approximately 200-202 bp. PCR reaction mixture was set in 0.2 ml PCR tube containing 2 U Taq DNA polymerase, 10 mM Tris-HCl (pH= 8.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 5μl each of forward (TCS1) and reverse (TCS2) primer at a final concentration of 30 pmol and 50 ng template genomic DNA in a final volume of 25μl. Each reaction mixture was overlaid with 50 μl mineral oil to prevent evaporation. Samples for enzymatic amplification were subjected to an initial denaturation programme of one cycle at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min. Finally the reaction mixture was allowed to complete an additional extension of 7 min at 72°C.

3.5.6. Fractionation of PCR product in 1% agarose gel

As described in section 3.2.11.

3.5.7. PCR product purification

As described in section 3.3.7.

3.5.8. DNA sequencing of caffeine synthase gene fragment

As described in section 3.4.7.

3.5.9. Analysis of caffeine synthase gene sequence

As described in section 3.4.8.

3.5.10. Submission to GenBank (Accession no. AY599069 and AY601112)

Caffeine synthase gene sequences of 202 bp (from clone HV39) and 200 bp (from clone T383) were submitted to the GenBank of NCBI. Two accession numbers were approved by the GenBank after the verification and annotation of the submitted DNA sequences. Accession no. AY599069 was given for 202 bp of tea clone HV39 and AY601112 for 200 bp of tea clone T383.

3.6. *In vitro* tissue culture

Plant tissues and organs are grown *in vitro* on artificial media, which supply the nutrients necessary for growth and development. The success of plant tissue culture as a means of plant propagation is greatly influenced by the nature of the culture medium and plant sources used.

The callus tissues from the stem explants and shoots regenerated from the somatic embryos were used in the differential gene expression in induced systemic resistance (ISR) following induction with an inducer, methyl jasmonate.

3.6.1. Plant material for *in vitro* tissue culture

Four tea clones, TV30, UPASI-26, T383 and HV39 were used for the present *in vitro* tissue culture for somatic embryogenesis and callusing. All the tea clones are being maintained in the Tea Germplasm Bank, Department of Botany, University of North Bengal. Somatic embryogenesis was initiated from the immature and mature cotyledons from tea seeds. Callus culture was initiated from the stem segment as explants. Green capsules were collected in September for immature cotyledon from immature seeds (9-11 mm diameter) and mature seeds from open mature capsules were collected in November for mature cotyledon. Mature seeds (13-18 mm diameter, Plate 8, fig. G & H) were extracted from the mature capsules and floated overnight in water to discern viable from nonviable seed. Seeds that floated were discarded whilst those that sank were used in the present study. Immature cotyledon was collected from the fruits of September. Mature and immature cotyledon was used for somatic embryogenesis and regeneration of plantlets. Plantlets were also regenerated from *in vitro* grown stem callus through morphogenesis.

3.6.2. Composition of tissue culture media

The principal components of most plant tissue culture media are inorganic nutrients (macronutrients and micronutrients), carbon source (s), organic supplements,

growth regulators and a gelling agent. For the induction of organogenesis and regeneration of plantlets from the cultured tissues, MS (Murashige and Skoog, 1962) and B5 medium (Gamborg *et al.*, 1968) was used in the present study. The B5 medium differs from MS medium in having much lower amounts of nitrate in the form of ammonium. The compositions of MS and B5 media are given in table 11 and 12 which are used in the present study.

Table 11. Composition of MS and B5 plant tissue culture media (mg/l).

Constituent	MS medium Murashige and Skoog, (1962)	B5 Gamborg *et al.* (1968)
Macronutrients (>0.5 mM /l)		
$MgSO_4\ 7H_2O$	370	250
$NaH_2PO_4.H_2O$	-	150
$CaCl_2.2H_2O$	440	150
KNO_3	1900	2500
NH_4NO_3	1650	-
KH_2PO_4	170	-
$Ca\ (NO_3)_2.\ 4H_2O$	-	-
$(NH_4)_2SO_4$	-	134
Micronutrients(< 0.5 mM/l)		
$FeSO_4.\ 7H_2O$	27.8	-
$MnSO_4.4\ H_2O$	22.3	-
$MnSO_4.H_2O$	-	10
KI	0.83	0.75
$CoCl_2.6H_2O$	0.025	0.025
$ZnSO_4.\ 7H_2O$	8.6	2
$CuSO_4.5H_2O$	0.025	0.025
H_3BO_3	6.2	3
$Na_2MoO_4.2H_2O$	0.25	0.25
EDTA disodium salt	37.3	-
EDTA-Na ferric salt	-	43
Organic Supplements		
Vitamins		
m-ionositol	100	100
Thiamine HCl	0.5	10
Pyridoxine HCl	0.5	1.0
Nicotinic acid	0.5	1.0
Others		
glycine	2	-
Cysteine	-	10
Sucrose	30,000	20,000
pH	5.8	5.5

Table 12. Concentration of different inorganic salts (mM) in MS and B5 media.

Salt	MS	B_5
Macronutrients (mM)		
Ca	3	1.0
Cl	6	2.2
K	20.1	25
NH_4	20.6	2
NO_3	39.4	25
PO_4	1.25	1.1
SO_4	1.8	-
Mg	1.5	1.0
Micronutrients (mM)		
B	100	50
C	0.11	1.0
Cu	0.1	0.1
Fe	1.0	1.0
I	5.0	4.5
Mn	92.5	60
Mo	1.0	1.0
Zn	30	7.0

3.6.3. Preparation of stock solution for MS medium (macro and microsalts)

Preparation of macro and micronutrient stock solutions: Four different stock solutions were prepared using double distilled water to prepare MS basal medium as shown in table 13.

a) Major salts stock solution I (20 X concentrated), b) Minor salts, stock solution II (200 X concentrated), c) Iron stock solution III (200 X concentrated) and d) Stock solution IV, organic nutrients except sucrose (200 X concentrated).

While dissolving the nutrients in double distilled water, one compound was added at a time to avoid precipitation. For macronutrient stock solution, calcium chloride was dissolved separately in water and then added to the rest of the solution to avoid precipitation. To prepare 1 l of medium, the following amount of stock solutions were taken, 50 ml of stock solution I, 5 ml of stock solution II, 5 ml of stock solution III and 5 ml of stock solution IV. The stock solution III was prepared freshly by dissolving $FeSO_4$ and $Na_2EDTA.\ 2H_2O$ separately in 450 ml distilled water by heating and constant stirring. Then mixed the two solutions, adjusted pH to 5.8 and added distilled water to make up the final volume to 1 l. The pH of the medium was adjusted to the required value (pH 5.8 for MS) by dropwise addition of 1N NaOH or 1N HCl with constant stirring.

3.6.4. Preparation of B5 medium

The B5 medium was prepared by making three different solution mixtures. Desired amounts of the B5 components were added into each of the three mixtures in a 500 ml flask containing 250 ml double distilled water. Mixture I, containing all the macronutrients, mixture II containing all the micronutrients and mixture III containing organic supplements including vitamins. Added all the three mixtures slowly in a 1l flask and then added 20g sucrose and 8.0 g Difco agar. The medium components were dissolved by heating them in a water bath. The medium was supplemented with the different plant growth regulators such as BAP, IAA, PBOA, NAA, 2,4-D, TDZ and Kin (1-5 mg/ml), or 10-15% CM either singly or in combinations, for induction of somatic embryos from mature and immature cotyledons and callusing from stem explants. After mixing well, the pH of the medium was adjusted to 5.5 using 1N NaOH and 1 N HCl. The final volume of the medium was made up to 1000 ml with double distilled water.

Table 13: Stock solutions for Murashige and Skoog's (1962) medium (MS).

Constituents	Amount (mg/l)
Stock solution I (20X)	
NH_4NO_3	33000
KNO_3	38000
$CaCl_2.2H_2O$	8800
$MgSO_4.7H_2O$	7400
KH_2PO_4	3400
Stock solution II (200X)	
KI	166
H_3BO_3	1240
$MnSO_4.4H_2O$	4460
$ZnSO_4.2H_2O$	1720
$Na_2MoO_4.7H_2O$	50
$CuSO_4.5H_2O$	5
$CoCl_2.6H_2O$	5
Stock solution III (200X)	
$FeSo_4.\ 7H_2O$	5560
$Na_2EDTA.\ 2H_2O$	7460
Stock solution IV (200X)	
myo-Inositol	20000
Nicotinic acid	100
Pyridoxine. HCl	100
Thiamine. HCl	20
Glycine	400

3.6.5. Preparation of stock solution for plant growth regulators

All the growth regulators are not soluble in water. Solubility of different growth regulators is given in Table 14. The compound was dissolved in few ml of solvent and then slowly added water to make up to the requisite volume.

Table 14. Preparation of stock solutions for plant growth regulators.

Compound	Abbreviation	Molecular weight(MW)	Solubility	mg/100ml (1 mM)
Auxins Indol-3 acetic acid	IAA	175.18	1N NaOH	17.51
Indole-3 butyric acid	IBA	203.23	1N NaOH	20.32
2,4-Dichlorophenoxy acetic acid	2,4-D	221.00	Alcohol	22.10
Naphthalene acetic acid	NAA	186.20	1N NaOH	18.62
Phenylboronic acid (Novel auxin)	PBOA	121.93	1N NaOH	12.19
Others: Gibberellic acid	GA_3	346.37	Alcohol	34.63
Brassin	Brassin	480.68	Alcohol	48.06
Cytokinins				
Benzyl adenine or 6-benzyl amino purine	BA or BAP	225.2	1N NaOH	22.52
Kinetin	KIN	215.2	1N NaOH	21.52
n-phenyl-N-1,2,3-thiadiazol-5-Urea (thidiazuron)	TDZ	220.2	1N NaOH	22.02

Table 15. Time duration for media sterilization at 15 psi

Volume (ml)	Sterilization time (min) at 121°C and 15 psi (pressure per square inch)
1 - 200	15
200 - 1000	30
1000 - 2000	40

3.6.6. MS medium preparation and sterilization

The sequences of steps involved in preparing MS medium were as follows: 30g sucrose and 8g agar powder were weighed and dissolved in 750 ml double distilled water in a 1 l flask, by heating them in a water bath. Appropriate quantities of the stock solutions were added (I, II, III and IV) sequentially. The medium was supplemented with the different plant growth regulators such as BAP, IAA, PBOA, NAA, 2,4-D and Kin (1-5 mg/ml), or 10-15% CM either singly or in combinations, for the induction of somatic embryos and embryo conversion to plantlets or effect on morphogenesis from callus. After mixing well, the pH of the medium was adjusted to 5.8 using 1N NaOH and 1 N HCl. The final volume of the medium was made up to 1000 ml with double distilled water. About 50 ml medium was dispensed into 250 ml Erlenmeyer flask and 15 ml in a 25 x 150 mm culture tube. Thermolabile plant growth regulators were filter sterilized through a 0.22 μm-0.45 μm disposable filter. Filter sterilized solutions were then combined with other nutrient substances sterilized in the autoclave to give a complete medium. Mouth of the culture vessels (flask) were closed with non-absorbent cotton wrapped in cheese-cloth (such closures exclude microbial contaminants but allow free gas exchange) and culture tube with plastic caps. The culture vessels containing medium were transferred to appropriate baskets, covered with aluminum foil to check wetting of plugs during autoclaving and sterilized by autoclaving at 121°C (15 psi) for 18 minutes (Table 15). The medium was allowed to cool at room temperature in 250 ml flasks but for slant preparation by keeping the tubes tilted during cooling in a slanted plastic rack. Such slants provide a larger surface area for tissue growth. The prepared medium was used 3-4 days after preparation, so that if it is not properly sterilized, contamination will start appearing and this medium can be discarded. Media were stored at 4°-10°C.

3.6.7. Preparation of undefined supplement (coconut milk)

The coconut milk (CM) was used to promote the growth of certain calli and organs. The liquid endosperm of *Cocos mucifera* fruits was used in the present investigation as an undefined organic supplement. Coconut milk from the fresh fruits was usually strained through cloth and deproteinized by being heated to 80-100°C for about 10 minutes while being stirred. It was then allowed to settle and the supernatant was separated from the coagulated proteins by filtration through paper. The liquid was stored frozen at-20°C and used as and when required.

3.6.8. Sterilization of explants

Mature and immature cotyledon of tea seeds from tea clone T383 was used for somatic embryogenesis and 5-7 mm long stem segment for callusing were used as explants in the present investigation from four tea clones, T383, TV30, HV39 and UPASI-26. Surfaces of the plant explants carry a wide range of microbial contaminants. To avoid this source of infection the plant explants were thoroughly surface sterilized before inoculation into the medium by different disinfectant (Table 16).

The following sequences of steps had been followed in the initiation of aseptic tissue culture from four tea clones. Mature and immature fruits were collected in a beaker and washed them in tap water with few drops of liquid detergent (Teepol) and then thoroughly washed in running tap water. After washing, fruits were treated with 3% hydrogen peroxide for 2 min and then with 70% alcohol for 2 min in a beaker, and then decanted the alcohol. Fruits were transferred to a screw cap bottle containing 20% solution of sodium hypochloride (1% active chlorine content) with a small amount of a suitable surfactant (2-3 drops of Triton-X100) and after capping the screw bottle was shaken gently for 5 minutes under Laminar Air flow. Then the cap of the bottle was removed and the liquid poured out and an adequate quantity of sterilized, distilled water was poured onto the seeds and the cap replaced. After shaking a few times, the water was discarded. Such washings with sterile distilled water were repeated 5 times. The seeds were removed from the fruits using sterile scalpel and forceps.

The seeds were then transferred to a pre-sterilized Petri-dish to remove the seed coat from the seeds and collected the cotyledon by removing the embryo and used as initiation explants. The intact mature de-embryonated cotyledonary endosperm cut into pieces (0.5-1.0 cm long) before placed into the somatic embryo induction medium. Green capsule collected in September were sterilized by the same procedure before the immature seeds (9-11 mm in diameter) were isolated and then each of immature de-embryonated cotyledon placed into the same medium for somatic embryogenesis. The cotyledon explants of various size and maturity are transferred onto the different medium in a culture tube of 25 x 150 mm in size and in 250 ml flask containing 50 ml medium, the neck of the culture vial flamed and the closure replaced in quick succession. For flame sterilization of the small instruments (Scalpels, forceps, razor) these were soaked in 95% ethyl alcohol followed by flaming

on a spirit lamp in the laminar airflow hood, which was repeatedly done while aseptic manipulation work was in progress. Young shoots of 10 cm long were also collected from the germplasm field of four tea clones for callus initiation and morphogenesis. Young shoots were washed in tap water with few drops of liquid detergent (Teepol). Young shoots were treated with 10% calcium hypochloride for 5 minutes. Then young shoot was cut into 5-7 mm long stem segment to be used as explants and treated with 70% ethanol in a screw cap bottle for 2 minutes. Stem explants was then transferred to sterile tissue paper in a petri plate and then inoculated into the MS and B5 medium with different concentration of plant growth hormones.

Table16: Disinfectants used for sterilizing explants material

Sterilizing agents	Concentration used (%)	Duration (minutes)
Calcium hypochloride	10	5
Sodium hypochloride	3% (of 20% v/v commercial bleach)	5
Hydrogen peroxide	3	3

3.6.9. Transfer of the explants

All kinds of transfer operations were carried out under strictly aseptic conditions in the laminar airflow hood, to prevent the entry of any contaminant into the culture vial when its months was opened either for sub-culture or for planting fresh tissues (inoculation). Laminar airflow hoods were sterilized by switching the UV light to expose the working area for 20 minutes and then wiping the working surface with 95% ethyl alcohol 15 minutes before initiating inoculation operation under the hood. The velocity of the air coming out of the fine HEPA filter was 27± 3 m per min (removes particles larger than 0.3 μm) which was adequate for preventing the contamination of the working area. Hands were washed with 95% ethyl-alcohol before starting the inoculation experiment as a measure of precaution.

3.6.10. Initiation of callus culture

Callus culture was initiated in both the basal media of MS and B5 taking the stem segment from four tea clones as explants, TV30, HV39, T383 and UPASI-26. After four weeks of culture the callus was transferred to the MS medium only because growth was optimum in MS medium. The MS medium was supplemented with

different concentration of plant growth hormones and coconut milk (CM) for the study of effect of various growth factors in callus growth, development and regeneration of plantlets. The cultures were incubated at 25± 2°C under 16 h photoperiod of 2000 lux light intensity (from Phillip's fluorescent lamps), at the level of the cultures.

3.6.11. Initiation of somatic embryogenesis

Both the cotyledonary tissues of immature and mature seeds (of tea clone T383) were placed onto the surface of semisolid, full strength Murashige and Skoog (1962) medium. The intact mature de-embryonated cotyledonary endosperm was cut into pieces (0.5-1.0 cm long) before placed into the somatic embryogenesis medium. Immature cotyledons from the immature seeds (9-11 mm in diameter) were isolated and then each of immature de-embryonated cotyledons placed into the medium. The medium was supplemented with the different plant growth regulators such as BAP, IAA, PBOA, NAA, 2,4-D and Kin (1 –5 mg/l), or 10% CM either singly or in combinations, for induction of somatic embryos. The medium was solidified with 0.8% agar after adjusting to pH 5.8 and sterilized at 121°C for 15-18 min. All the MS media contained Fe-EDTA and micronutrient formula described by Murashige and Skoog plus 0.5 mg/l thiamine-HCl, 0.5 mg/l pyridoxine-HCl, 0.05mg/l, nicotinic acid, 100 mg/l, m-inositol 30 g/l, sucrose 30g/l and 8g/l Difco-Bacto-Agar, The cultures were maintained for 14 weeks at 25± 2°C under a 12/12 (day/night) photoperiod with light provided by cool-fluorescent tubes at a photon flux density of 52-μmol $m^{-2}s^{-1}$ and 60-65% relative humidity.

3.6.12. Regeneration of plantlets from somatic embryos and calluses

For tea plant regeneration, somatic embryos (of tea clone T383) were removed from cotyledons and cultured in MS and Gamborg's B_5 (1968) basal medium impregnated with various growth regulators like BAP (5 mg/l), IAA (2 mg/l), Brassin (2 mg/l) and GA_3 (1.5 mg/l), either singly or in different combinations, or without growth regulators. Plantlets were regenerated from callus of tea clone T383 in MS medium containing different levels of cytokinin (TDZ, BAP) and auxin (IBA and NAA). Shoot formation was initiated in MS medium containing 1 mg/l IBA and 4 mg/l BAP. The shoot formation was also initiated from callus while cultured in MS medium with TDZ (cytokinin). MS medium with 1 mg/l NAA and 3 mg/l BAP was

shown shoot formation. The shoots were then transferred to ½ MS medium with 1 mg/l IBA in filter paper bridge in liquid medium for rooting. Kept as such for 4-6 weeks and then transferred to ½ MS medium for normal growth and development. All the experiments were repeated three times. Regenerated plants after 15-16 weeks old with well-developed roots and leaves were transferred into the hardening chamber for hardening the plantlets.

3.6.13. Hardening of *in vitro* regenerated plantlets

Selection and preparation of plantlets prior to their transfer to pots. 15-16 weeks old plantlets of *Camellia sinensis* were obtained through *in vitro* somatic embryo germination and morphogenesis from callus, were used as ready source of material for present study. Healthy plantlets having a height of 4-6 cm with 5-6 well developed leaves and 3-4 roots were deflasked and washed very carefully with double distilled water. Utmost care was taken during washing so that no agar or media should remain attached with the plantlets. After that they were treated with fungicide captan (4 mg/l) and kept on a sterile Petri dish.

3.6.14. Transfer of plantlets to potting mixture

Plastic bags measuring 2□ diameter X 2.5□ height were brought from the market. The bags were filled with a mixture of peat and soil at a ratio of 1:1. After filling up the plastic bags (pots) with potting material the plantlets were very carefully transferred and planted in the pots. The pots were then covered with inverted beakers. Distilled water was regularly sprayed in the beakers to keep high humidity around the plantlets. The pots were kept in the hardening chamber at culture room conditions *i.e.* at 25± 2°C under 16 h photoperiod for 3 weeks. After that the pots were transferred to greenhouse and the beakers were removed. In the greenhouse the pots with plants were kept for another 2 weeks under natural photoperiod and humidity. Finally after 2 weeks the plantlets were transferred to large earthen pots filled with garden soil. Watering was done at regular intervals. Results showed that the humidity played important role in the hardening of the regenerated plantlets. The highest survival rate 61% was found in clone T383 at the relative humidity of 95%.

3.7. Genomic constitution as revealed by chromosome

Cultivated tea (*Camellia sinensis*) has been maintained for centuries, by vegetative propagation. An immense heterogeneity is existed in the commercial tea

populations, because of the polymorphic origin of the latter. Cultivated tea generally is a mixture of species of tea *Camellia sinensis* (L) O. Kuntze (China type); *Camellia assamica* (Masters) (Assam type) and *Camellia assamica* sub-species *lasiocalyx* Planchon MS. (Cambod type), and other species of *Camellia*, including those fall outside the purview of *Thea* section. Most of the cultivated tea of the world are diploid 2n=30 and highly heterogeneous as a result of free natural hybridization between geographical races during cultivation. Moreover, continued development and release of cultivated varieties have added to the genomic diversity of commercial tea. There are no serious and systematic attempts have been made for gaining detailed information about the genomic constitution of cultivated tea.

3.7.1. Plant material

Karyotype analyses in ten cultivated tea clones [(*Camellia sinensis* (L.) O. Kuntze] were investigated for their characterization. Ten tea cultivars, T78, T383, TV30, HV39, TeenAli17/1/54, TV23, TV25, TV29, TV26, and UPASI-26, were used in the present chromosome study. Six months old tea cuttings were maintained in the earthen pot for the collection and availability of root tips. Root tips were collected by inverting the whole pot and removing the pot from soil clumps. And then fresh root tips of 5 mm size were collected in distilled water with a scissor from the surface of the soil clumps. Root tips were collected in a day light between 10.00 am to 12 noon.

3.7.2. Preparation of solution and cytological stain

2% (w/v) Aceto-Orcein stain

2 g Orcein powder was dissolved in 90 ml of 45% acetic acid in a 250 ml flask and heated the solution for 10 minutes at a simmering point. Then allowed to cool at room temperature and filtered through Whatman filter paper. Final volume 100 ml was made up by adding required amount of 45% acetic acid and stored at room temperature for cytological preparation.

Saturated solution of ρ-dichlorobenzene

The saturated solution of ρ-dichlorobenzene was prepared by adding at least 300g ρ-dichlorobenzene in 500 ml distilled water and allowed overnight for saturation and then stored at 4°C.

Fixative chemicals

Aceto: ethanol (1:3) mixture was prepared by adding 25 ml glacial acetic acid and 75 ml absolute ethanol and stored at 4°C for further use.

3.7.3. Somatic chromosome technique

The actively growing apical meristem part of the roots were collected between 10 am to 12 noon and properly washed in distilled water immediately after collection. About 5 mm long cut apical root meristems were pretreated in saturated solution of ρ-dichlorobenzene with a little amount of Aesculine for 3.3 h at room temperature and fixed in a fixative chemical, acetic: ethanol (1:3) for at least 24 h at room temperature. Following hydrolysis in 5N HCl for 1 h at room temperature, the root tips were transferred to 45% acetic acid for 10 min. The root tips were then stained in 2% aceto-orcein by heating the sample over spirit lamp for few seconds and allowed for 40 minutes to take the proper stain by the chromosomes. The temporary squash preparations were made in 45% acetic acid on grease free slide covered by square cover slips. The slides were sealed with wax to prevent air penetration into the chromosome preparation. The chromosome karyotypes from each of the clone were drawn using Camera-Lucida at the table magnification X 1500 and photomicrographs were taken in Lieze microscope at different magnifications and suitably enlarged and analyzed.

Each of the Karyotype was analyzed by the following index-

Centromeric index (F %), Total centromeric index (TF %), Disparity index (DI) and Total Haploid Chromosome Length (TCL) were calculated according to Huziwara (1962).

$$F\% = \frac{\text{Short arm length of the chromosome}}{\text{Total length of the chromosome}} \times 100$$

$$TF\% = \frac{\text{Total sum of short arm length}}{\text{Total sum of the chromosome length}} \times 100$$

$$D.I. = \frac{LCL - SCL}{LCL + SCL} \times 100$$

LCL = Longest chromosome length
SCL = Shortest chromosome length

3.7.4. *In vitro* cytology

Somatic chromosome constitution from the root tips of the regenerated plantlets (T383) was also studied to investigate the somaclonal variation at the chromosomal level. Same methods were applied as was *in vivo* cytological method. The apical meristems of root tips collected from one year old plantlets (T383) growing *in vitro* was used for the present cytological studies.

3.8. Abiotic induction of defense genes expression by methyl jasmonate (MeJa)

3.8.1. Preparation of test solution

A known plant defense inducer such as methyl jasmonate (0.2 mM) was prepared by dissolving 44.8 mg in 1000 ml distilled water. The solution was supplemented with a few drops of Tween-20 to ensure adhering to the plant leaves.

3.8.2. Foliar application

The 12 months old tea plants (clone T383) were grown in earthen pots and maintained in greenhouse in ambient light and temperature. Leaf development was separated into two stages- Stage I-young leaves, 2-4cm long (YL) and Stage II-mature leaves, 5-9cm long (ML). The above test solution, methyl jasmonate (MeJa) was sprayed as foliar application at a rate 40 ml/m^2 as to wet the leaves completely on both the ventral and dorsal surface using a hand sprayer.

Similarly tissues (tea clone T383) from different growth and developmental stages of *in vitro* grown calluses (2 months) and *in vitro* regenerated shoots of somatic embryos (6 months) were treated with test solution after filter sterilized through a 0.22 µm disposable filter. Controls were mock sprayed with distilled water plus Tween-20. Sprays were applied between 10.00 am to 11.00 am while stomata were remaining consistently open. The leaves, callus and shoots were harvested from different time courses (0, 0.3, 2, 6, 12, 16, 24, 36, 48, 72 h) and frozen in liquid nitrogen and stored at -80°C before RNA isolation. Experiments were repeated twice with 3 replicates.

3.9. Chitinase gene specific transcript accumulation

Differential gene expression (specifically chitinase gene, PR-3 protein gene) was studied during the induced systemic resistance (ISR) induced by methyl jasmonate. The above abiotically induced tea plants (T383) in different

developmental stages were used for mRNA transcript accumulation in different time course to observe the defense related gene expression levels.

3.9.1. Preparation of buffer for RNA isolation and gel electrophoresis

The following buffers for RNA isolation reactions were prepared by mixing appropriate amount of desired chemicals with distilled water and adjusted the desired pH using 1N HCl or 1N NaOH solution in all the cases.

10x MOPS electrophoresis buffer

0.2 M MOPS (pH 8.0)
20mM sodium acetate
10mM EDTA (pH 8.0)

RNA denaturing solution

10x MOPS electrophoresis buffer	2.0µl
Formaldehyde	4.0µl
Formamide	10.0µl
Ethidium bromide (200µg/ml)	1.0µl
RNA (up to 20µg)	2.0µl

10x Formaldehyde gel-loading buffer

50% glycerol (diluted in DEPC- water)
10mM EDTA (pH 8.0)
0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF

RNA extraction buffer (200ml)

2% CTAB(w/v)	4g
2% PVP K-30 (w/v)	4g
100mM Tris-HCl (pH 8.0)	2.42g
25mM EDTA	1.16g
2M NaCl	16.38g
Spermidine	0.1g
2% (v/v) β-mercaptoethanol	4ml

Lithium Chloride (LiCl, 7.5M)

LiCl 31.8g
Make final volume 100ml with H_2O

DNase I dilution buffer

10mM Tris-Cl (pH 7.5)
150mM NaCl
1mM $MgCl_2$

10x DNase I treatment buffer

100mM Tris-Cl (pH 8.4)
500mM KCl
15mM $MgCl_2$
0.01% gelatin

3.9.2. Total RNA isolation (transcripts)

Total RNA isolation was done according to Korban *et al.* (2004) from the above abiotically induced tea plant tissues. Briefly 1g leaf tissue was taken from each of the different tea samples (four treated and four controls). Samples were frozen and pulverized in liquid nitrogen, and added prewarmed (60°C) 5 ml RNA extraction buffer to make slurry using mortar and pestle. Vortex briefly and kept at 60°C for 15 minutes. Added equal volume of chloroform: isoamylalcohol (24:1) and immediately vortex for 2 minutes. Centrifuged at 10,000g for 10 minutes at 4°C and taken the upper aqueous supernatant into a fresh tube and repeated the extraction with an equal volume of chloroform: isoamylalcohol mixture. Again collected the upper supernatant carefully and transferred into a new tube. RNA precipitation was done by adding 1/3 volume of 7.5 M LiCl to each tube, mixed by inversion and kept at 4°C overnight for complete precipitation. Each of the RNA pellet was recovered by centrifugation at 12,000g at 4°C for 30 minutes and washed RNA pellet twice with 70% ethanol and air-dried at room temperature for 10 minutes. Each of the RNA samples was treated with DNase I, by the addition of 100µl DEPC water, 20µl 10x DNase I buffer and 4 µl DNase I to each tube. After a 30 minutes incubation at 37°C, the samples were extracted with an equal volume of 1:1 phenol:chloroform and centrifuged at 12,000g for 10 minutes at 4°C. Transferred the upper aqueous supernatant into a fresh tube and precipitated the RNA with 20µl 3M sodium acetate and 600µl absolute ethanol keeping at -80°C overnight. The samples were centrifuged 10 minutes at 12000g at 4°C. The RNA pellets were washed in 70% ethanol twice and air-dried at room temperature for 10 minutes. Dissolved the RNA in 80µl DEPC-treated distilled water and measured the quantity by UV-*vis* Spectrophotometer (Shimadzu, 160) at 260nm. Again RNA was precipitated by adding 2.5 volumes of 70% ethanol followed by addition of $1/10^{th}$ volume of 3M sodium acetate for 3 h at -80°C. RNA pellet was recovered by centrifugation at 12,000g for 30 minutes at 4°C and finally dissolved in 200µl DEPC-treated (diethylpyrocarbonate) distilled water and stored at -80°C.

3.9.3. Quantification of RNA by Spectrophotometry

The purified total RNAs were quantified with a UV-*vis* Spectrophotometer (Shimadzu, 160) at wavelength of 260nm and 280nm. The A_{260} value 1 corresponds to ~ 38µg/ml RNA. A pure preparation of RNA has $A_{260/280}$ value 2.0, which

indicates that the RNA is pure without any protein contamination. RNA concentration was adjusted at 20μg/μl with DEPC treated distilled H_2O. The integrity of total RNA was verified by running samples on 1.2% denaturing agarose gel.

3.10. Denaturing agarose gel electrophoresis for RNA

RAN was fractionated on 1.2% agarose gel containing 2.2 M formaldehyde (commercial formaldehyde is 12.3M) to check the quality and integrity.

3.10.1. Preparation of 1.2% denaturing agarose gels

To prepare 30 ml of a 1.2% agarose gel containing 2.2 M formaldehyde, added 360mg agarose to 21.7 ml of sterile distilled water. Dissolve the agarose by boiling in a microwave oven. Cool the solution to 55°C and added 3.0 ml 10x MOPS electrophoresis buffer and 5.3 ml of formaldehyde. The gel was set at room temperature for 1 h, and then transferred to the submarine gel electrophoresis tank for fractionation (according to Sambrook and Russell, 2001).

3.10.2. RNA sample preparation and electrophoresis

Before loading into the gel, RNA was denatured in a denaturing solution at 55°C for 60 minutes. Samples were chilled for 10 minutes in ice water then added 2μl of 10x formaldehyde gel-loading buffer to each sample. RNA samples (20 μg RNA) were loaded into the slots of the gel and turn on the power supply at 90 mA constant current and 75 Volt for electrophoresis in 1x MOPS buffer for 2 h. The RNA bands were visualized by placing the gel on a UV-transilluminator and photographed.

3.11. Reverse transcriptase- Polymerase Chain Reaction (RT-PCR)

The above RNA was taken from each of the treated and untreated (control) samples after induction with methyl jasmonate for the quantization of transcription accumulation (mRNA transcription levels). Defense system related gene expression was induced in the tea plant (clone T383) by the inducer, methyl jasmonate. In the present study, chitinase gene specific expression level (mRNA transcripts) was measured by RT-PCR (Innins *et al.*, 1990).

3.11.1 Preparation of RT-PCR buffers

The following buffers for RT-PCR reactions were prepared by mixing appropriate amount of desired chemicals with distilled water and adjusted the desired pH using 1N HCl or 1N NaOH solution in all the cases.

10x PCR Amplification buffer
500mM KCl
100mM Tris-HCl (pH 8.0)
15mM $MgCl_2$
0.1% Gelatin

Taq storage and dilution buffer
20mM Tris-HCl (pH 8.0)
0.1 mM EDTA
1mM dithiothreitol (DTT)
100mM KCl
0.5% Tween-20
0.5% Igepal
50% Glycerol (v/v)

One-step RT-PCR reaction mixture (25µl)

2x RT-PCR reaction mix.	12.5µl
10mM dNTPs (pH 8.0)	2.0µl
20µM Forward primer	2.5µl
20µM Reverse primer	2.5µl
Taq polymerase 2U	1.0µl
Denatured RNA (15µg)	2.0µl
Reverse transcriptase (100U)	1.0µl
RNasin	1.0µl
Sterile H_2O	0.5µl

10x Reverse transcriptase buffer
500 mM Tris-Cl (pH 8.0)
750 mM KCl
30 mM $MgCl_2$

Proteinase K (20 mg/ml) 10ml

Proteinase K 200mg
50mM Tris (pH 8.0)
1.5mM Calcium acetate
Make final volume 10ml
Use 50µg/ml in reaction

Proteinase K reaction buffer
0.01M Tris-Cl (pH 7.8)
0.005 M EDTA
0.5% SDS
50µg/ml Proteinase K

3.11.2. One-step RT-PCR for chitinase gene specific transcript accumulation

Chitinase gene specific transcript accumulation (mRNA transcription level) was obtained through RT-PCR method. The one step M-MuLV RT-PCR kit of Bangalore Genei was used in the present study and reaction was carried out according to the manufacturer's instruction manual. This one-tube method is designed for the

reverse transcription (RT) and polymerase chain reaction (PCR) amplification of a specific target RNA from either total RNA or mRNA. In the present investigation total RNA was added in the reaction mixture. Two-enzyme system was added at a time for sensitive, quick and reproducible analysis of even rare RNAs. The M-MuLV reverse transcriptase (M-MuLV-RT) from moloney murine leukemia virus was used for first strand cDNA synthesis and the *Taq* DNA polymerase from *Thermus aquaticus* was used for second-strand cDNA synthesis and DNA amplification with a fidelity enzyme. The kit consists of two major components: RT-PCR enzyme mixture and 2x RT-PCR reaction mixture. The M-MuLV RT enzyme in the mixture can synthesize cDNA at a temperature range of 40°C to 50°C and detect product upto 3 kb with varied amount of starting material (10 ng to 2 µg) of total RNA.

During reverse transcription, the HotStart *Taq* DNA polymerase is completely inactive and does not interfere with the reverse transcription reaction. For RT-PCR reaction, prepared the master mixture1, by adding RNasin, 2x RT-PCR reaction mixture, RT-PCR enzyme mixture including a fidelity enzyme, dNTPs, primer pair (chitinase specific) according to RT-PCR kit, and kept on ice. Master mixture 2 was prepared separately by adding denatured RNA at a final concentration 15µg/25µl RT-PCR reaction mixture. Heated master mixture 2 at 65°C for 5 minutes to denature the template RNA and kept on ice. The required volume of the master mixtures 1 and 2 were dispensed into each 0.2ml PCR tube on ice according to the manufacturer instruction manual for reverse transcription reaction. Placed the PCR tubes in the thermal cycler and started the One-step M-MuLV RT-PCR reaction.

The first strand cDNA synthesis was performed using the above One-step RT-PCR reaction mixture by incubating the reaction for 30 minutes at 50°C. After reverse transcription by M-MuLV reverse transcriptase, reaction was heated to 95°C for 10 minutes to activate HotStart *Taq* DNA polymerase and to simultaneously inactivate the reverse transcritase. The cDNA obtained was PCR amplified in 35 cycles consisting of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min and 72°C for 7 min for final extension in a thermocycler using the primers 5'-TTTTCGTCGGAAAATGGAAG-3' (forward primer) 5'-ACCAGCTTCTTCTCGTCCAA-3' (reverse primer). The primer sequence was the same as that used for obtaining the 201bp chitinase gene specific DNA fragment from genomic DNA (T383). The second DOP-primer pair was constructed from the

chitinase protein sequences was also used in the RT-PCR to quantify the chitinase gene specific transcript accumulation in treated (induced) and untreated samples. Forward DOP-primer was 5'-CTGCTGCGGCAAGTGGkgytggwrytg-3' and reverse primer was 5'-CAGTAGCAGCAGCAGTTGCArywccarcmsma-3'. In case of DOP-primer, the cycling temperature for 35 cycles was as follows- 94°C for 1 min, 60°C for 1 min and 72°C for 2 min and 72°C for 7 min for final extension.

3.11.3. RT-PCR product quantification on gel

The amplified cDNA product obtained from the RT-PCR was analyzed by electrophoresis on 1% agarose gel (described in section **3.2.11**) for visual quantification of the chitinase gene specific transcript accumulation in different samples (treated and control). 15µl aliquot of each of the RT-PCR product was taken and mixed with 3µl of 6x DNA loading buffer and then loaded into the slots of agarose gel. The gel was then run at voltage 75 and current 90 mA for 1.3 h until the bromophenol blue and xylene cyanol FF migrated at the end of the gel. The gel was taken out from the buffer tank and examined the gel by UV light on UV-Transilluminator and photographed.

3.11.4. RT-PCR product purification for transcript accumulation

The cDNA product of RT-PCR was purified according to Sambrook and Russell (2001). 150µl RT-PCR product was pooled up from the reaction tubes and added 0.2 volume of 5x proteinase K buffer to a final concentration of 50µg/ml. Incubated the mixture for 60 minutes at 37°C. Enzyme activity of the proteinase K was inactivated by heating the reaction mixture to 75°C for 30 minutes. Then extracted the solution once with equal volume of phenol: chloroform (1:1) and once with chloroform only. The upper supernatant was transferred to a fresh tube after centrifugation at 10,000g for 5 minutes. Added 0.2 volume of 10 M ammonium acetate and 2.5 volume of chilled (-20°C) ethanol for DNA precipitation. Mixed the solution by gentle shaking and kept at 4°C for 30 minutes for complete precipitation of RT-PCR product (cDNA fragments). The precipitated DNA was recovered by centrifugation at 5000 rpm for 5 minutes at 4°C in a microfuge. Discarded the supernatant and washed the DNA pellet twice with 70% ethanol. The DNA pellet was collected by centrifugation the solution at 5000 rpm in a microfuge at 4°C for 5 minutes. The DNA pellet allowed drying in the air in room temperature. Finally

purified RT-PCR product (DNAs) dissolved in 50µl TE buffer (pH 8.0). It was assumed that approximately 80% of the RT-PCR product was recovered by this method.

3.11.5. Quantification of purified RT-PCR product (cDNAs) to measure transcript accumulation

The purified RT-PCR products (cDNAs) from each of the treated and untreated samples (six samples) during induced systemic resistance by methyl jasmonate were measured by spectrophotometry. In RT-PCR, cDNA was synthesized by converting the mRNA molecules present in the starting RNA samples by reverse transcriptase and then the cDNA was amplified by normal PCR method. Quantification of RT-PCR product simultaneously indicates the quantity of transcription accumulation (mRNA transcription) in a given time. That means, the chitinase gene specific transcript accumulation has also been measured. The amount of the DNA concentration estimated in each sample was compared with the band thickness on the agarose gel. Bands were proportionately thick on the gel slots wherever the mRNA concentration was more within the starting samples of 15µg total RNA.

EXPERIMENTAL

4.1. Genomic fingerprinting analysis

After the amplification with the RAPD and ISSR primers, PCR products were separated on 1% agarose gel for visualization of the DNA banding patterns in all the 21 tea genotypes for genomic characterization. DNA bands were scored as 1s for presence of the band and 0s for absence of the band to develop binary data matrix. The binary data matrix was analyzed by statistical data analysis programs to construct a dendrogram for their phylogenetic relationships among the varieties of tea samples.

4.1.1. Statistical Data analysis

Only intensely stained, unambiguous and reproducible polymorphic bands were scored for data analysis. For considering a marker as polymorphic, the absence of an amplified product in at least one species was used as a criterion. As a dominant marker, RAPD and ISSR profiles were scored for each individual as discrete characters. All RAPD and ISSR products were scored for presence or absence of each amplicon evaluated. Only those bands that could be unequivocally scored across all samples were included in the analysis. Each polymorphic band was considered as a binary character and was scored as 1 for their presence or 0 for their absence across the clones for both RAPD and ISSR markers to generate a binary data matrix. Two matrices, one for each marker (RAPD and ISSR), were generated. The binary data was used to calculate genetic similarity matrix among samples using two different methods of coefficient such as Jaccard's coefficient (1908) and Nei and Li's (1979) coefficient. Based on the similarity matrix, a dendrogram showing the genetic relationships between genotypes was constructed using the UPGMA (unweighted pair group method with arithmetic average) (Sneath and Sokal, 1973) algorithm in SAHN clustering module from NTSYS-pc software version 1.5 (Rohlf, 1989). The Pearson's correlation coefficients between the similarity matrices based on different marker system (RAPD and ISSR data) were calculated using the standardized Mantel coefficient (Mantel, 1967). The significance level for the correlation coefficient was calculated following Sokal and Rohlf (1995). In a genetic study of substructured populations, Wright (1943) showed that the variation in gene frequency among subpopulations may be analyzed by the fixation indices or *F*-statistics. The *F*-statistics are applicable to any population if there are only two alleles at a locus. In the presence

of multiple alleles, however, *F*-statistics equation is no longer good fit. In this situation, Nei's (1973) method is used to evaluate the genetic diversity, heterozygosity and the degree of gene differentiation between a pair of populations. This method is based on the identities of two randomly chosen genes within and between populations and independent of the number of alleles. The probability of nonidentity, *H*, is a measure of genic variation of a population and usually called heterozygosity ($H = 1 - J$; *J*: gene identity), and value ranges from 0 to 1. This word, heterozygosity, however, is not appropriate for a nonrandom mating population. Therefore, *gene diversity* (*H*) can be used instead of heterozygosity. In contrast to gene diversity, the word *'J'* can be used for *gene identity.* In case of random mating population, the words gene diversity and gene identity may be replaced by heterozygosity and homozygosity, respectively. G_{ST} is equivalent to Wright's F_{ST}, and called as the coefficient of gene differentiation.

The POPGENE freeware software (Yeh *et al.*, 1997) was used to partition genetic diversity into within and between populations according to Nei's formula (1973). The diversity within population (*Hs*), total gene diversity (*Ht*) and coefficient of gene differentiation (*Gst*) are calculated on the basis of Nei's (1973) method in POPGENE software. The same software was also used to calculate the Shannon's gene diversity index. Significance of the genetic differences ($P < 0.001$) was measured by using a third approach, Analysis of Molecular Variance (AMOVA) with a software program WINAMOVA 1.55 (Excoffier *et al.*, 1992). Analysis of Molecular Variance (AMOVA) is a method of estimating population differentiation directly from molecular data and testing hypotheses about such differentiation. Because the molecular data consist of Euclidean distances derived from vectors of 1s and 0s, the data are unlikely to follow a normal distribution. The significance level of this *F*-statistics analogue was determined by 1000 random permutations. Treating the ISSR and RAPD profile as a haplotype, AMOVA allows the estimation of population genetics parameters at the genotypic level.

4.1.2. Genetic relationship among genotypes

The genetic relationships and their grouping were analysed on the basis of band matching similarity coefficients of the ISSR and RAPD profiles using the two methods-

1. Nei and Li's (1979) coefficients ($GSij = 2Nij/(Ni + Nj)$, where, Nij is the number of bands that are shared by genotypes i and j, Ni is the number of bands present in genotypes i, and Nj is the number of bands present in genotype j.

2. Jaccard's (1908) coefficients ($GS_{ij} = 2a/(2a + b + c)$, where GS_{ij} is the measure of genetic similarity between individuals i and j, a is the number of polymorphic bands that are shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i.

These methods incorporate only bands that are present. The coefficients of Jaccard (1908) and Nei and Li (1979) [same as Dice (1945) compare the number of bands shared between individuals or populations. Distances were calculated from the Nei & Li's coefficient. Jaccard's coefficient is the most simple, but the coefficient of Nei and Li puts more weight on positive matches.

4.1.3. Genetic relationships among the varieties

Genetic relationships among the varieties were also investigated by analyzing the genotypes of each varietiey as a single population. The inter and intragenetic divergence was estimated using ISSR and RAPD markers. All the above mentioned coefficients were also worked out for each variety and dendrograms were constructed to find out the phylogenetic relationships among the varieties. The genetic variability in the population was calculated using Nei's (Nei 1973) coefficient of gene differentiation (G_{ST}) in POPGENE freeware program (Yeh *et al.*, 1997). In POPGENE, the genetic divergence among different populations was calculated using a multiallelic analogue of F_{ST} among a finite number of populations, which was otherwise called the coefficient of gene differentiation (Nei 1973). This is stated in the following equation:

$G_{ST} = D_{ST}/H_t = (H_t - H_s)/H_t$, where, D_{ST} is the average gene diversity between subpopulations, including the comparisons of subpopulations with themselves, with

$D_{ST} = (H_t - H_s)$. G_{ST} is an extension of Nei's (1972) genetic distance between a pair of populations to the case of hierarchical structure of populations (Nei 1973). H_t is defined by the following equation:

$$H_t = 1 - \Sigma p_i^2$$

where, p_i is the frequency of ith allele at a locus in a population. Hence, H_s was defined in terms of gene diversities.

However, for random mating subpopulations, gene diversities can be defined as expected heterozygosities under Hardy–Weinberg equilibrium averaged among subpopulations (H_s) and of the total population (H_t). The main difference between G_{ST} and F_{ST} (Wright 1943) is that in G_{ST} the estimation of the heterozygosities relies on allele frequencies (Nei 1987), whereas in F_{ST} to estimate the H_S the individual genotypes have to be known. The estimate of gene flow from G_{ST} was calculated as

$$N_m = 0.5(1 - G_{ST})/G_{ST}.$$

Assuming that each RAPD band represents a single diallelic locus in Hardy–Weinberg (H–W) equilibrium, the p/a data set can be converted into allele frequencies (Apostol *et al.*, 1996; Lu and Rank, 1996). However, because RAPDs behave as dominant markers (*i.e.* 1=*AA* or *Aa* and 0=*aa*), allele frequency estimates are less accurate than with codominant markers (Lynch and Milligan, 1994). Both distance matrices were subjected to UPGMA clustering and matrix correlations were tested with Mantel's statistic (permutation level=1000) as implemented by the NTSYSpc ver. 1.5 program (Rohlf, 1989).

4.1.4. DNA polymorphism within the genotypes

DNA extracted from three individuals of single genotypes which were clonally propagated from each variety was tested with three RAPD (OPB10, OPA18 and OPA19) and three ISSR primers (ISSR13, ISSR14 and ISSR18) for the polymorphism analysis among the same genotypes. Results showed no DNA polymorphism among the clones of the same genotype. Therefore, DNA from a single clonal plant was used for each genotype for the present studies.

4.1.5. DNA polymorphism among the genotypes

On the basis of the DNA markers generated by the primers, considerable genetic diversity was observed among the different tea varieties. The band profiles

were generated by the RAPD primers clearly reveal such variability among the genotypes (Plate 2, fig. A & B; Plate 3, fig. B; Plate 4, fig. B; Plate 5, fig. B). The twelve RAPD primers generated a total of 72 bands (table 17 and 18), of which 56 were polymorphic. The maximum number (9) of bands was produced by OPB10, whereas the minimum number (4) was generated by L5. A total of 77.77% polymorphic markers were generated by the 12 RAPD primers used in this study. Similarly, out of 70 bands generated by the ISSR primers (Tables 19 & 20), 62 were polymorphic, thus generating 88.57% polymorphism among the 21 tea genotypes. The maximum number of bands (11) was observed in ISSR14 (Plate 4, fig. A). The minimum number (9) was observed in ISSR18 (Plate 5, fig. C.).

Primer	Total no. of RAPD bands (a)	Number of Polymorphic bands (b)	Polymorphism= b/a x 100 (%)	Approx. band size (bp) Mini	Maxi
OPA-1	5	3	60.00%	250	2500
OPA-4	4	3	75.00%	250	2500
OPA-9	6	5	83.33%	250	2500
OPA-11	5	4	80.00%	250	2500
OPA-12	7	6	85.71%	250	2500
OPA-13	6	4	66.66%	250	2500
OPA-16	7	6	85.71%	250	2500
OPA-18	6	4	66.66%	250	2500
OPA-19	8	7	87.50%	250	2500
L5	4	3	75.00%	250	2500
OPB-10	9	7	77.77%	250	2500
OPB-20	5	4	80.00%	250	2500
Total: 12	Total: 72	Total: 56	Average: 77.77%		

Table-17. Analysis of the polymorphisms obtained with RAPD primers in twenty one clonal tea cultivars [*Camellia sinensis* (L.) O. Kuntze].

Total number of RAPD primers screened - 12
Number of primers producing polymorphism –12
Total number of loci screened –72
Total number of polymorphic loci- 56
Total number of monomorphic loci-16
Average markers produced by individual primers – 6.5
Size of amplified bands ranges between 250 and 2500bp.
Percent of total bands, which are polymorphic 77.77%

Table-18. Short summary of the table 17, providing information about the total number of bands and average percentage of polymorphism prevailed in the RAPD profiling.

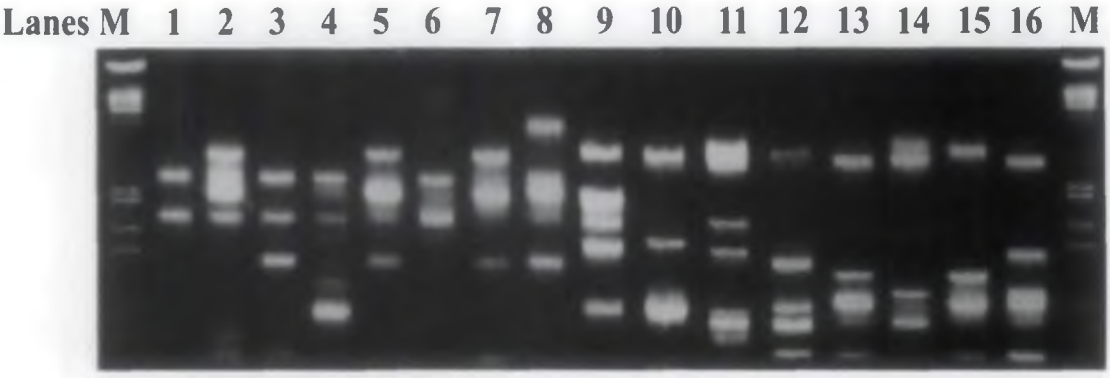


Fig. A: RAPD profile of the four selected tea genotypes generated by four different primers (Lanes 1-4 for L5; 5-8 for OPA-16; 9-12 for OPA-18 and 13-16 for OPA-19). Lanes 1,5,9 & 13 for clone HV39; 2,6,10 & 14 for clone T383; 3,7,11 & 15 for clone TeenAli17; 4,8,12 & 16 for clone T78. Lane M, Lambda DNA marker (*Eco*RI & *Hin*dIII cut).

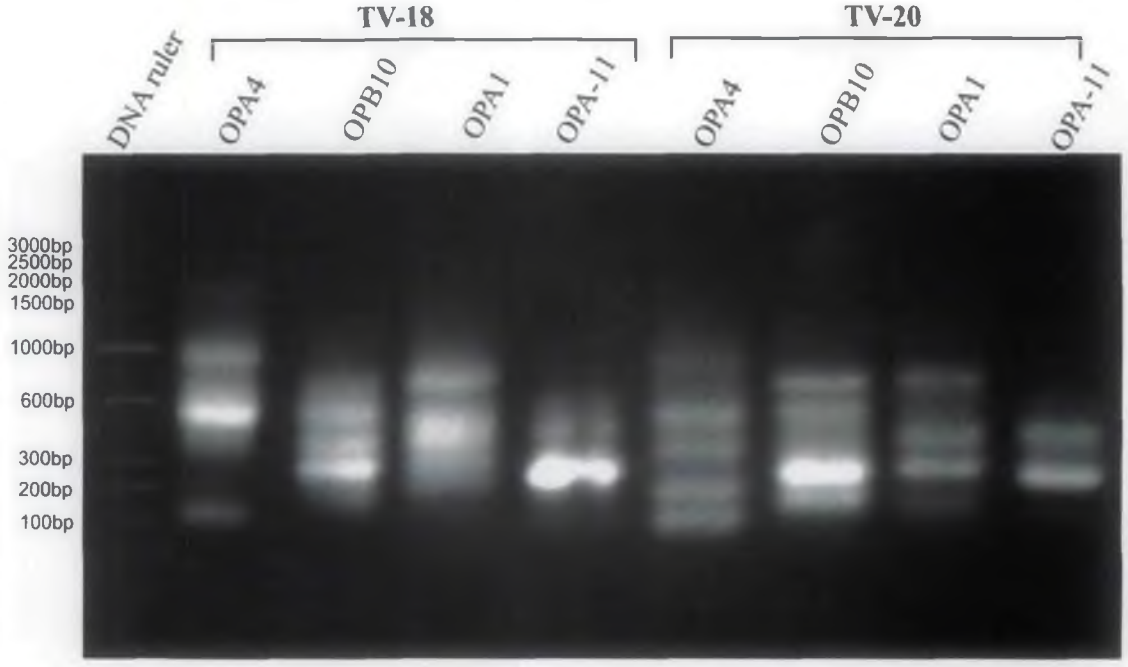


Fig. B. RAPD based DNA fingerprinting of two tea cultivars *viz.*TV18 and TV20 using four random primers of 10-mer.

Plate 2 [Figs A & B]: PCR amplified products of RAPD based DNA fingerprinting was fractionated in 1% agarose gel electrophoresis.

Table 19. Analysis of the polymorphism obtained with seven ISSR markers in twenty one clonal tea cultivars [*Camellia sinensis* (L.) O. Kuntze].

primer	Sequence 5' to 3'	Total no. of ISSR bands (a)	Number of Polymorphic bands (b)	Polymorphism = b/a x100 (%)	Approx. band size (bp) Mini	Maxi
ISSR13	$(AC)_8C$	10	8	80.00%	300	2500
ISSR14	$(TG)_8G$	11	10	90.90%	300	2500
ISSR17	$(TC)_8C$	10	9	90.00%	300	2500
ISSR18	$(TG)_8G$	09	8	88.88%	300	2500
ISSR814.1	$(CT)_8TG$	10	9	90.00%	300	2500
UBC830	$(GA)_8C$	10	9	90.00%	300	2500
UBC825	$(AC)_8Y^*T$	10	9	90.00%	300	2500
Total: 7		Total: 70	Total: 62	Average: 88.54%		

- Y=G/C

Total number of ISSR primers screened - 7
Number of primers producing polymorphism –7
Total number of loci screened –70
Total number of polymorphic loci- 62
Total number of monomorphic loci-8
Average markers produced by individual primers - 10
Size of amplified bands ranges between 300 and 2500bp.
Percent of total bands, which are polymorphic 88.54%

Table-20. Short summary of the table 19, providing information about the total number of bands and average percentage of polymorphism prevailed in the ISSR profiling.

4.2. Genetic similarity among genotypes

4.2.1. RAPD analysis

The protocol of the sensitive RAPD technique was carefully optimized for various experimental parameters and a subset of three replicas from each of the clones were used to select the reproducible bands with each primer. Only clear, repeatable and reproducible bands were scored and used in further genetic diversity analysis.

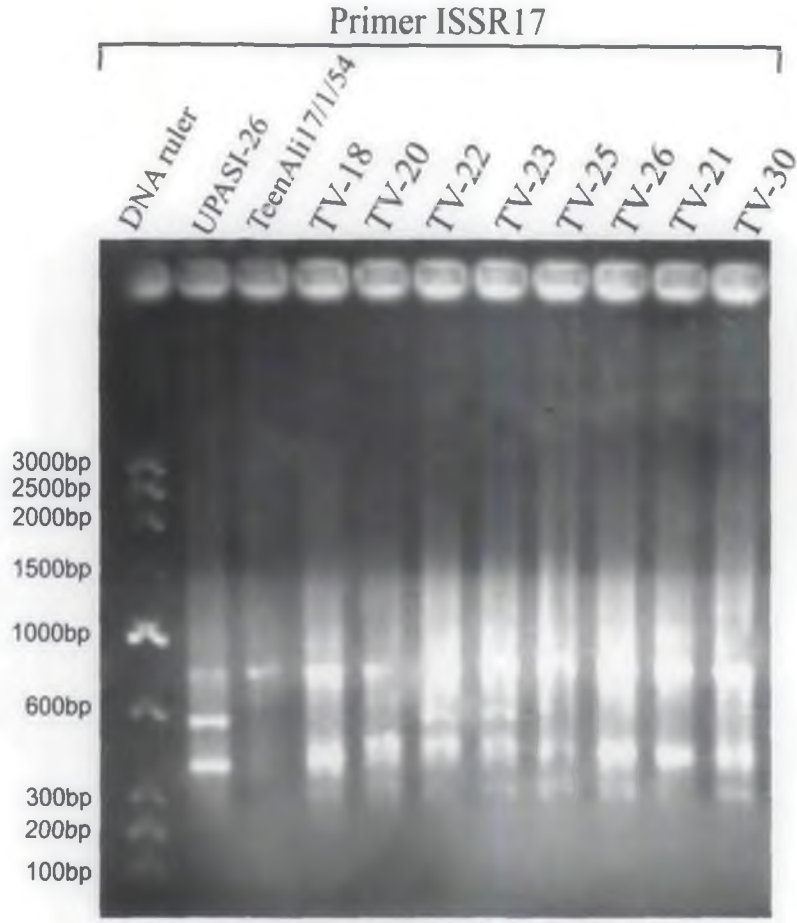


Fig. A: DNA fingerprinting of ten tea cultivars viz. UPASI-26, TeenAli17/1/54, TV-18,TV-20,TV-23,TV-25,TV-26, TV-21 and TV-30 using ISSR17 primer.

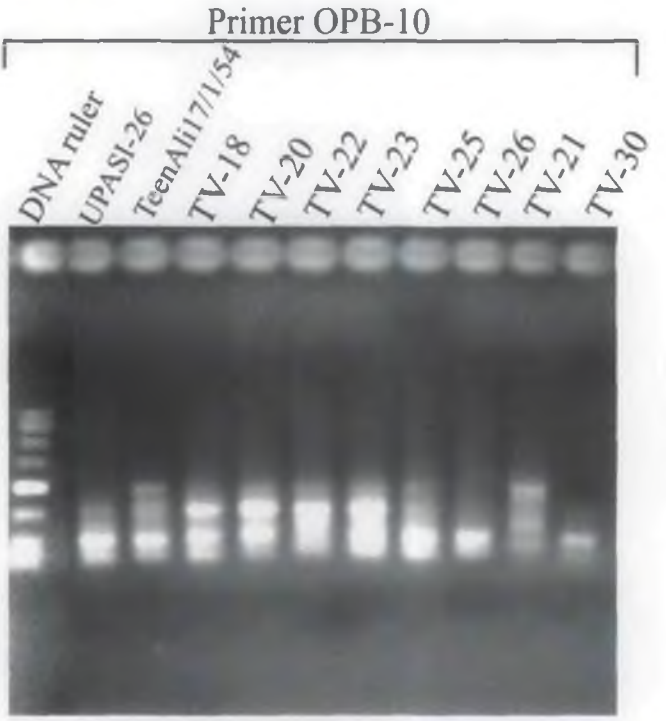


Fig. B: RAPD based DNA fingerprinting of ten tea cultivars *viz.* UPASI-26, TeenAli17/1/54, TV-18, TV-20, TV-22, TV-23, TV-25, TV-26, TV-21 and TV-30, using one random 10-mer primer OPB-10.

Plate 3 [Fgs. A & B]: RAPD and ISSR based DNA fingerprinting products have been separated in 1% agarose gel electrophoresis

Twenty-one cultivars of tea were subjected to RAPD analysis. RAPD assay of the total genomic DNA from *Camellia sinensis* was performed using 12 random 10-mer primers such as OPA-1, OPA-4, OPA-9, OPA-11, OPA-12, OPA-13, OPA-16, OPA-18, OPA-19, OPB-10, OPB-20 and L5 (table 17 and 18). In RAPD analyses, 56 out of 72 bands (77.77%) were polymorphic. The number of alleles ranged from 4 to 9 per primer, with an average of 6.5 per primer. The assay revealed a large amount of polymorphism, and the size of amplification product ranged between 250-2500 bp. Each of the random primers produced distinct polymorphic banding patterns in all the varieties tested. Typical results obtained with the RAPD primers are shown in plate 2, fig. B; plate 3, fig. B; plate 4, fig. B and plate 5, fig. B.

The bands were scored as either monomorphic or polymorphic. Primers L5 and OPA-4 were generated a total of 4 products of which 3 (75%) were scored as polymorphic, primer OPA-1 generated a total of 5 products of which 3 (60%) were scored as polymorphic. With primer OPA-11 and OPB-20, 4 bands out of 5 bands (80%) were polymorphic and with primer OPA-9, 5 out of 6 bands (83.33%) were polymorphic and OPA-13, OPA-18 produced 6 bands out of which 4 bands (66.66%) were polymorphic. Primer OPA-19 generated 8 bands out of which 7 bands (87.50%) were polymorphic and primer OPB-10 produced highest number of bands 9, out of which 7 bands (77.77%) were polymorphic. Using 12-decamer RAPD primers, a total of 72 bands were scored (an average of 6 bands per primer) and about 77.77% were found to be polymorphic between the tea cultivars (table 17 and 18).

The number of scorable bands per primer ranged between 4 to 9 with an average 6.5. The percentage of polymorphism varied from 60 % (OPA-1) to 87.50% (OPA-19). All the tea clones could be distinguished from one another based on these polymorphic bands. Similarities among the test samples calculated with Nei and Li's formula indicated that the highest similarity (0.85) between T135 and T383 while the lowest (0.65) between TV21 and UPASI-26. The similarity matrix obtained was used in an UPGMA cluster analysis. The dendrogram (Figure 2) showed that three groups could be recognized at a similarity index of 0.5. The first group consisted of all cultivars of China tea. The second group consisted of seven cultivars of Assam tea while the third group consisted of 7 samples of Cambod tea. The dendrogram was constructed using the same data of RAPD profiling through Jaccards' coefficient also given the similar result with that of Nei and Li's coefficient (Figure 3).

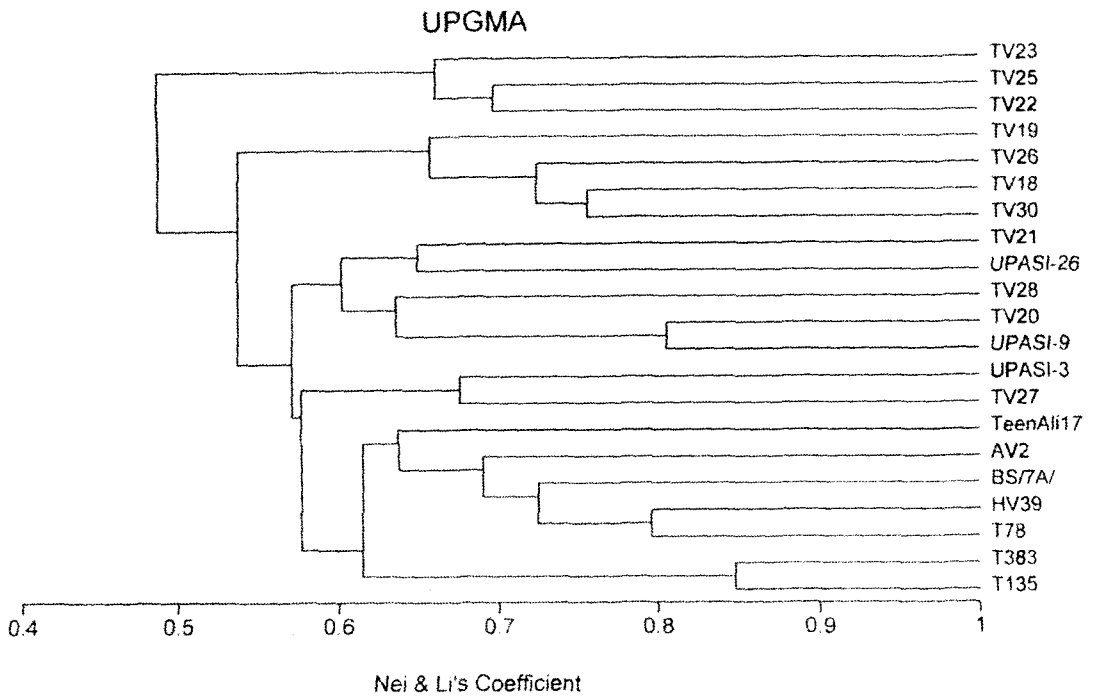


Figure 2. Dendrogram showing the genetic relationship among the twenty one tea cultivars based on RAPD markers using Nei & Li's (1979) coefficient.

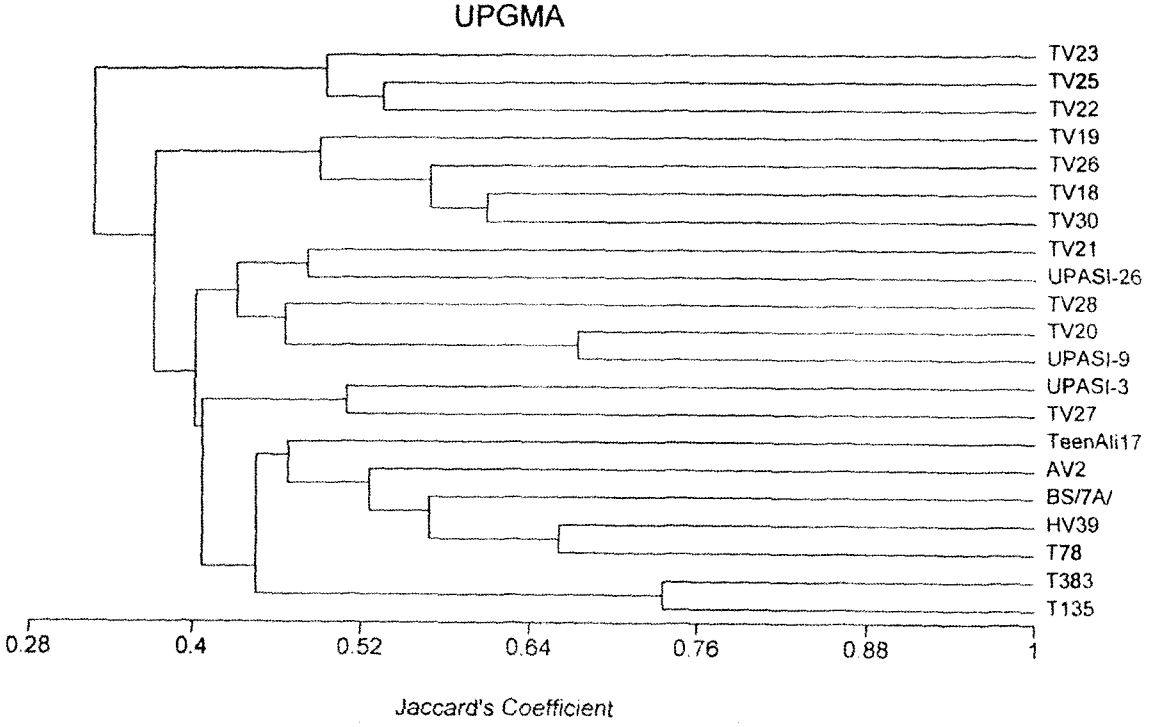


Figure 3. Dendrogram showing the genetic relationship among the twenty one tea cultivars based on RAPD markers using Jaccard's (1908) coefficient.

4.2.2. ISSR analysis

In ISSR analysis, using seven primers, a total of 70 bands (70 alleles) were observed, out of which 62 bands (alleles) were polymorphic producing 10 bands per primer (Table 19 and 20). The average polymorphism was 88.54%, and the lowest polymorphism was 80% in ISSR13 (Plate 5, fig. A) and highest polymorphism was 90.90% in ISSR14 (Plate 4, fig. A). The number of scorable alleles (bands) per primer ranged between 9 to 11 with an average of 10 alleles (bands) per ISSR primer. An example of the polymorphisms detected among some test samples by 3' anchored primer ISSR14 [$(TG)_8G$] was shown in plate 4, fig. A. The lowest bands 9 in number were produced by primer ISSR18 (Plate 5, fig. C.) and primer ISSR14 produced highest bands 11 in number (Plate 4, fig. A.).

The ISSR primers UBC830 and UBC825 also produced polymorphic bands in all the materials tested (Plate 6, fig. A-B). All the tea clones could be distinguished from one another based on these polymorphic bands. Similarities among samples calculated with Nei and Li's formula indicated that the highest similarity (0.90) was between HV39 and T383 while lowest similarity (0.67) was between TV18 and TV27. The similarity matrix was used in an UPGMA cluster analysis. The dendogram constructed on the basis of ISSR profiling by using Nei and Li's coefficient (Figure 4) and Jaccard's coefficient (Figure 5) showed that three groups could be recognized at the similarity coefficient of 0.77. The first group consisted of all China and hybrids cultivars. The second group consisted of genotypes of Assam tea and the third group consisted with all the cultivars of Cambod tea. The similarity matrix of Nei and Li's coefficient was shown in table 21 on the basis of ISSR fingerprinting.

4.2.3. Genetic similarity matrix and cluster analyses

The genetic similarity coefficients among genotypes estimated on the basis of Nei and Li (1979) varied from 0.898 to 0.673 with an average genetic similarity of 0.785 in ISSR markers. The same was in the range of 0.848 and 0.194 with an average of 0.521 in RAPD and from 0.873 to 0.434 with a mean coefficient of 0.653 when the pooled data of both marker system were used (Table 22). The similarity coefficients among the genotypes estimated on the basis of Jaccard (1908) was between 0.821 and 0.643, 0.733 and 0.346 and 0.777 and 0.424 in ISSR, RAPD, and in the pooled data of ISSR + RAPD markers, respectively.

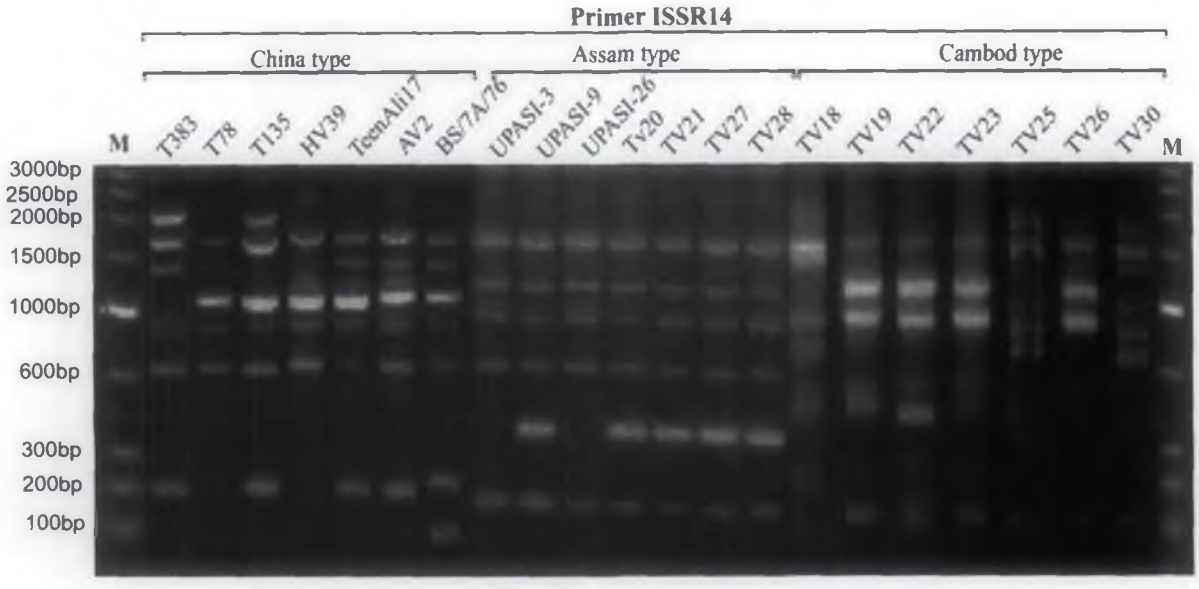


Fig.A: DNA fingerprinting of 21 tea genotypes based on ISSR markers. Lane, M for low range DNA ruler marker.

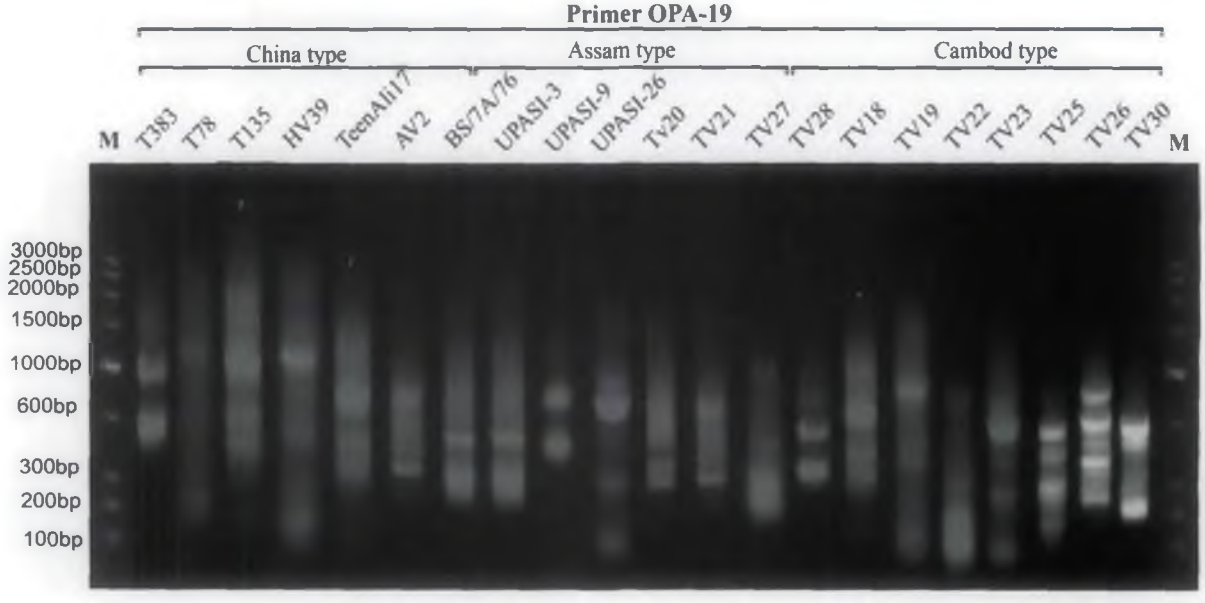


Fig.B:DNA fingerprinting of 21 tea genotypes based on RAPD markers. Lane, M for low range DNA ruler marker.

Plate 4 [Figs. A & B]:DNA fingerprinting of 21 tea genotypes based on ISSR marker (A) and RAPD markers (B).

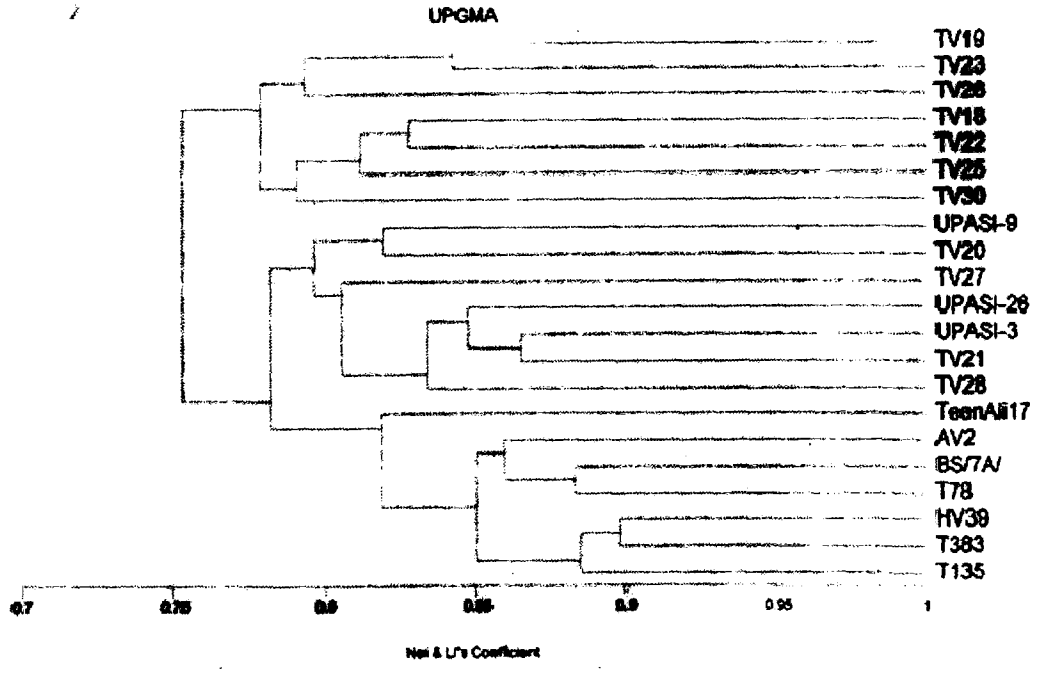


Figure 4. Dendrogram showing the genetic relationship among the twenty one tea cultivars based on ISSR markers using Nei & Li's coefficient.

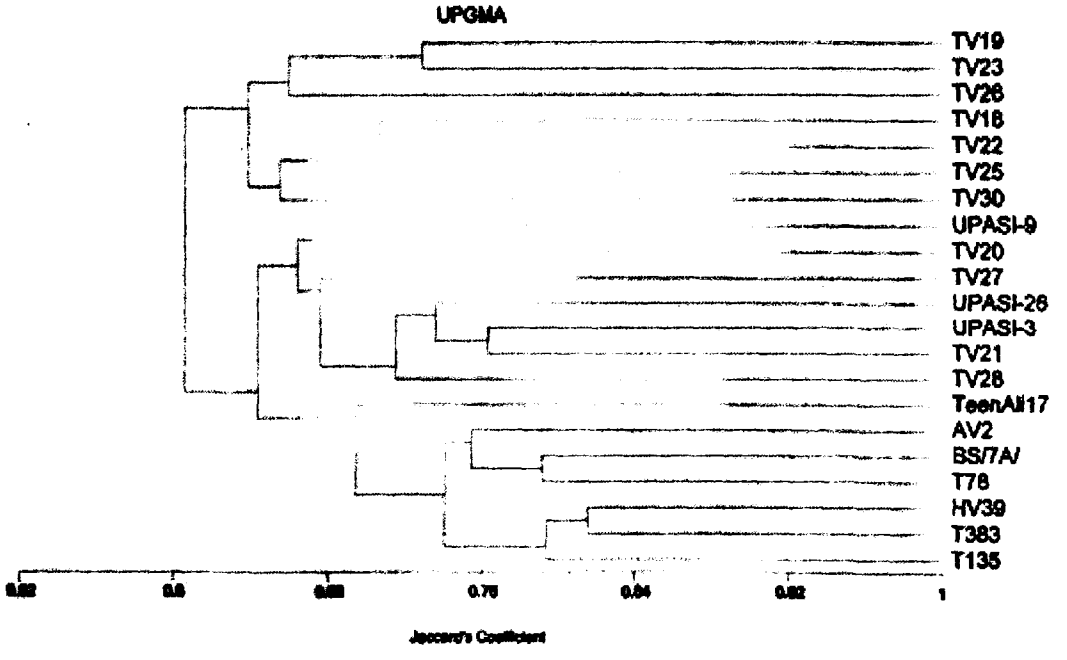


Figure 5. Dendrogram showing the genetic relationship among the twenty one tea cultivars based on ISSR markers using Jaccard's coefficient.

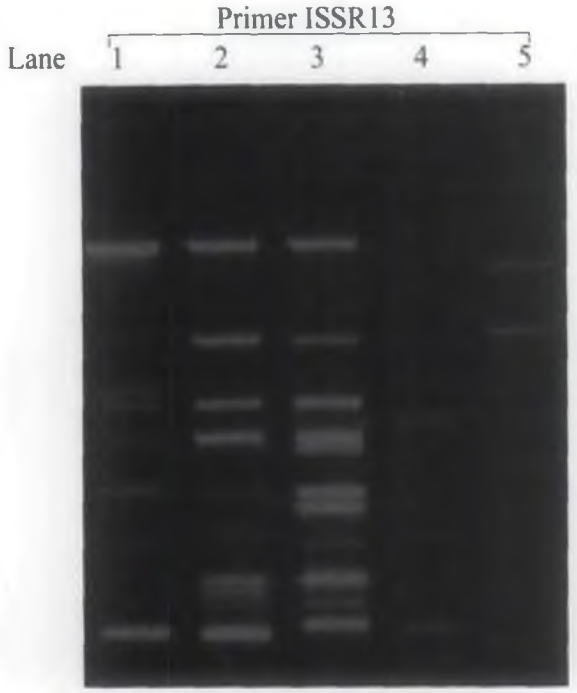


Fig. A. ISSR based DNA fingerprinting in five tea genotypes. Lanes 1-5, BS/7A/76, AV2, TeenAli/17/1/54, TV20 & HV39, respectively.

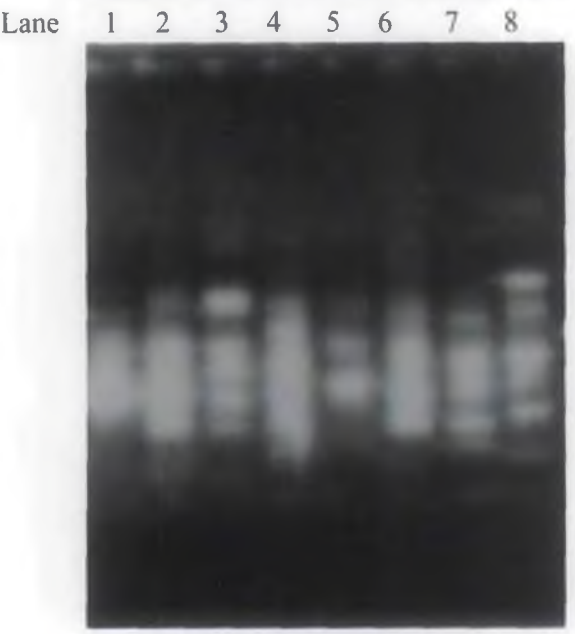


Fig. B. RAPD based DNA fingerprinting in eight tea genotypes using random 10-mer primers. Lanes 1-8; BS/7A/76, T135, T78, TV27, TV20, TV28, TV25 & TV18, respectively. Primers, lane 1-2, OPA-9; lane 3-4, OPA-12; lane 5-6, OPA-13 and lane 7-8, OPB-20.

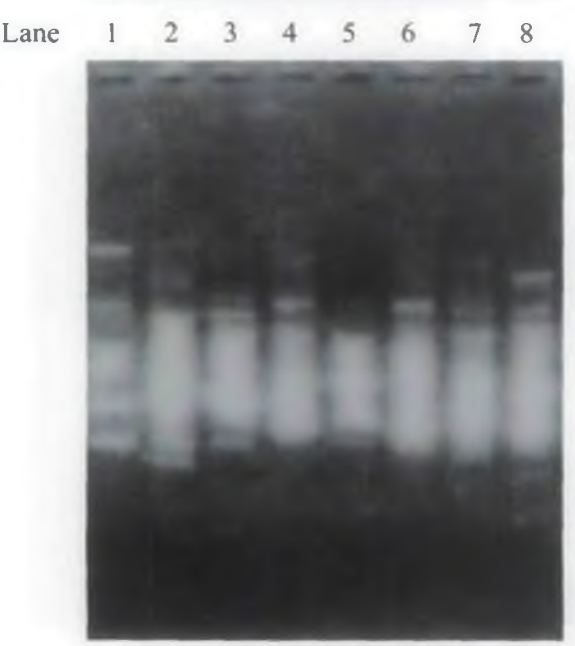


Fig. C. DNA fingerprinting of eight tea genotypes using ISSR primers. Lanes 1-8; UPASI-26, TeenAli17, T383, TV22, TV23, AV2, UPASI-9 and UPASI-3, respectively. Primers, lane 1-4, ISSR18 and lane 5-8, ISSR814.1.

Plate 5 [Figs. A-C]: RAPD & ISSR products separated in 1% agrose gel.

The Pearson's correlation coefficients between different matrices subjected to the Mantel test (Mantel 1967) were found to be highly significant ($r = 0.579 - 0.879$, $p < 0.001$).

The bands obtained with RAPD and ISSR profiling was used to compute similarity coefficients with Jaccard's and Nei and Li's coefficient using NTSYSpc software program. The data set with 72 bands for RAPD were employed. With RAPD data matrix, the highest genetic similarity value of 0.848 was obtained between T135 and T383. The lowest value obtained was 0.194 between TV22 and TV18. The genetic similarity between T383 and HV39 was 0.898 according to Nei and Li's coefficient when the ISSR markers were considered and lowest was 0.673 between TV18 and TV27. From table 23, the value of the genetic similarity coefficient of the three tea populations varied between 0.47 and 0.67, with an average of 0.57. In order to clarify the correlation between populations, UPGMA algorithm was used to draw a dendrogram of the three tea populations (Figure 6) on the basis of Nei's genetic idendity (Table 23). Dendogram showing the close relationship between Assamica and Cambod variety than China and Assamica variety.

Populations	China tea	Assam tea	Cambod tea
China	***	0.59	0.47
Assam	0.42	***	0.67
Cambod	0.52	0.34	***

Table 23. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) of the three populations of Tea *viz.* China type, Assam type and Cambod type (Nei, 1972).

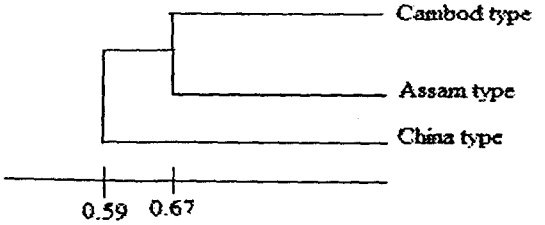


Figure 6. Phenetic dendogram constructed on the basis of genetic distance shown in table 23 based on ISSR markers among the three populations of tea varieties [China type, Assam type and Cambod type] to show their relationships.

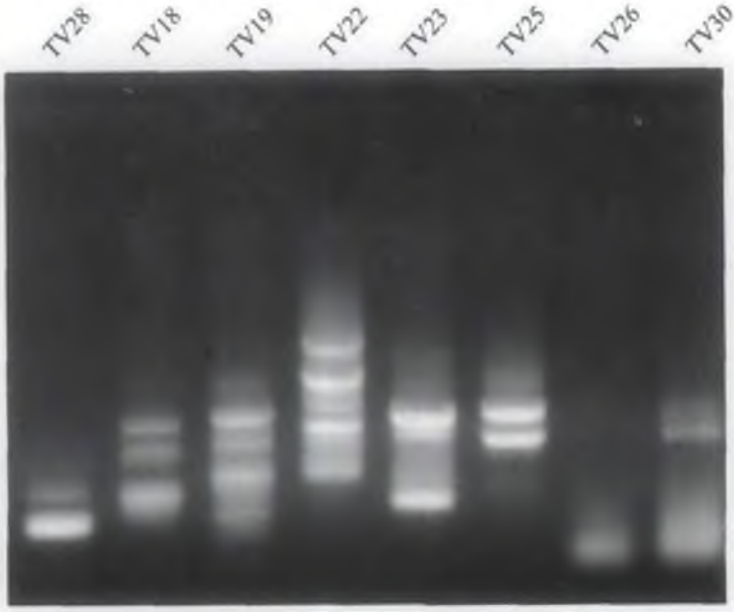


Fig.A: DNA fingerprinting of the eight tea clones were generated using ISSR primer UBC830.

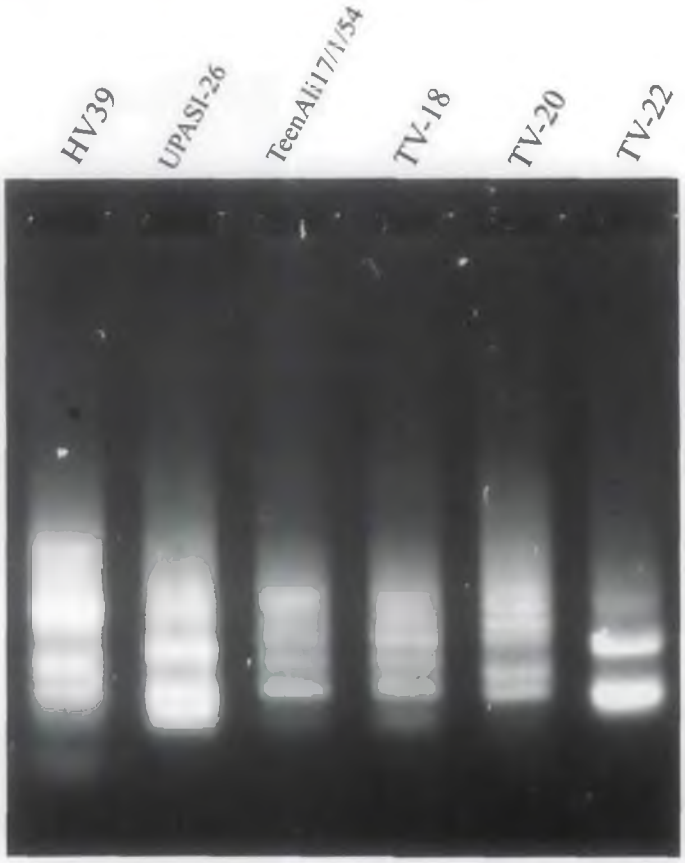


Fig. B: DNA fingerprinting of the six tea clones were generated using ISSR primer UBC825.

Plate 6 [Fgs. A & B]:ISSR based DNA fingerprinting products has been separated in 1% agarose gel electrophoresis.

Table 22. The genetic similarity coefficients estimated from the ISSR, RAPD, and pooled markers in twenty one genotypes of tea.

Coefficients	Mean	Maximum	Minimum
ISSR			
Nei and Li	0.785	0.898	0.673
Jaccard	0.732	0.821	0.643
RAPD			
Nei and Li	0.521	0.848	0.194
Jaccard	0.539	0.733	0.346
ISSR + RAPD			
Nei and Li	0.653	0.873	0.434
Jaccard	0.600	0.777	0.424

The dendrograms realized from the above matrices of ISSR and RAPD marker systems using UPGMA method grouped the twenty one clonal tea genotypes into three clusters (Figure. 2 & 3 and 4 & 5). The first group comprised seven genotypes from china type tea cultivars, the second cluster, seven genotypes from Assam tea cultivars and the rest seven tea genotypes grouped into a separate third cluster. The third cluster contained seven genotypes, all from Cambod type tea genotypes. Another important point noticed from the dendrogram is that the high genetic distance enjoyed by different types of tea (China type, Assam type and Cambod type). The Mantel tests (Mantel 1967) between cophenetic correlation matrix and its corresponding similarity matrix were found highly significant (r = 0.678–0.849, P < 0.001) for all dendrograms.

T135 T78 T383 TeenAli17 AV2 BS/7A HV39 TV28 TV27 TV21 TV20 UPASI-9 UPASI-3 UPASI-26 TV30 TV26 TV25 TV23 TV22 TV19 TV18
T135 1.000
T78 0.862 1.000
T383 0.887 0.843 1.000
TeenAli 0.838 0.842 0.817 1.000
AV2 0.871 0.843 0.885 0.783 1.000
BS/76 0.862 0.883 0.876 0.825 0.876 1.000
HV39 0.883 0.821 0.898 0.811 0.814 0.821 1.000
TV28 0.796 0.745 0.811 0.769 0.757 0.764 0.804 1.000
TV27 0.832 0.764 0.775 0.750 0.775 0.782 0.766 0.820 1.000
TV21 0.817 0.786 0.832 0.736 0.779 0.750 0.844 0.843 0.765 1.000
TV20 0.789 0.811 0.768 0.743 0.768 0.811 0.796 0.752 0.792 0.757 1.000
UPASI90.804 0.771 0.782 0.738 0.745 0.752 0.811 0.788 0.788 0.812 0.820 1.000
UPASI30.817 0.768 0.814 0.755 0.779 0.768 0.826 0.824 0.824 0.865 0.854 0.832 1.000
UPASI260.828 0.779 0.789 0.729 0.789 0.779 0.782 0.835 0.816 0.838 0.788 0.804 0.857 1.000
TV30 0.825 0.739 0.804 0.724 0.804 0.775 0.796 0.733 0.733 0.757 0.706 0.760 0.757 0.750 1.000
TV26 0.807 0.757 0.768 0.781 0.750 0.811 0.778 0.713 0.713 0.699 0.725 0.760 0.718 0.769 0.784 1.000
TV25 0.750 0.789 0.745 0.699 0.764 0.771 0.755 0.727 0.707 0.772 0.740 0.755 0.733 0.765 0.780 0.780 1.000
TV23 0.828 0.796 0.789 0.766 0.789 0.779 0.800 0.718 0.699 0.724 0.692 0.725 0.724 0.774 0.808 0.827 0.804 1.000
TV22 0.789 0.757 0.768 0.743 0.804 0.793 0.778 0.752 0.733 0.757 0.745 0.760 0.757 0.750 0.784 0.784 0.820 0.788 1.000
TV19 0.768 0.789 0.782 0.738 0.727 0.734 0.774 0.747 0.687 0.792 0.680 0.755 0.752 0.745 0.760 0.760 0.755 0.843 0.760 1.000
TV18 0.775 0.741 0.752 0.745 0.771 0.778 0.762 0.694 0.673 0.740 0.707 0.742 0.740 0.752 0.808 0.788 0.804 0.792 0.828 0.742 1.000
T135 T78 T383 TeenAli17 AV2 BS/7A/ HV39 TV28 TV27 TV21 TV20 UPASI-9 UPASI-3 UPASI-26 TV30 TV26 TV25 TV23 TV22 TV19 TV18

Table 21. Genetic similarity matrix calculated on the basis of ISSR markers using Nei & Li's (1979) coefficient.

4.2.4. Genetic variability among the different types of tea varieties

The genetic distances between different species of tea estimated through ISSR, RAPD, and pooled data from both marker systems showed that Cambod tea type enjoyed a high genetic distance from the rest of the tea types (China and Assam type), which was also observed when individual genotypes were analyzed. Similarly, the close genetic relationship between China and Assam type is evident from the matrices. The matrix generated with Nei and Li (1979) and Jaccard's (1908) showed almost the same pattern of genetic distance among the varieties, where maximum genetic distance was observed between varieties of China type and Cambod type and the minimum between China and Assam type. In the Jaccard coefficients (Jaccard 1908), the highest genetic distance was observed between China and Cambod tea varieties and the least genetic distance was between China type and Assam type tea varieties. The genetic distance calculated with ISSR and pooled data of both ISSR and RAPD once again confirmed the higher genetic distance of China type from the rest of the varieties and the close genetic similarity of China and Assam type varieties. The Pearson's correlation coefficients between different matrices analyzed by the Mantel test (Mantel 1967) were found to be highly significant (r = 0.678–0.849, $p <$ 0.001). The dendrograms realized from the matrices generated from the ISSR+RAPD pooled data using both Nei and Li (1979) and Jaccard's (1908) coefficients generated exactly the same pattern of clustering of the varieties. The genetic analysis of the population and subpopulation of tea varieties through the software POPGENE revealed that the average number of observed alleles (*Na*) per population was two in each ISSR and RAPD markers (Table 24). The number of effective alleles (*Ne*) varied from 1.46±0.33 in China, 1.68±0.44 in Assam and 1.45±0.17 in Cambod type tea for ISSR markers.

Diversity measures were calculated by Nei's (1973) index and genetic heterozygosity ranged from H = 0.129 to H = 0.435. Averaged over all markers, Cambod variety was found to be least diverse 0.25 (Table 25). The China variety displayed the highest level of variability (0.33) while Assam variety revealed intermediate diversity value 0.30 (Table 25). Nei's total genetic heterozygosity varied from 0.139 to 0.316 and on an average 0.25 in Cambod variety which showed least variability while Assam variety displayed heterozygosity from 0.177 to 0.376 with an average 0.30. The heterozygosity ranges from 0.129 to 0.435 in China variety with an

average 0.33. The Shannon's information index (*I*) was 0.31, 0.23 and 0.20 for China, Assam and Cambod variety, respectively while both the RAPD and ISSR data was analyzed (Table 24). The average gene diversity within populations (H_S) was 0.27 and the total diversity (H_T) amounted to 0.38 (Table 26). The value of H_S ranges from 0.178 to 0.364 and value of H_T ranges from 0.254 to 0.497. The mean level of genetic differentiation (G_{ST}) between populations over all loci was 0.25. This indicated that only a low (25%) proportion of diversity is observed between populations as compared with diversity within populations (75%). The markers contributed differently to the observed degree of population differentiation, varying from a low differentiation of 18% for OPA-1 to a high of 32% for OPA-19.

Table 24. The Nei's genetic heterozygosity (*H*) estimated among three varieties of tea.

Tea Varieties	No. of observed alleles (*Na*)	No. of effective alleles (*Ne*)	Genetic hererozygosity (*H*)	Shannon's information index (I)
ISSR				
C.sinensis var. *sinensis*	2	1.46±0.33	0.33±0.22	0.30±0.34
C.sinensis var. *assamica*	2	1.68±0.44	0.30±0.18	0.26±0.11
C.sinensis spp. *lasiocalyx*	2	1.45±0.17	0.25±029	0.23±0.22
RAPD				
C.sinensis var. sinensis	2	1.34±0.17	0.28±0.31	0.29±0.23
C.sinensis var. *assamica*	2	1.44±0.27	0.26±0.20	0.27±0.26
C.sinensis spp. *lasiocalyx*	2	1.37±0.33	0.23±0.19	0.21±0.31
ISSR + RAPD				
C.sinensis var. sinensis	2	1.24±0.27	0.31±0.23	0.31±0.22
C.sinensis var. *assamica*	2	1.31±0.32	0.26±0.25	0.23±0.19
C.sinensis spp. *lasiocalyx*	2	1.41±0.19	0.22±0.22	0.20±0.24

Na = Observed number of alleles, Ne = Effective number of alleles, H = Nei's (1973) gene diversity, I = Shannon's Information index.

The estimated gene flow from one variety to the other over generations (*N*m) on an average was 0.769 while lowest (0.673) was between China and Cambod varieties and highest (0.878) between Assam and Cambod variety (Table 27). Interpopulation gene flow [$N_m = 0.5(1 - G_{ST})/G_{ST}$] was 0.76, $N_m < 1.0$ showing the limited genetic exchange among populations (fewer than one migrant per generation into a population. In table 26, the genetic diversity result indicated that Nei's total gene diversity (H_T) was 0.38,

within population genetic diversity (*Hs*) was 0.27, and coefficient of gene differentiation (G_{ST}) was 0.25, 75% of which was within population genetic diversity while 25% was among population variation, *i.e.* most variation existed within the community. In table 28, gene diversity (*Hs*) was calculated according to Nei's formula and it was 0.285, 0.241 and 0.207 respectively in China, Assam and Cambod varieties for RAPD markers while on an average it was 0.24 overall and 0.291, 0.223 and 0.199 for ISSR markers respectively. Genetic diversity (Nei's 1973) was 0.33, 0.30 and 0.25 in China, Assam and Cambod varieties respectively (Table 25). The Nei's (1972) genetic identity and distance were shown in table 23, indicating that the highest identity (0.67) observed between Assam and Cambod type tea clones while lowest identity (0.47) was between China and Cambod type tea clones. The Nei's gene diversity measure for each group is listed in table 28. The China type had the largest within group diversity (*Hs* = 0.285 - 0.291) while the Cambod tea had the least diversity (*Hs* = 0.193 - 0.207) but moderate diversity was observed in Assam tea (*Hs* = 0.223 – 0.241).

Highly significant differences ($P < 0.001$) were detected among populations when the data were submitted to an analysis of molecular variance using WINAMOVA software ver.1.55 (Table 29). AMOVA (Table 29) revealed that the variance components among groups and among individual samples within groups were 29% (28.87) and 71% (71.13), respectively. Nevertheless, and in agreement with the G_{ST} result, 71.13% of the total diversity was attributed to variation among individuals within the populations and only 28.87% to population differences.

AMOVA (table 29) revealed that the variance components among groups and among individual samples within groups were 3.535 (31.07%) and 7.763 (68.93%), respectively in case of RAPD markers and 3.227(28.87%) and 7.876 (71.13%) in case of ISSR markers, respectively. The average diversity within populations (*Hs*) was 0.27 and the total diversity (H_T) accounted to 0.38 (Table 26). The mean level of genetic differentiation (G_{ST}) between populations over all loci was 0.25, which indicates that only a low (25%) proportion of diversity is observed between populations as compared with diversity within populations (75%). The markers contributed differentially to the observed degree of population differentiation, varying from a low differentiation of 18% for OPA-1 to a high of 32% for OPA-19 primer and ISSR17 as high as 31% and low for ISSR13 only 21%.

The coefficient of gene differentiation, G_{ST}, an analogue of F_{ST}, was 0.25 for the variety variability assessed from the both RAPD and ISSR markers (Table 26). The gene diversities among the subpopulations (Hs) were 0.27 from both the ISSR and RAPD markers. The same in the total population (H_T) was 0.38 when the pooled data from both marker systems were used for analysis. The gene flow (Nm) between China and Assam varieties was 0.878 and 0.673 between China and Cambod and 0.743 between Cambod and Assam tea (Table 27). The highest population heterozygosity (H_T) was observed between China and Assam tea 0.245. Similarly, the second highest H_T was observed in cases where Assam and Cambod pairs 0.233 (Table 27).

Table 25. Genetic diversity within tea populations estimated by Nei's (1973) diversity measure for 12 RAPD and 7 ISSR markers.

Markers	China type	Cambod type	Assam type
OPA-1	0.129	0.189	0.177
OPA-4	0.209	0.177	0.276
OPA-9	0.309	0.211	0.286
OPA-11	0.254	0.267	0.294
OPA-12	0.367	0.315	0.303
OPA-13	0.421	0.139	0.288
OPA-16	0.361	0.305	0.346
OPA-18	0.272	0.293	0.376
OPA-19	0.349	0.288	0.322
L5	0.312	0.271	0.342
OPB-10	0.267	0.316	0.331
OPB-20	0.377	0.289	0.344
ISSR13	0.405	0.275	0.287
ISSR14	0.352	0.286	0.324
ISSR17	0.347	0.247	0.303
ISSR18	0.435	0.231	0.321
ISSR814.1	0.385	0.247	0.297
UBC830	0.394	0.299	0.307
UBC825	0.387	0.243	0.302
Average *SD*	**0.33** *0.078*	**0.25** *0.050*	**0.30** *0.040*

Table 26. Apportionment of gene diversity index (Nei's 1973) within and between tea populations for 12 RAPD and 7 ISSR markers.

Markers	*Ht	*Hs	*Gst
OPA-1	0.278	0.227	0.186
OPA-4	0.381	0.245	0.189
OPA-9	0.309	0.283	0.211
OPA-11	0.254	0.298	0.199
OPA-12	0.367	0.211	0.251
OPA-13	0.379	0.178	0.254
OPA-16	0.361	0.267	0.217
OPA-18	0.483	0.293	0.232
OPA-19	0.497	0.367	0. 321
L5	0.367	0.364	0.308
OPB-10	0.338	0.297	0.271
OPB-20	0.388	0.237	0.254
ISSR13	0.304	0.286	0.211
ISSR14	0.453	0.286	0.233
ISSR17	0.377	0.247	0.318
ISSR18	0.342	0.271	0.277
ISSR814.1	0.476	0.247	0.305
UBC830	0.456	0.283	0.301
UBC825	0.423	0.266	0.299
Average *SD*	**0.38** *0.069*	**0.27** *0.045*	**0.25** *0.043*

**Ht:* Genetic diversity over all groups; **Hs*: Genetic diversity within populations; **Gst*: Coefficient of gene differentiation (*i.e.* proportion of genetic diversity between populations).

Table 27. Gene differentiation coefficients and genetic diversity in the populations of three tea varieties.

Varieties		*C. sinensis* var. *sinensis*	*C. sinensis* var. *assamica*	*C. sinensis* spp. *lasiocalyx*
C.sinensis var.*sinensis*	H_t		0.245	0.221
	H_S		0.231	0.213
	D_{ST}		0.098	0.048
	G_{ST}	****	0.329	0.285
	N_m		0.878	0.673
C.sinensis var.*assamica*	H_t	0.118		0.233
	H_S	0.144	****	0.209
	D_{ST}	0.063		0.041
	G_{ST}	0.223		0.253
	N_m	0.838		0.743
C.sinensis spp.*lasiocalyx*	H_t	0.205	0.234	
	H_S	0.211	0.213	****
	D_{ST}	0.052	0.046	
	G_{ST}	0.259	0.247	
	N_m	0.691	0.769	

N_m = estimate of gene flow from G_{ST}. *e.g.*, $N_m = 0.5(1 - G_{ST})/G_{ST}$. H_t = Total genetic diversity (heterozygosity) over all groups; H_s = genetic diversity within population and D_{ST} = heterozygosity between subpopulations and G_{ST} = proportion of genetic diversity between populations or (coefficient of gene differentiation).

Table 28. Gene diversity (H_S) within China, Assam and Cambod type tea clones.

	China tea	Assam tea	Cambod tea
RAPD	0.285	0.241	0.207
ISSR	0.291	0.223	0.193

Table 29. Results of analysis of molecular variance (AMOVA) of 21 tea genotypes sampled from three tea populations. Statistics include degree of freedom (d.f.), mean squared deviations (MSDs), variance component estimates, the probability (*P*) of obtaining a more extreme component estimate by chance alone after 1,000 permutations and the percentage of total variance contributed by each component.

Sources of variation	RAPD					ISSR				
	df	MSD	Variance comp-onent	% of total variance	P-value	df	MSD	Variance comp-onent	% of total variance	P-value
Among groups	2	27.52	3.535	31.07	<0.001	2	28.11	3.227	28.87	<0.001
Among individuals within groups	18	7.76	7.763	68.93	<0.001	18	7.87	7.876	71.13	<0.001

4.3. Cloning and sequencing of chitinase gene fragment of tea

4.3.1. Primer designing

The chitinase gene specific primer pair (forward and reverse) was designed from the available gene sequences of Arabidopsis from the GenBank. The primer pair was constructed from the known chitinase gene sequence of *Arabidopsis* (Accession no. M38240), using Primer 3 program, which was designed by Steve Rozen and Whitehead Institute for Biomedical Research. The primer sequences was as follows, forward sequence 5'-TTTTCGTCGGAAAATGGAAG-3' and reverse sequence was 5'-ACCAGCTTCTTCTCGTCCAA-3'. The same primer also was used in chitinase gene specific transcript accumulation in reverse transcriptase-polymerase chain reaction (RT-PCR reaction).
(http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi,
http://www.basic.nwu.edu/biotools/Primer3.html).

Degenerate oligonucleotide primer (DOP primer) has been designed from the chitinase protein sequence information of 5 plant species, Solanum, Rice, Coffee, *Arabidopsis*, and Brassica using CODEHOP program (CODEHOP Version 10/14/04.1, Fred Hutchinson Cancer Research Center, Seattle, WA, USA). Chitinase protein sequences of the five plant species first aligned by ClustalW v.1.83 (Figure 7) multiple sequence alignment algorithm as given in figure 7, then made the block map and lastly run in CODEHOP program for degenerate oligonucleotide primer construction. Result showed single DOP-primer that was used for the quantification of chitinase gene mRNA in RT-PCR reaction.

4.3.2. Result of CODEHOP run for DOP-primer construction

Forward DOP-primer sequence was 5'-CTGCTGCGGCAAGTGGkgytggwrytg-3' and reverse primer was 5'-CAGTAGCAGCAGCAGTTGCArywccarcmsma-3'. For the good out put the following parameters were considered during CODEHOP run: Amino acids PSSM calculated with odds ratios normalized to 100 and back-translated with Standard genetic code and codon usage table "../docs/equal.codon.use" Maximum core degeneracy 256, Core strictness 0.25, Clamp strictness 1.00, Target clamp temperature 60°C, DNA Concentration 40 nM, Salt Concentration 50 mM, and Codon boundary 1.

IUPAC codes: M= A/C, R= A/G, W =A/Y, Y = C/T, S= C/G, K = G/T, H =A/C/T, V= A/C/G, D= A/G/T, B= C/G/T, and N= A/C/G/T.

CLUSTALW (v.1.83) multiple sequence alignment:

```
Brassica    ----------MKTYLLLLLIFSLLLSFSSGEQCGSQSIPEGALCPNGLCCSEAGWCGT 48
Arabidopsis ----------MKTNLFLFLIFSLLLSLSSAEQCGRQAG--GALCPNGLCCSEFGWCGN 46
Solanum     ---------MRLSEFTTLFLLFSVLLLSASAEQCGSQAG--GALCASGLCCSKFGWCGN 48
Rice        MSTPRAAASLAKKAALVALAVLAAALATAARAEQCGAQAG--GARCPNCLCCSRWGWCGT 58
coffea      ----------MTACLRPLFLAIISLLMISSLTRSSEGAG-------IAVYWGQNGNEGS 42
                         :   : : :     :

Brassica    TEAYCGHGCQSQCNPGPYPPPPTPQCGRQSIPAGALCPNGLCCSEAGWCGTTEAYCGHGC 108
Arabidopsis  TEPYCKQ----------------------------P-------------------GC 56
Solanum      TNEYCGPGN-------------------------------------------------C 58
Rice        TSDFCGDG---------------------------------------------------C 67
Coffea       LEEACRSG---------------------------------------------------Y 51


Brassica    QSQCTPIPTPPAPTPTPPTPTPPSPTPPGPTPPGPSGDLSGIISRDQFYKMLKHMNDNDC 168
Arabidopsis  QSQCT--------------------PGGTPPGPTGDLSGIISSSQFDDMLKHRNDAAC 94
Solanum      QSQCPGG----------------------PGPSGDLGGVISNSMFDQMLNHRNDNAC 93
Rice        QSQCSGCGPT---------------PTPTPPSPSDGVGSIVPRDLFERLLLHRNDGAC 110
Coffea       YDYVNIAFLVS---------------FGSGQTPELNLAGHCIPSPCTFLSSQIEVCQS 94
                                       :     : : :

Brassica    HAVG-FFTYDAFITAAKSFPSFGNTGDLAMRKKEIAAFFGQTSHETTGGWSGAPDGANTW 227
Arabidopsis  PARG-FYTYNAFITAAKSFPGFGTTGDTATRKKEVAAFFGQTSHETTGGWATAPDGPYSW 153
Solanum      QGKNNFYSYNAFVTAAGSFPGFGTTGDITARKREIAAFLAQTSHETTGGWPTAPDGPYAW 153
Rice        PARG-FYTYEAFLAAAAAFPAFGGTGNTETRKREVAAFLGQTSHETTGGWPTAPDGPFSW 169
Coffea       LGIKVLLSLGGGGAGAGRGPILASPEDARGVAAYLWNNYLGGQSDSRPLGAAVLDG---I 151
             :: :'*  : :   :    ::

Brassica    GYCYKEEIDKSDPHCDSNNLEWPCAPGKFYYGRGPMMLSWNYNYGPCGRD-----LGLEL 282
Arabidopsis  GYCFKQEQNPASDYCEP-SATWPCASGKRYYGRGPMQLSWNYNYGLCGRA-----IGVDL 207
Solanum      GYCFLREQGSPGDYCTP-SSQWPCAPGRKYFGRGPIQISHNYNYGPCGRA-----IGVDL 207
Rice        GYCFKQEQNPPSDYCQP-SPEWPCAPGRKYYGRGPIQLSFNFNYGPAGRA-----IGVDL 223
Coffea       DFDIEYGSNLYWDDLARALSGYSTAERKVYLSAAPQCFFPDYYLDVAIRTGLFDFVWVQF 211
          :          :  :    : ::      : :::

Brassica    LKNPDVASSDPVIAFKTAIWFWMTPQAPKPSCHDVITDQWEPSAADISAGRLPGYGVITN 342
Arabidopsis  LNNPDLVANDAVIAFKAAIWFWMTAQPPKPSCHAVIAGQWQPSDADRAAGRLPGYGVITN 267
Solanum      LNNPDLVATDPVISFKSAIWFWMTPQSPKPSCHDVITGRWQPSGADQAANRVPGFGVITN 267
Rice        LSNPDLVATDATVSFKTALWFWMTPQGNKPSSHDVITGRWAPSPADAAAGRAPGYGVITN 283
Coffea       YNNPPCQYGTSTGNADNLLNSWSNDWAPHPGVNKLFLGLPAAPEAAPSGGYIPPEVLINQ 271
                    :   : : ::     :   : :

Brassica    IINGGLECAGRDVAKVQDRISFYTRYCGMFGVDPGSNIDCDNQRPFNEGSNVFLDAAI 400
Arabidopsis  IINGGLECGRGQDGRVADRIGFYQRYCNIFGVNPGGNLDCYNQRSFVNG---LLEAAI 322
Solanum      IINGGLECGHGSDSRVQDRIGFYRRYCGILGVSPGENLDCGNQRSFGNGLLVDIM--- 322
Rice        IVNGGLECGHGPDDRVANRIGFYQRYCGAFGIGTGGNLDCYNQRPFNSGSSVGLAEQ- 340
Coffea       ILP--VVQSYPKYGGVMLWSRFYDRNYSPAIIRPYVNGDPLTYTTKSVKKSHAVA--- 324
          : :                 :     :
```

9 distinct blocks in 5 sequences

```
Brassica    (408) -AAA-BB---------CCCDDDDDEEFFF-GGGGGGHIIII
Arabidopsis (408) -AAA-BB---------CCCDDDDDEEFFF-GGGGGGHIIII
Solanum     (408) -AAA-BB---------CCCDDDDDEEFFF-GGGGGGHIIII
Rice        (408) -AAA-BB---------CCCDDDDDEEFFF-GGGGGGHIIII
Coffea      (408) -AAA-BB---------CCCDDDDDEEFFF-GGGGGGHIIII
                  ---- 40 amino acids
```

Figure 7. Chitinase protein sequences of five plants were aligned through ClustalW multiple sequence alignment program to make specific block and then the block was used in CODEHOP program for the generation of DOP-primer.

4.3.3. Primer for caffeine synthase gene amplification

The caffeine synthase gene specific primer was designed from the GenBank database of caffeine synthase gene (cDNA) of *Camellia sinensis* containing 1438bp coding sequence (GenBank Accession no. gi|9967142|dbj|AB031280.1|) by using the Primer3 program. The sequence of the forward caffeine synthase gene specific primer (TCS1) was 5'-TCTTCAAAGGCCTGTCGTCT-3' and a reverse (TCS2) primer was 5'-TCCCCTTGTTTAATGCCAAG-3', which was used for caffeine synthase gene specific amplification in PCR reaction.

4.3.4. Analysis of chitinase gene specific PCR amplification product

Chitinase gene specific PCR amplified product was fractionated in 1% agarose gel electrophoresis after completion of 35 cycles in thermal cycler and visualized the PCR products on UV-transilluminator and photographed. Chitinase gene specific PCR product of about 201 bp was observed on the gel (Plate 7, fig. B). A single distinct DNA band of about 201 bp was observed in lane 1, which was chitinase gene specific DNA band amplified by chitinase specific primer from the genomic DNA of tea clone T383 (Plate 7, fig. B). Lane-2 in the gel was indicating the low range DNA ruler as marker (Bangalore Genei) and lane-3, genomic DNA of tea clone T383. The PCR product was purified according to the protocol of section 3.3.7 of material and methods and used in TA cloning procedure. The TA cloning vector pGEM-T *Easy* of Promega was utilized in the present PCR cloning system because it is most suitable for PCR product cloning (Plate 7, fig. A & C.). Since Taq DNA polymerase has a non-template dependent terminal transferase activity, which adds a single 3′ deoxyadenosine (A) to the ends of PCR products, this linearized pGEM-T *Easy* vector has a single overhanging 3′ deoxythymidine residue (T), which allows for increased ligation efficiency with the vector (Plate 7, fig. A & C). It is always prefer to use new PCR product during cloning otherwise the terminal 3′ deoxyadenosine residue has a tendency to fall off over time (from 3-4 days old PCR product). The cloning efficiency was ranging between 60-80 clones per plate (Plate 7, fig. D). The optimal ratio of PCR/vector is 3:1. The pGEM-T *Easy* vector is 3015 bp in size. PCR amplified chitinase gene specific DNA fragment of about 201 bp was cloned into pGEM-T *Easy* vector in *E. coli* host strain JM109. Transformed cells are selected on LB-ampicillin medium with chromogenic substrate X-gal and IPTG (isopropyl-β-D-thiogalactoside, inducer of LacZ gene). Blue colonies were with non-recombinant

vector without PCR insert but white colonies were with recombinant vector with PCR insert (Plate 7, fig. D.). Blue colonies were produced because the intact LacZ gene on the plasmid synthesized active β-galactosidase enzyme, which converted the colourless chromogenic substrate, X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactoside) to blue indolyl compound and helped to distinguish the recombinant vector from non-recombinants producing blue colour.

4.3.5. Purification of recombinant pGEM-T *Easy* plasmid for sequencing the insert

White colonies were picked from the LB-ampicillin agar plate (plus X-gal and IPTG) with sterilized toothpick and added in a 1.5 ml eppendorf tube containing LB medium with ampicillin only for the multiplication of the plasmid copy number including the DNA insert. The cultured was kept overnight at 37°C with 200 rpm agitation for adequate aeration. Then the plasmid vector was purified from the host cell according to the description of material and methods (3.4.5.).

4.3.6. Sequencing of 201 bp chitinase gene specific PCR product

The purified recombinant pGEM-T Easy vector with insert (chitinase gene fragment of 201 bp) was sequenced bi-directionally using the T7 and SP6 vector specific primer by Big Dye Terminators method (Applied Biosystems by Bangalore Genei, India). The partial electropherogram of the chitinase gene sequence was represented in figure E of plate 7.

4.3.7. DNA sequence information of 201 bp of chitinase gene

The 201 bp DNA sequence information was analyzed through the sequence alignment software of BLAST algorithm. The 201 bp chitinase gene specific sequence was deposited to the GenBank of NCBI (Accession no.EF673751). The 201 bp sequence was as follows- tttttcgtcg gaaaatggaa gctacacatt ggcttgatcg ttggaaaagt cgtggacaag ctggaggtgg tgcaaattac attgggttgt ttgggtttaa tgaagattca tccgagctag cagaattttc attagcagat ctgcaaactg cgacttgcaa tttctcggag agcttcaaga ttggacgaga agaagctggt a.

4.4. PCR amplified product of caffeine synthase gene

4.4.1. Caffeine synthase gene of tea was amplified by PCR reaction using gene specific 20-mer primer. The 200 bp product was observed on the 1% agarose gel in case of tea clone T383 and 202 bp in case of tea clone HV39. PCR product of 200 bp

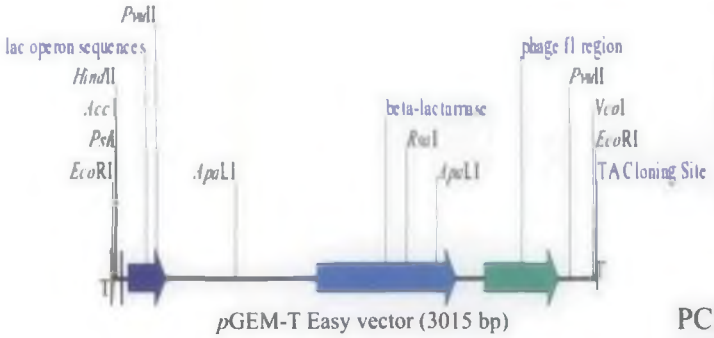


Fig. A. Structural features of *p*GEM-T Easy vector in linear state. PCR product with 3′ overhang of A-residue can easily be ligated into this vector in presence of T4 DNA ligase.

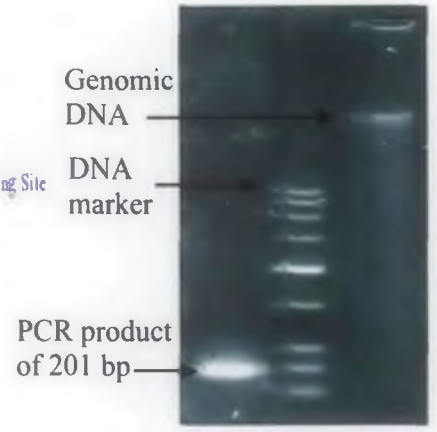


Fig.B. Chitinase gene specific 201 bp PCR product of tea clone T383 has been fractionated in 1% agarose gel electrophoresis.

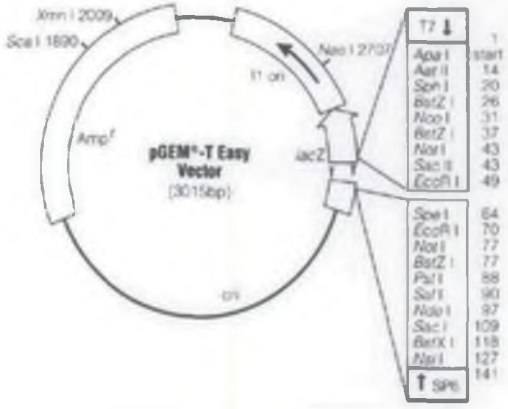


Fig. C. pGEM-T Easy vector circularized after ligation with 201 bp chitinase DNA of tea clone T383.

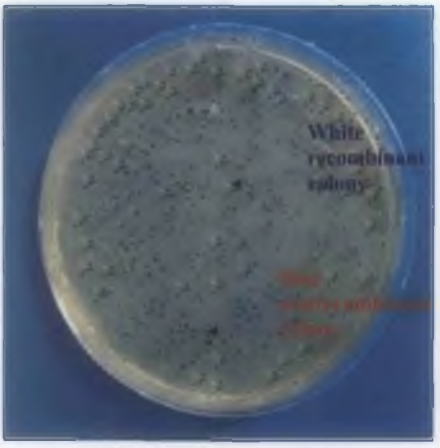


Fig. D. Transformed *E. coli* host JM109 with pGEM-T Easy vector was selected on X-gal and IPTG medium. White colonies were with recombinant vector.

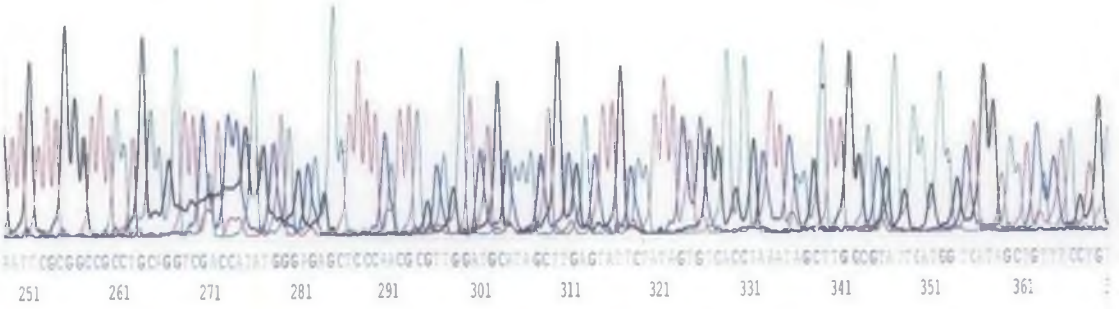


Fig. E. Electropherogram showing the partial DNA sequence of chitinase gene (201 bp) of tea clone T383.

Plate 7 [A-E]: Chitinase gene of tea clone T383 (201 bp) was cloned in T/A cloning (pGEM-T Easy vector) vector and sequenced.

and 202 bp were purified according to Sambrook and Russell (2001) (section 3.3.7. of material and methods) and sequenced by single pass analysis. DNA sequencing reactions were carried out with an automated DNA sequencer (ABI Prism 377, PE-Applied Biosystems) using Big Dye terminator kit (at the Bangalore Genei, Bangalore, India).

Nucleotide sequence comparison was performed using BLAST program. Default setting was used with these programs. The BLAST program was run for the processing of primary data sequences and annotation. Nearly exact short sequence similarity search of 202bp of HV39 was carried out by using BLAST program with the GenBank database and showed exact short sequence matching of 17bp (123-GACGACAGGCCTTTGAA-139) at the region of 123bp to 139bp with the 1438bp caffeine synthase gene (cDNA) coding sequences (cds) of the GenBank accession no. gi|9967142|dbj|AB031280.1| around the region at 442bp to 426bp (Figure 8-A). This 17 bp (GACGACAGGCCTTTGAA) nucleotide was considered as a unique sequence of HV39 clone. The 17 bp unique sequence also matched with another partial cDNA coding sequence of 716 bp of caffeine synthase gene of the GenBank database containing accession no. gi|46242825|gb|AY570929.1|(Figure 8-B). Consequently the 103bp to 125bp (GACGACGGGCCTTTGAAGAGGGT) of 200bp sequence of T383 clone matched with GenBank database. While the nucleotide sequences of 202bp of HV39 and 200bp of T383 was aligned with the help of BLAST 2 sequence program, it was given 85% similarity of 200bp with plus/plus strand shown in figure 8-E. The gene finder analysis program, GRAIL was run for the identification of genes, exons, and other features in DNA sequences of 202 bp of HV39 (GenBank accession no. AY599069). It was given protein translation features with partial Open Reading Frame (ORF) at the region at 78 bp to 152 bp, which encoded 25 amino acids containing truncated protein (AASPALSLLDHGPFHTDDRPLNNFH) (Figure 8-C). A different result was observed while ran for 200 bp DNA sequence of T383 (GenBank accession no.AY601112). It was given partial Open Reading Frame (ORF), which has encoded 24 amino acids (WEFFKGRSAVFKGPWSSDKPGEAA) containing truncated protein at the region at 52 bp to 126 bp (Figure 8-D).

A)

```
Query: 123 gacgacaggcctttgaa 139
           ||||||||||||||||
Sbjct: 442 gacgacaggcctttgaa 426
```

B)

```
Query: 1   gacgacaggcctttgaa 17
           ||||||||||||||||
Sbjct: 418 gacgacaggcctttgaa 402
```

C) .Result of GRAIL analysis, Exon and feature list of 202 bp (gi|46411104|gb|AY599069.1|)

Position	Str	Fr	ORF	Qlt
Protein translation				
75 - 150	F	2	78 - 152	0.80E
Amino acid sequences (25 in Numbers): AASPALSLLDHGPFHTDDRPLNNFH				

D) Result of GRAIL analysis, Exon and feature list of 200bp (gi|46561812|gb|AY601112.1|)

Position	Str	Fr	ORF	Qlt
Protein translation				
51 – 101	R	0	52 - 126	0.73G
Amino acid sequences (24 in numbers): WEFFKGRSAVFKGPWSSDKPGEAA				

E)

```
Query: 1   atnccaggcctttgaagaagatncagccacccngaccccgacggaaaaaacaaagccaac 60
           |||||||||||||| |||||||||||| |||||||| |||||||| | || ||||| ||
Sbjct: 1   atnccaggcctttgnagaagatncagccncccngaccncgacggaananacnaagccnac 60

Query: 61  gtgnccgatttgtgttaagccgcctcccggggtttgtcgttggacaacgggcctttgaaa 120
           |||| ||| ||||| |||||||||| | |||||||||| | ||| ||||||||||||
Sbjct: 61  gtgnncganttgtgntaagccgcctcncnggGtttgtcgntngacnacgggcctttgaan 120

Query: 121 agggccgacgggcctttgaaaaattnccattaattttcgaagacnggatcgtccgtcgtt 180
           | || |||| |||||||||||||| || |||||||||| ||| | | ||||||||||||||
Sbjct: 121 anggncgacnggcctttgaaaaantnncattaattttngaanannngatcgtccgtcgtt 180

Query: 181 ccccgctnttgancacaccc 200
           ||| ||||| |||| || ||
Sbjct: 181 cccngctntngancncancc 200
```

Figure 8, (A-E). A) Search result of 202bp for short, nearly exact matches in the GenBank Databases using BLAST program. Identity was 17/17 (100%) with the mRNA of caffeine synthase gene of *Camellia sinensis* (gi|9967142|dbj|AB031280.1|). B) Search result of 200bp for short, nearly exact matches in the GenBank Databases using BLAST program matching with mRNA of Caffeine synthase gene of Camellia sinensis with 17/17 (100%), (gi|46242825|gb|AY570929.1|). C). Result of GRAIL of 202 bp, D). Result of GRAIL of 200 bp, E). Result of the alignment of two sequences of 202 bp of HV39 with 200 bp of T383 using Blast 2 sequence program (version Blastn 2.2.6.). Identities = 171/200 (85%).

4.5. Growth pattern of callus tissues in different culture media.

The calluses were weighed by an electric single pan balance in aseptic condition. Initial weight was recorded at the time of inoculation. Final weight was taken after 8 weeks. Weighing was done at a regular interval of two weeks. Growth index is expressed as the ratio of increase in fresh weight of the callus to the initial fresh weight of the callus. Each treatment has 5 replicates and repeated twice.

The growth index (GI) of the investigated materials was calculated as

$$GI = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}}$$

The stem part of 5-8 mm from the young shoot of four tea clones (T383, TV30, HV39 and UPASI-26) were used as explants for callus initiation. The each stem part was inoculated into two media MS and B5 for callus initiation (Figure 9, A & B and Plate 8, figs. I-P). The results are compiled in tables 30, 31, 32 and 33. From the growth index study (Table 31, Figure 9, A) it was evident that the optimal callus growth of tea plants has been on MS medium and the minimal growth has been in B5 medium. The growth index studies of callus tissues also suggest the suitability of MS medium for better callus growth. The clone T383 has shown the better response in callus growth and regeneration in the media tested. Hence, clonal cultivar T383 had been used for all sorts of investigation. The growth and development of callus was better in the genotype of T383 on both MS and B5 media. The growth pattern of callus from each of the four materials was recorded on two different media, MS and B5. Though all the tea clones TV30, T383, HV39 and UPASI-26, readily grew on MS and B5 medium and initiated callus formation. Growth index was 1.48 after 8 weeks of culture in MS but 1.00 in case of B5 medium of the genotype T383. Other three genotypes were given good growth of callus but less than that of T383 genotypes.

4.5.1 Effect of hormones, and organic additives on the growth of callus

Calluses were derived from Tea clones TV30, T383, UPASI-26 and HV39. The present investigation was directed at elucidating the effect of incorporating different growth substances, vitamins and organic additives alone or in combinations into the culture medium. From the growth index study (Table 32, 33 & 34) it was evident that the calluses of T383 grow well on the medium supplemented with 2,4-D (4 mg/l) and

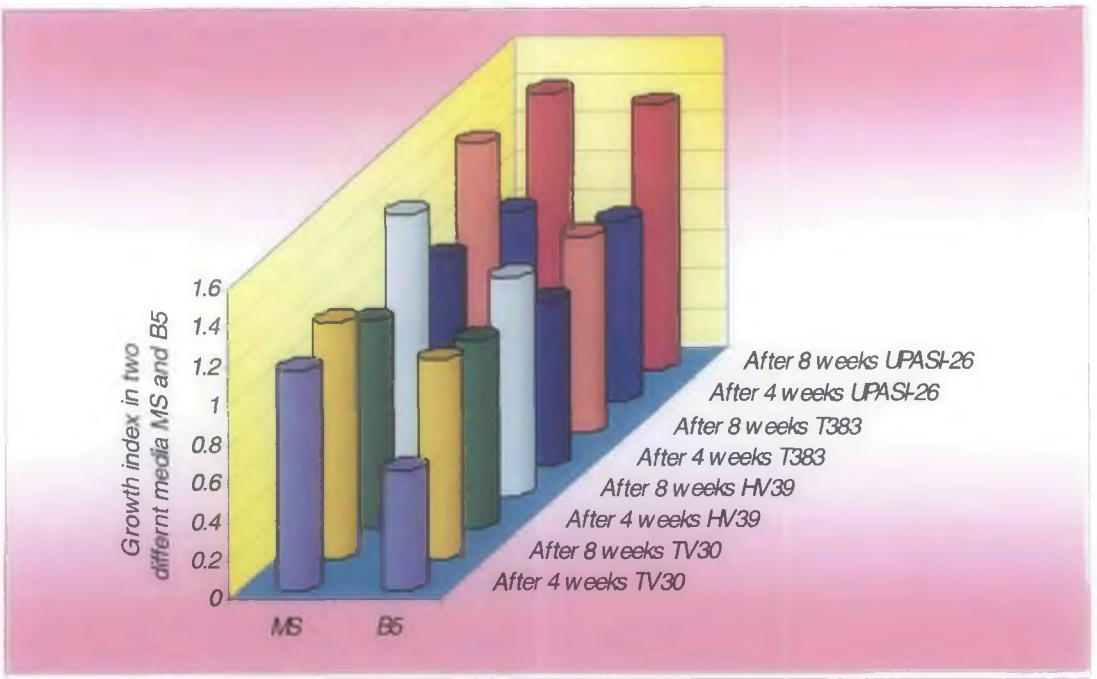


Fig. A: Growth index (GI) of material T383, HV39, TV30 and UPASI-26 on different media

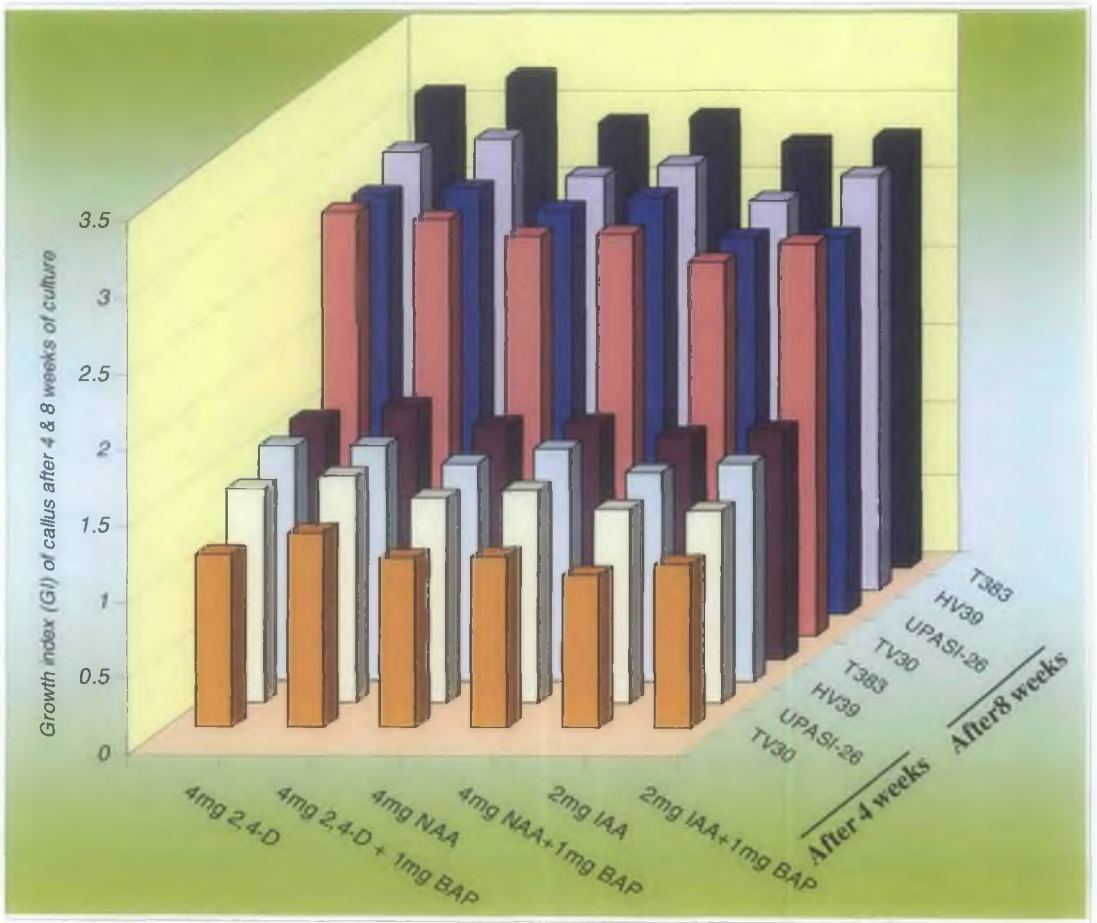


Fig. B: Growth index (GI) of 4 materials on different hormonal composition in MS medium after 4 and 8 weeks of initiation.

Fig. 9[A & B]: Growth index (GI) of 4 materials on different hormonal medium.

BAP (1 mg/l) (Figure 9, B.). The comparative study of the growth rates reveals that the growth rate of calluses on the medium containing IAA (2 mg/l) and BAP (1 mg/l) was less in all the genotypes. The organic additive such as coconut milk (CM) was used in the present investigation. The rate of callusing was different in different concentration of coconut milk (CM) (Figure 10, A.). The effect of the CM in callus growth and development was comparable with the medium containing different growth hormones. The growth index 2.94 was achieved in tea clone T383 in medium containing 15% coconut milk (Table 35). Callus growths of four tea clones in 15% coconut milk were better after 4 weeks (Plate 8, figs. I-L, for TV30, HV39, UPASI-26 and T383 respectively) and after 8 weeks of culture, calluses were increased in diameter and showed different colouration and the fresh weight was also taken for GI calculation (Plate 8, figs, M-P, for TV30, HV39, UPASI-26 and T383 respectively). The callus growths of four tea clones were tabulated in tables 32, 33 and 34 after 4 and 8 weeks of cultute initiation in different combination of plant growth hormones.

4.5.2. Effect of auxin and cytokinin on callus growth.

To observe the effect of auxins (IAA, NAA, 2,4-D) on the growth of the calluses all the four tea clones were investigated. The callus was inoculated in the medium containing different concentrations of IAA, NAA and 2,4-D. Callus growth was optimum while 2mg/l IAA, 4mg/l NAA and 4mg/l 2,4-D were used alone or in combination (Table 36). The effect of 2,4-D on callus growth and development was remarkable. Callus growth was very fast in the medium containing 4 mg/l 2,4-D in case of tea tissue culture (Figure 10, B). For studying the effect of cytokinins, kinetin and TDZ for the callus growth and regeneration were tested. The callus showed different growth rates in different levels of cytokinins used (Figure 10, C). The optimum callus growth was observed in the medium containing 4 mg/l Kn. Shooting formation was achieved in high rate in the medium containing 3 mg/l TDZ and 4 mg/l BAP (Table 37). Percentage of shoot formation was highest 74% in medium with TDZ (3mg/l) (Table 37). After 4 weeks and 8 weeks of culture days of incubation the calluses were weighed and recorded. Growth index was 3.68 after 8 weeks in 3 mg/l TDZ + 2 mg/l NAA and 2.91 in 4 mg/l BAP with 2 mg/l NAA. Shoot regeneration was obtained from the callus of T383 using 3 mg/l TDZ and 2 mg/l BAP (Table 37, Plate 8, figs. A-F).

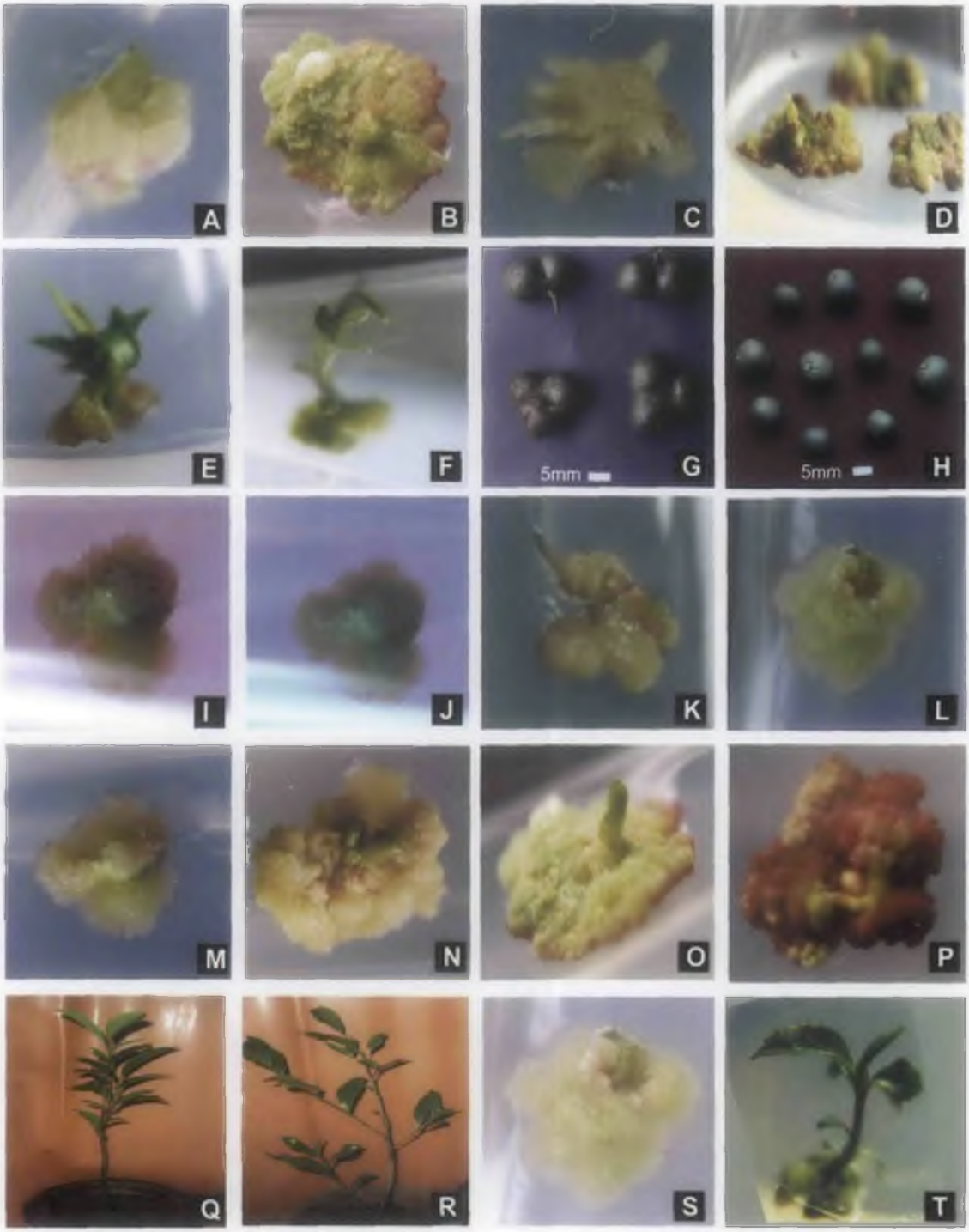


Plate 8 [Figs. A-T]: (A-F) Plantlet regenerated from callus culture of T383; (G) Mature tea fruits collected in November; (H) Tea seeds; (I-L) Four weeks old callus in T383, TV30, UPASI-26 and HV39 respectively was initiated from stem explants; (M-P) Callus growth after eight weeks of culture initiation in T78, TV30, UPASI-26 and HV 39 respectively; (Q-T) Young tea plant, callus and shoot were induced with methyl jasmonate (inducer), Q for young leaf, R for mature leaf, S for two months old callus and T for six months old shoot regenerated from somatic embryo of T383.

In different concentration of IAA, the callus growth of tea clone T383 showed a gradual increase followed by gradual decrease. The growth reaches its highest peak at 2 mg/l with gradual fall. The findings have been presented in table 36 and in figure 10, B. However, the callus growth of the tea clone T383 on Kn containing medium showed its highest growth rate when 4 mg/l of Kn was used. The growth rate decreases in higher concentrations of Kn (Figure 10, C).

4.5.3. Effect of coconut milk on callus growth

Addition of 15% coconut milk in the MS basal medium enhanced the callus growth and development in tea clone T383 (Table 35). The remarkable growth stimulating property of coconut milk had led to attempts to isolate and identify the active principles. Coconut milk contains all the necessary organic additives required for callus growth and development.

Table 30. Growth of callus of tea clones T383, HV39, TV30 and UPASI-26 on MS and B5 medium

Media	Initial fresh weight* of the callus (in gm)		Fresh Wt.* (gm) after 4 weeks		Fresh wt.* (gm) after 8 weeks	
	T383	**HV39**	**T383**	**HV39**	**T383**	**HV39**
MS	0.112±0.17	0.112±0.11	0.249±0.81	0.231±0.15	0.278±0.31	0.267±0.13
B5	0.116±0.24	0.108±0.09	0.213±0.17	0.212±0.17	0.233±0.04	0.230±0.24
Media	**TV30**	**UPASI-26**	**TV30**	**UPASI-26**	**TV30**	**UPASI-26**
MS	0.109±0.02	0.106±0.20	0.234±0.12	0.209±0.14	0.342±0.09	0.256±0.54
B5	0.116±0.12	0.114±0.17	0.118±0.21	0.219±0.06	0.236±0.07	0.269±0.28

* Means of 5 samples.

Table 31. Growth index (GI) of material T383, HV39, TV30 and UPASI-26 on different media

Media	After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks	After 4 weeks	After 8 weeks
	T383	T383	HV39	HV39	TV30	TV30	UPASI-26	UPASI-26
MS	1.14	1.22	1.06	1.44	1.07	1.48	0.97	1.41
B5	0.62	1.03	0.96	1.12	0.83	1.00	0.92	1.35

4.5.4. Plantlet regeneration from callus

Well developed shoot was transferred to ½ MS medium with 1 mg/l IBA in filter paper bridge in liquid medium for rooting. Incubated for 6 weeks and then transferred to ½ MS basal medium for normal growth and development. Regenerated plantlets after 15-16 weeks old with well developed roots and leaves were transferred into the hardening chamber to acclimatize the plantlets before transfer to greenhouse.

Table 32. Effect of hormones on callus growth of material TV30 and HV39 (*Means of 5 samples).

Media + hormones mg/l	TV30			HV39		
	Initial fresh weight* (gm)	Fresh weight* after 4 weeks (gm)	Fresh weight* after 8 weeks (gm)	Initial fresh weight* (gm)	Fresh weight* after 4 weeks (gm)	Fresh weight* after 8 weeks (gm)
MS + 4mg 2,4-D	0.152±0.07	0.323±0.21	0.574±0.12	0.153±0.15	0.389±0.07	0.592±0.10
MS + 4mg 2,4-D+ Img BAP	0.154±0.15	0.351±0.33	0.573±0.23	0.155±0.07	0.396±0.31	0.612±0.13
MS + 4mg NAA	0.148±0.03	0.311±0.15	0.535±0.19	0.156±0.12	0.378±0.06	0.578±0.13
MS + 4mg NAA + 1mg BAP	0.150±0.13	0.317±0.11	0.545±0.22	0.151±0.01	0.381±0.19	0.570±0.27
MS + 2mg IAA	0.151±0.13	0.301±0.05	0.522±0.02	0.155±0.14	0.368±0.34	0.549±0.33
MS + 2mg IAA + Img BAP	0.148±0.08	0.306±0.27	0.528±0.44	0.153±0.05	0.371±0.23	0.567±0.11

Table 33. Effect of hormones on callus growth of material T383, and UPASI 26.

Media + hormones mg/l	T383			UPASI-26		
	Initial fresh weight* (gm)	Fresh weight* after 4 weeks (gm)	Fresh weight* after 8 weeks (gm)	Initial fresh weight* (gm)	Fresh weight* after 4 weeks (gm)	Fresh weight* after 8 weeks (gm)
MS + 4mg 2,4-D	0.152±0.07	0.393±0.12	0.621±0.20	0.153±0.15	0.369±0.30	0.572±0.17
MS + 4mg 2,4-D+ 1mg BAP	0.150 ±0.25	0.399±0.23	0.627±0.07	0.151±0.17	0.370±0.11	0.592±0.23
MS + 4mg NAA	0.151±0.15	0.384±0.23	0.587±0.11	0.152±0.02	0.358±0.16	0.553±0.07
MS + 4mg NAA + 1mg BAP	0.152±0.03	0.387±0.22	0.596±0.08	0.151±0.01	0.361±0.29	0.563±0.05
MS + 2mg IAA	0.151±0.13	0.374±0.23	0.568±0.19	0.153±0.21	0.348±0.17	0.529±0.08
MS + 2mg IAA + 1mg BAP	0.151±0.12	0.378±0.15	0.573±0.06	0.155±0.12	0.351±0.07	0.537±0.33

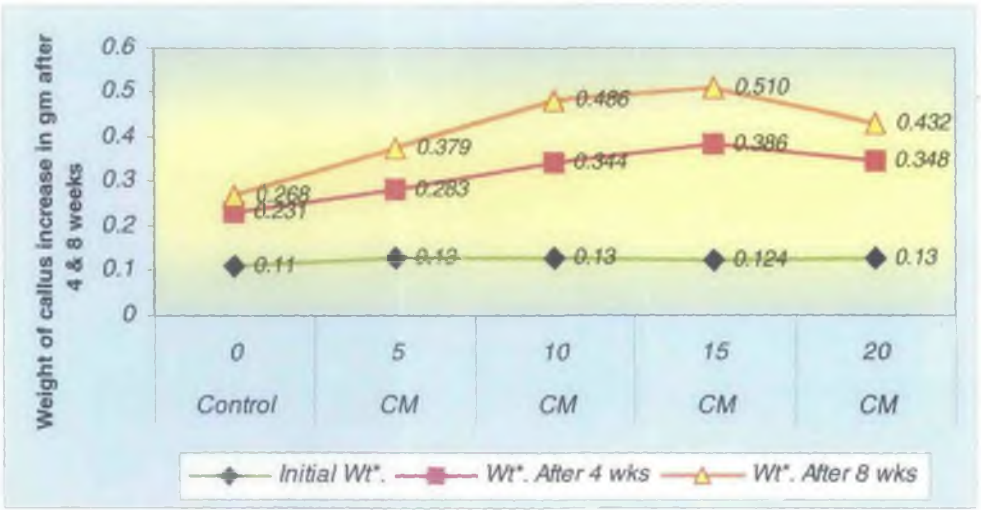


Fig. A: Effect of coconut milk (CM) as organic additives on growth and development of callus in *C. sinensis* clone T383 on MS medium.

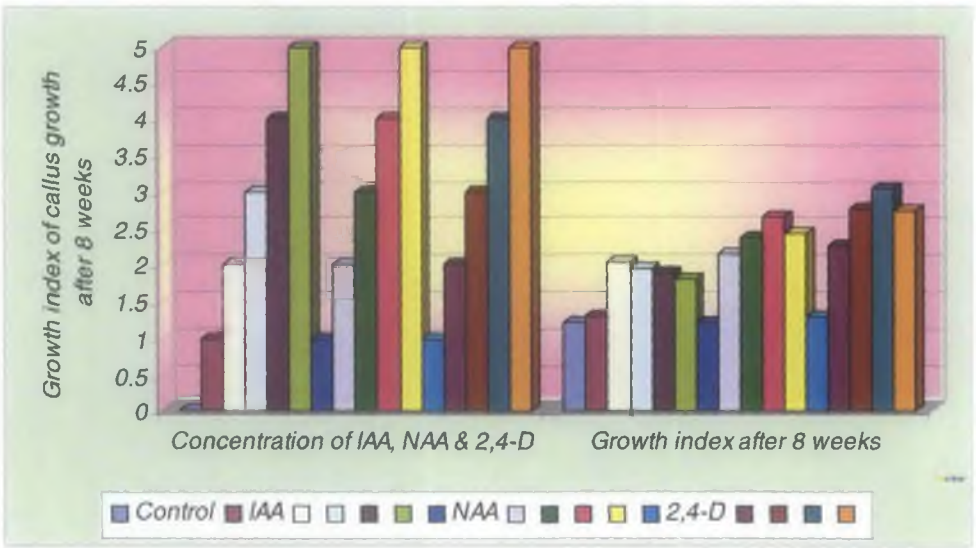


Fig. B: Effect of different concentration of growth regulators (IAA, NAA and 2,4-D) on callus growth and development of tea clone T383 on MS.

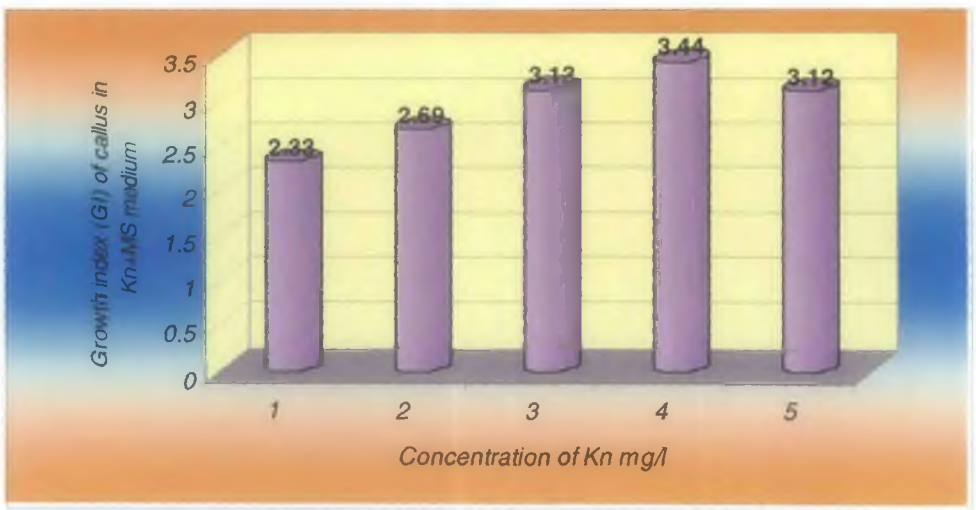


Fig. C: Effect of Kinetin on callus growth in tea clone T383.

Fig. 10 [A – C]: Callus growth and development of tea clone T383 has been studied in different combinations of plant growth hormones and coconut milk in MS medium.

Table 34. Growth index (GI) of materials on different hormonal medium

Media + hormones mg/l	GI. after 4 weeks				GI. after 8 weeks			
	TV30	UPASI-26	HV39	T383	TV30	UPASI-26	HV39	T383
MS + 4mg 2,4-D	1.13	1.41	1.54	1.58	2.77	2.73	2.86	3.08
MS + 4mg 2,4-D+1mg BAP	1.27	1.49	1.55	1.66	2.72	2.77	2.94	3.18
MS + 4mg NAA	1.10	1.35	1.42	1.54	2.61	2.63	2.70	2.88
MS + 4mg NAA + 1mg BAP	1.11	1.39	1.52	1.55	2.63	2.72	2.77	2.92
MS + 2mg IAA	0.99	1.27	1.37	1.47	2.45	2.45	2.54	2.76
MS + 2mg IAA + 1mg BAP	1.06	1.26	1.42	1.50	2.56	2.46	2.70	2.79

Table 35. Effect of coconut milk (CM) as organic additives on growth anddevelopment of callus in *C. sinensis* clone T383 on MS medium (* Means of 5 samples)

Treatment	Concen-tration (in % v/v)	Characteristics of callus				
		Initial Wt*. (gm)	Wt*. After 4 weeks	Wt*. After 8 weeks	Colour of Callus	Growth index after 8 weeks
Control	Nil	0.110	0.231±0.21	0.268±0.11	Light green	1.43
CM	5	0.130	0.283±0.23	0.379±0.25	Greenish	1.91
CM	10	0.130	0.344±0.19	0.486±0.22	Pinkish-red	2.73
CM	15	0.124	0.386±0.24	0.489±0.07	Redish-green	2.94
CM	20	0.130	0.348±0.06	0.432±0.15	Redish-yellow	2.32

Table 36. Effect of different concentration of growth regulators (IAA, NAA and 2,4 D) on callus growth and development of tea clone T383 on MS.

	Concentration (mg/l)	Initial Wt*. of callus (gm)	Wt*. After 4 weeks	Wt*. After 8 weeks	Growth index after 8 weeks
Control	0	0.120	0.234±0.05	0.266±0.31	1.21
IAA	1	0.120	0.246±0.22	0.277±0.25	1.30
	2	0.120	0.298±0.34	0.365±0.03	2.04
	3	0.120	0.247±0.14	0.355±0.45	1.95
	4	0.120	0.241±0.08	0.347±0.24	1.89
	5	0.120	0.236±0.33	0.338±0.27	1.81
NAA	1	0.120	0.231±0.21	0.268±0.11	1.23
	2	0.120	0.283±0.23	0.379±0.25	2.15
	3	0.120	0.344±0.19	0.406±0.22	2.38
	4	0.120	0.386±0.24	0.439±0.07	2.65
	5	0.120	0.348±0.06	0.412±0.25	2.43
2,4-D	1	0.120	0.239±0.27	0.277±0.28	1.30
	2	0.120	0.286±0.33	0.391±0.13	2.25
	3	0.120	0.351±0.08	0.453±0.25	2.77
	4	0.120	0.398±0.11	0.486±0.12	3.05
	5	0.120	0.379±0.35	0.450±0.44	2.75

* Means of 5 samples

Table 37. Effect of various combination of BAP, TDZ and NAA on growth, development and regeneration of shoot from callus in tea clone T383 on MS medium.

Hormone concentration (mg/l)		Initial weight* of callus (gm)	Wt*. After 8 weeks	Growth index	% of callus forming shoots
BAP	NAA				
1	2	0.125	0.353±0.38	1.82	34
2	2	0.125	0.399±0.43	2.19	39
3	2	0.125	0.463±0.70	2.70	47
4	2	0.125	0.489±0.54	2.91	65
5	2	0.125	0.465±0.82	2.72	53
TDZ	NAA				
1	2	0.125	0.413±0.48	2.30	45
2	2	0.125	0.523±0.63	3.13	66
3	2	0.125	0.586±0.43	3.68	74
4	2	0.125	0.563±0.44	3.50	68
5	2	0.125	0.445±0.56	2.56	50

* Means of 5 samples

Table 38. Effect of humidity on acclimatization and hardening in *Camellia sinensis* clone.

Initial humidity (%) at the time of transfer	Humidity % after 1 week	% of plant survival (four clones) , T383*
95	95	54±3.45
95	90	53±1.09
95	85	45±3.41
95	80	38±1.22
95	70	23±2.47
95	60	10±2.34
95	50	03±1.51

*Each treatment had 5 replicates and repeated twice.

4.6. Induction of somatic embryos

4.6.1. Somatic embryo development on immature cotyledons

The immature white cotyledon explants enlarged while in culture turned greenish in colour and developed translucent outgrowths. After 4 weeks of culture, somatic embryos began to appear on 10-15% of the explants. Then numerous primary somatic embryos developed directly from the adaxial surface of the cotyledons in 40% of the explants (Plate 9, figs, A, B, C). During the 4 weeks, various embryo

stages were observed: globular, heart, torpedo. When these structures enlarged they developed morphologically distinct somatic embryos without subculturing (Plate 9, figs. A, B, C & H). The immature cotyledonary tissues were on average more productive than that of mature cotyledons (Table 39.). The embryogenic competence was greatest on culture medium impregnated with plant growth regulators. The number of somatic embryos produced per embryogenic explant ranged from 3 to 45 (Table 39).

Seeds collected in November

Only 18 % of the mature cotyledonary tissues from seeds collected in November were embryogenic. The other 82 % enlarged slightly, and some of ` them were developed non-embryogenic callus (Plate 9, fig, G). Mature cotyledonary explant were green and swollen after two weeks in culture. Somatic embryos continually arose on cotyledon explants between weeks 5 and 6. The overall embryogenesis rates were afforded by medium with PBOA 1 mg/l + BAP 5 mg/l or PBOA 1 mg/l (18 %), on the contrary the lowest rates being obtained by medium with BAP 5 mg/l (3%) or 2,4-D 4 mg/l + IAA 1mg/l (13.28 %). The mean number of embryos per embryogenic explant varied greatly from 2 to 22 (Table 39).

4.6.2. Morphology of somatic embryos

Major types of somatic embryos produced were morphologically clearly distinct types. These were as follows.

i) 'Seed-like' embryos (Plate 9, fig. H), which were yellowish-white with large cotyledons, like those of the zygotic embryos. These turned green at maturity, and so far the most abandoned in most media. Two cotyledons were usually produced of different size and partially or wholly fused together. Sometimes also formed more than two cotyledons. They germinate into whole plants in MS medium with 1.5 mg/l GA_3 + IAA (1 mg/l) or MS + 2 mg/l Brassin (Table 40, Plate 9, figs. D & F).

ii) 'Cup-shaped' embryos, which were pale yellow to creamy-greenish in colour. These emerged as small and rounded structures. The embryo underwent a typical embryogenic pathway as evident cup-shaped structures. Bipolar nature rarely observed (Plate 9, fig. F).

iii) 'Bud-like' embryos (Plate 9, fig. A), which were green, with cotyledons like true leaves. They were bipolar nature developing whole plantlets with shoots and root poles, and generally occurred in media with relatively moderate concentrations of PBOA (1 mg/l) and Kin (5 mg/l). Germinated well in MS with 2 mg/l Brassin (Table 40).

4.6.3. Secondary embryogenesis and germination to plantlets

When somatic embryos were excised and transferred singly or in embryo-clusters on to embryo germination medium (EGM), either MS or B5 supplemented with different combinations of BAP (5 mg/l), IAA (2 mg/l), IBA (1 mg/l), Brassin (1 mg/l) and GA_3 (1.5 mg/l), there was negligible amount of embryo conversion in some combinations (3-9%). However, secondary somatic embryogenesis had been occurred within 5-6 weeks of transfer (Plate 9, fig, H). It has been observed that maximum number of secondary embryogenesis were obtained in MS/B5 medium in presence of BAP (5 mg/l) + IBA (1 mg/l) or IAA (2 mg/l) (Plate 9, figs. D & F). The secondary somatic embryos were located on the hypocotyls region of the both 'Bud-like' and 'seed-like' primary somatic embryos. The embryogenic potential has maintained for over 20 months by successive 6-weekly subcultures. 'Bud-like' embryos had a 42.78% germination rate and subsequent root and shoot development in MS-medium with GA_3 (1.5 mg/l) + IAA (1 mg/l), whereas only 12% of 'seed-like' embryos germinated in this medium. It was observed that when embryos transferred to MS or B5 medium, there was negligible embryo conversion (3%) to whole plants. Embryo conversion rate little bit increased while transferred in MS medium in presence of BAP (5 mg/l) (6%) or BAP (5 mg/l) + IAA (2 mg/l) (8%), or BAP (5 mg/l) + IBA (1 mg/l) (9%).

The somatic embryo germination frequency was enhanced in both the cases ('Bud-like', and 'seed-like') to 51% in MS medium containing Brassin (2 mg/l) and 1% agar (Table 40). Plantlets regenerated from both the type of embryos ('Bud-like', and 'seed-like') were strong enough for transfer to pots after 6-8 weeks in germination medium (Plate 9, fig. I). The success rate in the acclimatization process was 54% (Table 38).

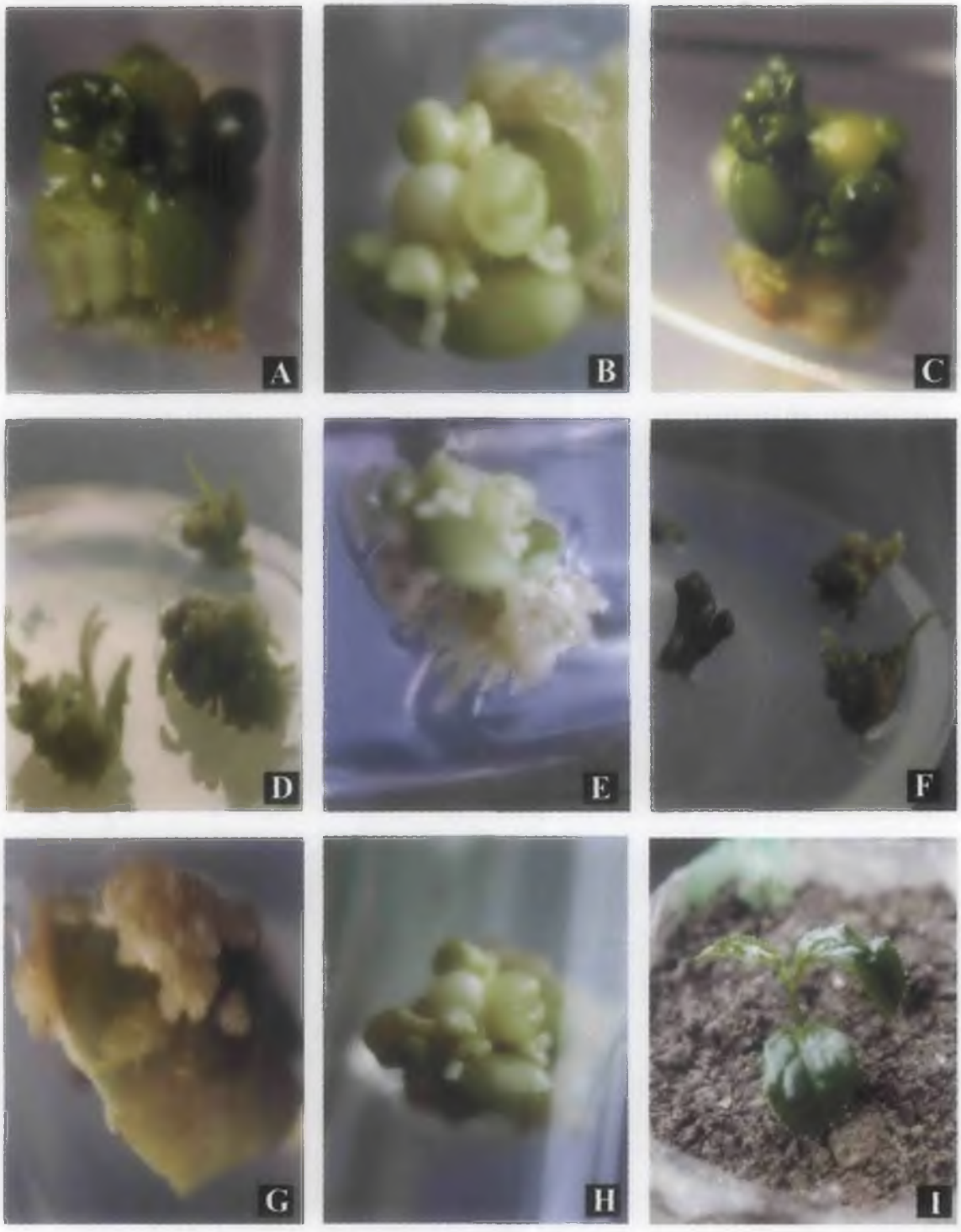


Plate 9 [Figs. A-I]: Somatic embryogenesis and plantlet regenerated from tea clone T383 using immature cotyledon as explants. (A) Bud-like somatic embryo which was green in colour; (B) Cup shaped somatic embryo which was pale yellow to creamy greenish in colour; (C) Seed-like somatic embryo with large cotyledon like those of zygotic embryo; (D & F) Shoots were developing either singly or multiple in number on the somatic embryo during germination; (E) Somatic embryo were developed along with rhizogenesis; (G) Non-embryogenic friable callus were developed on the upper surface of the mature cotyledon; (H) Secondary somatic embryogenesis were initiated and (I) Regenerated plantlet (15 weeks old) transferred to potted soil.

Table 39. Percentage of somatic embryo induction from immature and mature cotyledons of *C. sinensis* var. T383, after 14 weeks of culture on different hormone containing MS basal medium.

Medium (MS) + hormones mg/l	Immature cotyledons		Mature cotyledons	
	% embryogenic cotyledons[a] ± S.E	No. of somatic Embryos/explants[b] ± S.E.	% embryogenic cotyledons[a] ± S.E	No. of somatic Embryos/explants[b] ± S.E.
MS basal	3.02 ± S.E. 1.12	2.2 ± S. E. 1.23	3.02 ± S. E.1.02	1. 5 ± S.E. 1.23
BAP 5mg	5.09 ± S.E. 2.01	3.89 ± S.E. 1.67	3.0 ± S.E. 1.02	2.0 ± S.E. 1.06
4mg 2,4-D 4 + IAA 1mg	12.07 ± S.E 2.01	8.12 ± S.E 2.10	9.50 ± S.E 2.01	9.56 ± S.E 1.56
CM (10%)	15.13 ± S.E 2.03	22.80 ± S.E 2.01	10.12 ± S.E 1.89	10.25 ± S.E 2.34
PBOA 1mg	30.20 ± S.E 1.02	40.25 ± S.E 1.58	18.00 ± S.E 2.00	22.23 ± S.E 2.06
PBOA 1mg + BAP 5mg	40.40 ± S.E 1.58	45.29 ± S.E 2.24	18.00 ± S.E 2.00	22.23 ± S.E 2.06
PBOA 1mg + Kin 5mg	40.25 ± S.E 2.04	43.55 ± S.E 2.04	17.89 ± S.E 2.35	20.22 ± S.E 1.79

[a] Based on 20 explants per treatment after 14 weeks of culture.

$$\% \text{ embryogenic cotyledones} = \frac{\text{No. of cotyledon explants showing induction of somatic embryos}}{\text{No. of explants cultured}} \times 100$$

[b] Based on 20 explants per treatment ± S.E.

Table 40. Effect of hormones and basal medium on embryo conversion frequency in *C. sinensis* clone T383 after 8 weeks of culture.

Medium + hormone mg/l	% Embryo conversion ± S.E.
MS basal	3.03 ± 0.68
B5 basal	3.30 ± 0.99
MS + BAP 5mg	6.24 ± 1.04
MS + BAP 5mg + IAA 2mg	8.67 ± 1.07
MS + BAP 5mg + IBA 1mg	8.88 ± 2.11
B5 + BAP 5mg + IBA 1mg	9.12 ± 1.98
B_5 + BAP 5mg+ IAA 2mg	23.93 ±1.08
MS + GA_3 1.5mg + IAA 0.5mg	42.78 ±1.03
MS + Brassin 2mg	51.09 ±1.78

4.7. Karyotype analysis of the somatic chromosome complement of tea

Somatic chromosome analysis has been made in ten cultivars of Tea *in vivo* and one cultivar (T383) *in vitro*. The cultivars consist of 30 chromosomes in their somatic complements. The taxa are characterized by a gradate karyotype with median to nearly subterminal chromosomes varying in length from 1.24 μm to 4.20 μm. Micro-chromosome or B chromosomes is found in cultivar TV25 only.

Centromeric index (F%), total centromeric index (TF%), disparity index (DI) and total haploid chromosome length (TCL) were calculated as given by Huziwara (1962). The chromosome types encountered in the taxa studied have been described under the section 3.7.3.

$$F\% = \frac{\text{Short arm length of the chromosome}}{\text{Total length of the chromosome}} \times 100$$

$$TF\% = \frac{\text{Total sum of short arm lengths}}{\text{Total sum of chromosome lengths}} \times 100$$

$$DI = \frac{\text{Longest chromosome length (–) shortest chromosome length}}{\text{Total length of largest and shortest chromosome}} \times 100$$

4.7.1. Chromosome morphology in relation to Karyotype and idiogram

Karyotypes of the chromosomes (2n=30) were grouped arbitrarily on the basis of their length and position of the centromere into four types (Type A-D).

Type A: Comparatively long chromosomes (4.2 μm to 2.8 μm) each with two constrictions, primary and secondary, one of them nearly median (nM) and the other nearly sub-terminal (nST).

Type B: Medium to small chromosomes (2.8 μm) with median (M) centromeric constriction.

Type C: Medium sized chromosomes (3.54 μm) with nearly submedian (nSM) centromeric constriction.

Type D: Medium to small chromosomes (1.24 μm) with nearly subterminal (nST) centromeric constriction.

The detailed karyotype analyses of the ten cultivated varieties of tea *viz*- T78, T383, TV30, HV39, TeenAli17/1/54, TV23, TV25, TV29, TV26, and UPASI-26, were done according to Huziwara (1962).

1. *Camellia sinensis* cv. TV23

Karyotype formula: 2n = 30=A3 + B5 + C3 + D4.

The somatic complement of the taxon possesses 3 pair of chromosomes with secondary constructions (plate 10, fig. B). The chromosomes are medium sized varying in length from 2.8 μm to 2.1 μm, Total centromeric index (TF %), disparity index (DI) and total haploid chromosome length (TCL) are 51.08, 29.41 and 39.90 μm respectively. The camera lucida drawing and the idiogram of the karyotype was given in plate 12, fig. H.

Salient karyotypic features of *Camellia sinensis* cv.TV23

Type	No. of Chromosome Pair	Chromosome length (μm)			F%	Special features.
		Long arm (μm)	Short arm (μm)	Total length (μm)		
A	3	2.54-2.80	0.74-1.05	3.15-3.85	22-27	nST $^{Sat+}$
B	5	1.05-1.40	1.05-1.40	2.10-2.80	50.00	M
C	3	1.40-1.40	1.05-1.05	2.45-2.45	42.85	nM
D	4	1.62-1.75	0.98-1.05	2.68-2.80	37.50	nSM

M-median, nSM-nearly sub-median, nST-nearly sub-terminal, nT-nearly terminal. TCL- Total Haploid Chromatin Length; Sat^{+} = Satellite chromosome with secondary constriction.

2. *Camellia sinensis* cv. TV25

Karyotype formula: 2n = 30= A3 + B2 + C8 + D2.

The somatic complement of the taxon possesses 3 pair of chromosomes with secondary constrictions (Plate 10, fig. C). The chromosomes are median to small sized ranges in lengths 2.1μm to 3.1μm. Total centromeric indix (TF%), disparity index (DI) and total haploid chromosome length (TCL) are 38.67, 20.00, and 35.35 μm, respectively. The camera lucida drawing and the ideogram of the karyotype was represented in plate 12, fig. I.

Salient karyotypic features of *Camellia sinensis* cv.TV25

Type	No of Chromosome Pair	Chromosome length (μm)			F%	Special features.
		Long arm (μm)	Short arm (μm)	Total length (μm)		
A	3	1.75-2.45	0.70-0.83	2.45-3.15	22-28	nST $^{Sat+}$
B	2	0.70-1.05	0.70-1.05	1.40-2.10	50.00	M
C	8	1.75-1.75	1.40-1.40	3.15-3.15	44.00	nM
D	2	1.40-1.75	1.05-1.05	2.45-2.80	37- 42	nSM

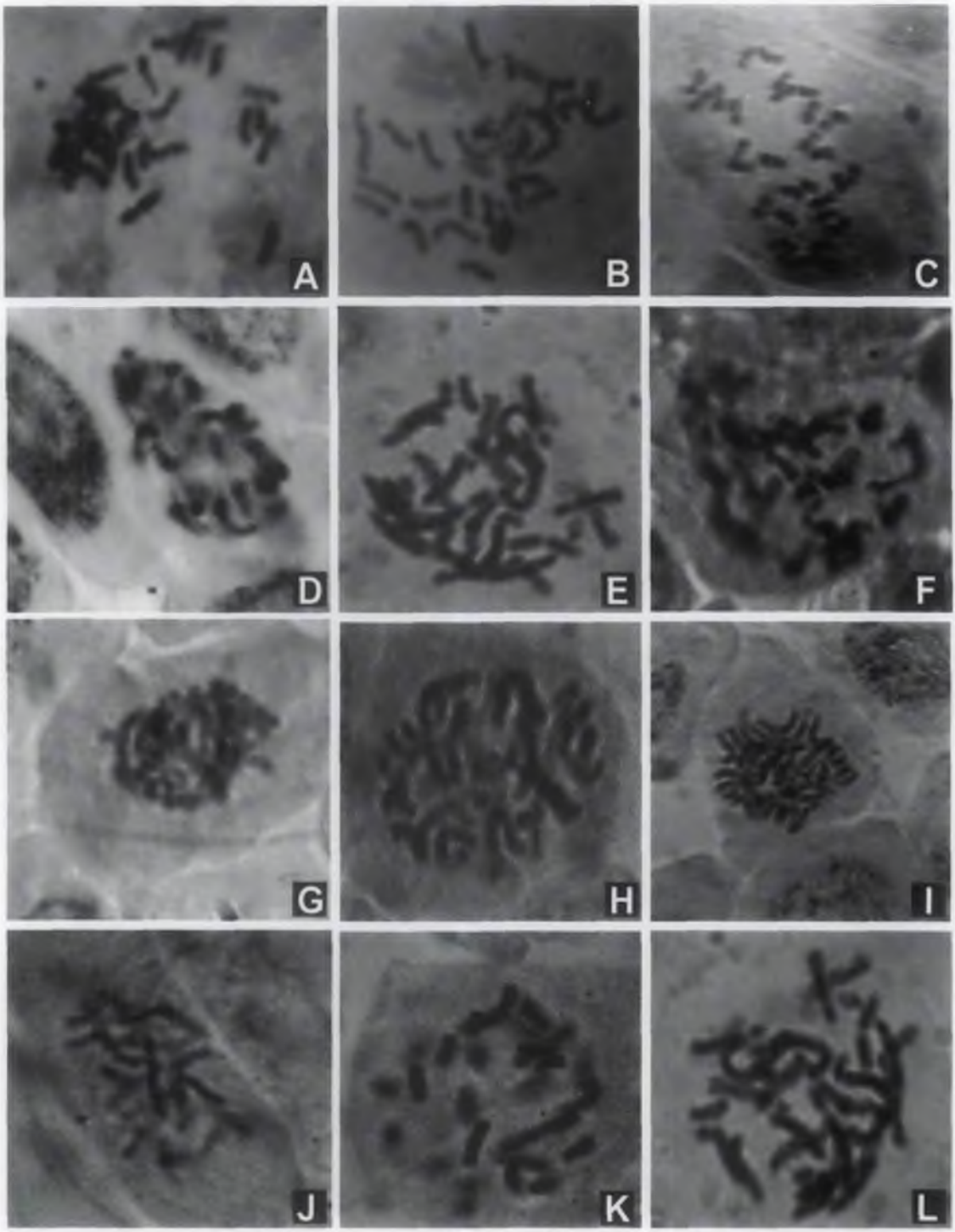


Plate 10 [A-L]: Photomicrographs showing 2n=30 chromosomes in the somatic complement in different cultivars of *Camellia sinensis*. (A) Tv26; (B) TV23; (C) TV25; (D) TV29; (E) T78; (F) T383; (G) HV39; (H) TV30; (I) UPASI-26; (J) TeenAli17/1/54; (K & L) *In vitro* regenerated plantlet showing true-to-type of somatic chromosome complement in T383.

3. *Camellia sinensis* **cv.**TV26

Karyotype formula: 2n = 30= A3 + B7 + C3 + D2.

The somatic complement of the taxon posses 5 pair of chromosomes with secondary constrictions (Plate 10, fig. A). The chromosomes are medium to small sized varying in lengths 3.15µm to 2.8 µm. Total centromeric index (TF%), disparity index (DI) and total haploid chromosome length (TCL) are 37.03, 20.00, and 48.3µm, respectively. The camera lucida drawing and the ideogram of the karyotype was represented in plate 12, fig. J.

Salient karyotypic features of *Camellia sinensis* cv.TV26

Type	No. of Chromosome Pair	Chromosome length (µm)			F%	Special features.
		Long arm (µm)	Short arm (µm)	Total length (µm)		
A	3	2.45-3.15	0.70-1.05	3.15-4.20	25.00	nST $^{Sat+}$
B	7	1.40-1.40	1.40-1.40	2.80-2.80	22.22	nST
C	3	2.14-2.14	1.40-1.40	3.54-3.54	39.54	nSM
D	2	1.75-2.14	0.93-1.05	2.87-3.19	32.91	nSM

4. *Camellia sinensis* cv.TV30

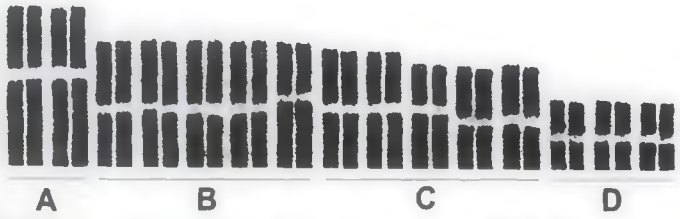
Karyotype formula: 2n = 30 = A4 + B5 + C4 + D2

The somatic complement of the taxon posses 2 pair of chromosomes with secondary constriction (Plate 10 , fig. H). The chromosomes are medium to small sized varying in length 1.24 µm – 3.12 µm. Total centromeric index (TF%), disparity index (DI) and total haploid chromosome length (TCL) are 45.12, 43.11 and 64.44 µm, respectively. The camera lucida drawing and the ideogram of the karyotype was represented in plate 11, fig. D.

Salient karyotypic features of *Camellia sinensis* cv.TV30

Type	No. of Chromosome Pair	Chromosome length (µm)			F%	Special features.
		Long arm (µm)	Short arm (µm)	Total length (µm)		
A	4	1.25-1.87	1.25-1.25	2.50-3.12	40.06	nM $^{Sat+}$
B	5	1.25-1.25	0.93-1.25	2.18-2.50	48-50	M
C	4	0.62-1.25	0.62-0.93	1.24-1.87	33.15	nSM
D	2	0.62-0.62	0.82-0.82	1.44-1.44	43.05	nM

A



Karyotype of *C. sinensis* cv. HV39 2n=30

Idiogram of *C. sinensis* cv. HV39.

B

Karyotype of *C. sinensis* cv. UPASI-26. 2n=30

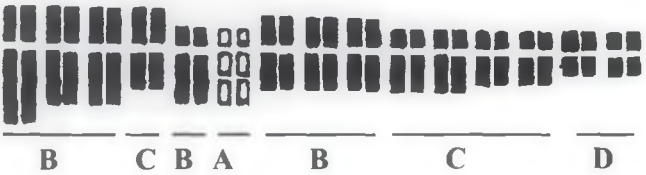
Idiogram of *C. sinensis* cv. UPASI-26.

C

Karyotype of *C. sinensis* cv. TeenAli17. 2n=30

Idiogram of *C. sinensis* cv.TeenAli17

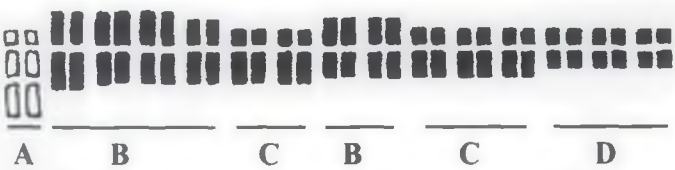
D



Karyotype of *C. sinensis* cv. TV30. 2n=30

Idiogram of *C. sinensis* cv.TV30.

E



Karyotype of *C. sinensis* cv. TV29. 2n=30

Idiogram of *C. sinensis* cv.TV29.

Plate 11 [Figs. A-E]: Karyotypes and Idiograms of five tea cultivars.

5. *Camellia sinensis* cv.T78

Karyotype formula: 2n=30= A1 + B6 + C5 + D3

The somatic complement of the taxon posses 3 pair of chromosomes with secondary constriction (Plate 10, fig. E). The chromosomes are medium to small sized varying in length 1.24 μm – 3.75 μm. Total centromeric index (TF%), disparity index (DI) and total haploid chromosome length (TCL) are 37.35, 50.30 and 35.36 μm, respectively. The camera lucida drawing and the ideogram of the karyotype was represented in plate 12, fig. F.

Salient karyotypic features of *Camellia sinensis* cv.T78

Type	No. of Chromosome Pair	Chromosome length (μm)			F%	Special features.
		Long arm (μm)	Short arm (μm)	Total length (μm)		
A	1	1.87-2.50	0.62-1.25	2.49-3.75	33.33	nSM^{Sat+}
B	6	1.87-2.50	0.62-0.62	2.49-3.12	33.21	nSM
C	5	0.62-1.25	0.62-1.25	1.24-2.50	50-50	M
D	3	1.25-1.25	0.93-0.93	2.18-2.18	42.66	nM

6. *Camellia sinensis* cv.T383

Karyotype formula: 2n = 30= A2 + B8 + C3 + D2

The somatic complement of the taxon posses 4 pairs of chromosomes with secondary constriction (Plate 10, fig. F). The chromosomes are medium to small sized varying in length 1.24 μm – 3.75 μm. Total centromeric index (TF%), disparity index (DI) and total haploid chromosome length (TCL) are 38.25, 50.30 and 33.33 μm, respectively. The camera lucida drawing and the ideogram of the karyotype was represented in plate 12, fig. G.

Salient karyotypic features of *Camellia sinensis* cv.T383

Type	No. of Chromosome Pair	Chromosome length (μm)			F%	Special features.
		Long arm (μm)	Short arm (μm)	Total length (μm)		
A	2	1.25-2.50	0.62-1.25	1.87-3.75	33.33	nSM^{Sat+}
B	8	0.93-1.87	0.62-1.25	1.55-3.12	42.66	nM
C	3	0.62-1.25	0.62-1.25	1.24-2.50	50-50	M
D	2	1.87-1.87	0.62-0.62	2.49-2.49	24.89	nST

7. *Camellia sinensis* cv.TV29

Karyotype formula: 2n = 30 = A1 + B6 + C5 + D3

The somatic complement of the taxon posses one pair of chromosomes with secondary constriction (Plate 10, fig. D). The chromosomes are medium to small sized varying in length 1.24 μm – 3.12 μm. Total centromeric index (TF%), disparity index (DI) and total haploid chromosome length (TCL) are 41.87, 43.12 and 28.94 μm, respectively. The camera lucida drawing and the ideogram of the karyotype was represented in plate 11, fig.E.

Salient karyotypic features of *Camellia sinensis* cv.TV29

Type	No. of Chromosome Pair	Chromosome length (μm)			F%	Special features.
		Long arm (μm)	Short arm (μm)	Total length (μm)		
A	1	2.50-2.50	0.62-0.62	3.12-3.12	19.87	nSM $^{Sat+}$
B	6	0.93-1.56	0.62-1.25	1.55-2.82	42.66	nM
C	5	0.62-1.25	0.62-1.25	1.24-2.50	50.00	M
D	3	1.25-1.25	0.62-0.62	1.87-1.87	33.15	nSM

8. *Camellia sinensis* **cv.**TeenAli17/1/54

Karyotype formula: 2n = 30 = A3 + B3 + C6 + D3

The somatic complement of the taxon posses one pair of chromosomes with secondary constriction (Plate10, fig. J). The chromosomes are medium to small sized varying in length 1.24 μm – 3.75 μm. Total centromeric index (TF%), disparity index (DI) and total haploid chromosome length (TCL) are 45.60, 50.30 and 32.06 μm, respectively. The camera lucida drawing and the ideogram of the karyotype was represented in plate 11, fig.C.

Salient karyotypic features of *Camellia sinensis* cv.TeenAli17/1/54

Type	No. of Chromosome Pair	Chromosome length (μm)			F%	Special features.
		Long arm (μm)	Short arm (μm)	Total length (μm)		
A	3	2.50-2.50	1.25-1.25	3.75-3.75	33.33	nSM $^{Sat+}$
B	3	0.93-1.87	0.93-1.87	1.87-3.74	50.00	M
C	6	0.62-0.93	0.62-0.93	1.24-1.86	50.00	M
D	3	0.93-1.56	0.62-1.25	1.55-2.18	44.48	nM

9. *Camellia sinensis* **cv.**UPASI-26

Karyotype formula: 2n = 30 = A3 + B6 + C5 + D1

The somatic complement of the taxon posses three pairs of chromosomes with secondary constriction (Plate 10, fig. I). The chromosomes are medium to small sized varying in length 1.24 μm – 2.50 μm. Total centromeric index (TF%), disparity index (DI) and total haploid chromosome length (TCL) are 42.20, 33.68 and 29.39 μm, respectively. The camera lucida drawing and the ideogram of the karyotype was represented in plate 11, fig.B.

Salient karyotypic features of *Camellia sinensis* cv.UPASI-26

Type	No. of Chromosome Pair	Chromosome length (μm)			F%	Special features.
		Long arm (μm)	Short arm (μm)	Total length (μm)		
A	3	0.62-1.25	0.62-1.25	1.24-2.50	50.00	M^{Sat+}
B	6	1.87-1.87	0.60-0.62	2.49-2.49	24.89	nSM
C	5	0.62-0.93	0.93-1.25	1.55-2.18	42.66-50.00	nM
D	1	1.25-1.25	0.62-0.62	1.87-1.87	33.15	nSM

10. *Camellia sinensis* **cv.** HV39

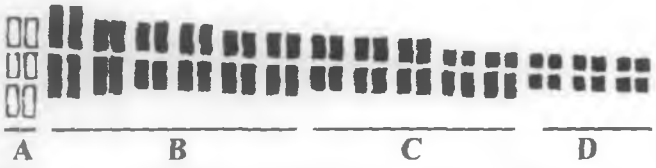
Karyotype formula: 2n = 30 = A4 + B6 + C3 + D2

The somatic complement of the taxon posses four pairs of chromosomes with secondary constriction (Plate 10, fig. G). The chromosomes are medium to small sized varying in length 1.55 μm – 3.12 μm. Total centromeric index (TF%), disparity index (DI) and total haploid chromosome length (TCL) are 43.51, 43.11 and 62.92 μm, respectively. The camera lucida drawing and the ideogram of the karyotype was represented in plate 11, fig. A.

Salient karyotypic features of *Camellia sinensis* cv. HV39

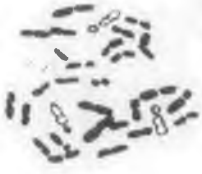
Type	No. of Chromosome Pair	Chromosome length (μm)			F%	Special features.
		Long arm (μm)	Short arm (μm)	Total length (μm)		
A	4	1.25-1.87	1.25-1.25	2.25-3.12	40.06	nSM^{Sat+}
B	6	1.25-1.83	0.62-1.25	1.87-2.50	48-50	M
C	3	0.93-0.93	0.62-0.93	1.55-1.87	33.81	nSM
D	2	1.25-1.25	0.62-0.62	1.87-1.87	33.15	nSM

F

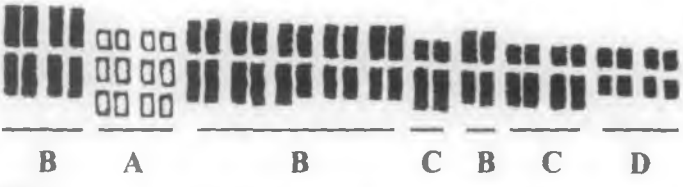


Karyotype of *C. sinensis* cv. T78. 2n=30

Idiogram of *C. sinensis* cv. T78.



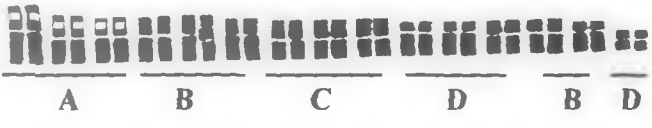
G



Karyotype of *C. sinensis* cv. T383. 2n=30

Idiogram of *C. sinensis* cv. T383.

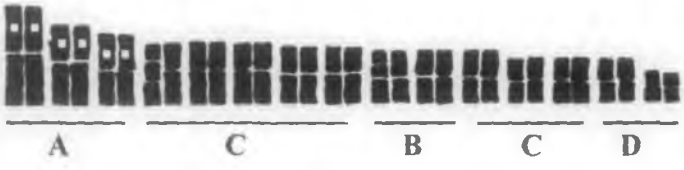
H



Karyotype of *C. sinensis* cv. TV23. 2n=30

Idiogram of *C. sinensis* cv. TV23.

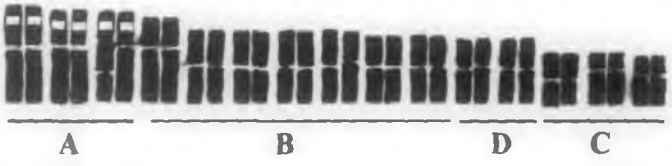
I



Karyotype of *C. sinensis* cv. TV25. 2n=30

Idiogram of *C. sinensis* cv. TV25.

J



Karyotype of *C. sinensis* cv. TV26. 2n=30

Idiogram of *C. sinensis* cv. TV26.

Plate 12 [Figs. F-J]: Karyotypes and Idiograms of five tea cultivars.

4.8. Transcript accumulation in induced systemic resistance

4.8.1. Differential gene expression after induction with Meja

Four different tissues (Mature leaf, Young leaf, Callus and Shoot) of the tea clone T383 were used for the study of induced systemic resistance after induction with inducer, methyl jasmonate (Plate 8, figs. Q, S, R & T). Young plant of 12 months old was considered for the *in vivo* induction and callus tissues of 2 month old and 6 month old shoots regenerated from the somatic embryos were considered for *in vitro* induction. All the four material were induced with methyl jasmonate according to the protocol described in the section 3.8.2. After the foliar application, the plant material harvested from the different time courses one for control and other for treatment.

4.8.2. Quantification of RNA in treated and control samples

High quality total RNA was obtained by using the protocol of Korban *et al* (2004). The yield of total RNA (µg/g fresh weight, FW) was as follows: 657, 660, 645 and 651 for young leaf, mature leaf, callus tissues and regenerated shoots, respectively. For all samples, the $A_{260/280}$ ratios ranged from 1.88 to 1.93, indicating a lack of protein contamination. For all samples, the $A_{260/230}$ ratio was higher than 2.0. This indicated that the RNA was of high purity and without polyphenol and polysaccharide contamination (Table 41). The RNA integrity was assessed by the sharpness of RNA bands visualized on a denaturing 1.2% agarose gel (Plate13,fig. A).

Total gene expression pattern (RNA transcriptions) was increased in the treated samples which were judged by the increasing amount of RNA synthesis (Table 42). RNA transcriptions accumulation was increased from 2-6 times to that of the control transcription level. Highest rate of transcription (698 µg RNA/g FW) was found in treated mature tea leaves followed by young leaves (684 µg RNA/g FW). Genes transcription rate were very slow in both treated and control samples of callus tissues. The relative transcription rate varied from 645 to 662 (RNA µg/g FW). Transcripts accumulation was moderate in case of shoots regenerated from somatic embryos, varied from 651 to 680. It was noticed that young leaves were not induced by the elicitor, methyl jasmonate at a rate which can be comparable with the induction level of mature leaves (657-684). Mature leaves showed quite high rate of transcript accumulation induced by treating with methyl jasmonate (660 to 698), which is 4.24 times more than that of the control transcription level. The present results indicated

that any how some of the genes expressed in higher transcription rate in the treated samples than controlled one. The expression may occur in some of the defense related genes *i.e.* pathogenesis related genes. From the various literature reviews it was found that methyl jasmonate can induce defense system in various plant species. Induction level was highest at time course of 16 h from the time of treatment and gradually reduced the induction level on both the side of 16 h time course. After 72 h of treatment the level of induction was similar with the level of the control conditions (Figure 11, A).

4.8.3. Quantification of cDNAs in treated and control samples after RT-PCR

Plant has its own defense system to protect from being attack of any pathogen fungi or bacteria or insect pests. During pathogenic attack plants released some signaling molecules to enhance its defense mechanism. The defense signaling pathway induces some specific genes to express at a higher rate to combat the pathogenic invasion. As a result of induction of the defense system, some proteins/enzymes are synthesized and express at a higher levels. The proteins are considered as pathogenesis related proteins (PRs). Chitinase is a pathogenesis related protein its expressing is high during infection due to the induction by the pathogen itself or its derivatives components (elicitor). The differential expression after induction with an inducer (MeJa) was measured by reverse transcription-polymerase chain reaction.

Table 41. Estimation of total RNA yield and quality using UV-*vis* Spectrophotometer

Sample source	Yield, µg/g FW*	$A_{260/280}$	$A_{260/230}$
Young leaf (12 month old plant)	657	1.88	2.13
Mature leaf (12 month old plant)	660	1.90	2.23
In vitro grown callus tissues (2 month old)	645	1.93	2.31
In vitro regenerated shoots of somatic embryo (6 month old)	651	1.89	2.18

*FW: Fresh weight

Table 42 . Quantification of total transcripts accumulation (RNA µg/g FW) in young, mature green leaf and *in vitro* grown tissues after treatment with methyl jasmonate in different time courses.

Time course (h)	Tea plant (12 months old)				*In vitro* grown callus tissues		*In vitro* regenerated shoots of somatic embryos	
	Young Leaf (YL)		Mature leaf (ML)					
	Control	Induced	Control	Induced	Control	Induced	Control	Induced
0	657	657	660	660	645	645	651	651
0.3	657	660	660	665	645	645	651	654
2	657	664	660	670	645	645	651	659
6	657	673	660	677	645	645	651	663
12	657	679	660	684	645	653	651	672
16	657	684	660	698	645	662	651	680
24	657	674	660	678	645	661	651	676
36	657	662	660	669	645	647	651	669
48	657	663	660	669	645	646	651	664
72	657	658	660	667	645	646	651	653

Table 43. Quantity of purified RT-PCR products (cDNA fragments) were estimated using UV-*vis* Spectrophotometry.

Test materials	Starting sample 15µg RNA in RT-PCR	RT-PCR products (µg amount cDNAs)		$A_{260/280}$
		Control	Treated (after 16h)	
Young leaf	15µg	134µg	203 µg	1.98
Mature leaf	15µg	143 µg	253 µg	1.99
In vitro grown callus tissues	15µg	121µg	154µg	2.00
In vitro regenerated shoots of somatic embryo	15µg	124µg	183µg	2.01

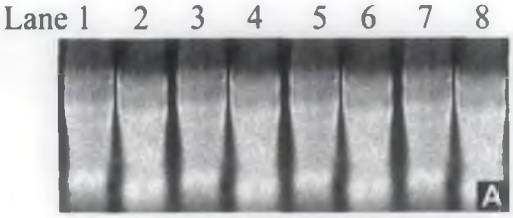


Fig.A. Equal RNA (15µg) from each sample was loaded and fractionated in 1.2% denaturing agarose gel. Lane 1-4 (control) and 5-8 (induced), mature leaf, young leaf, callus tissue, shoot respectively.

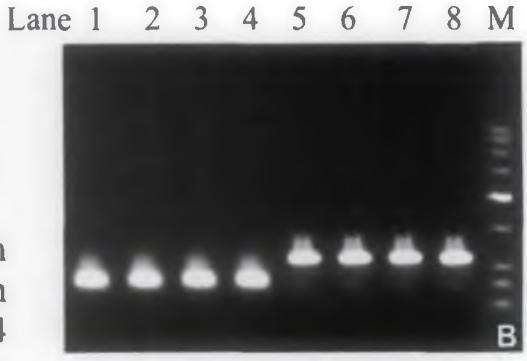


Fig.B. Chitinase gene specific transcript accumulation in RT-PCR. Lane 1-4, RT-PCR product of 254 bp using chitinase gene primer; lanes 5-8, RT-PCR product of 366 bp using DOP-primer in tea clone T383.

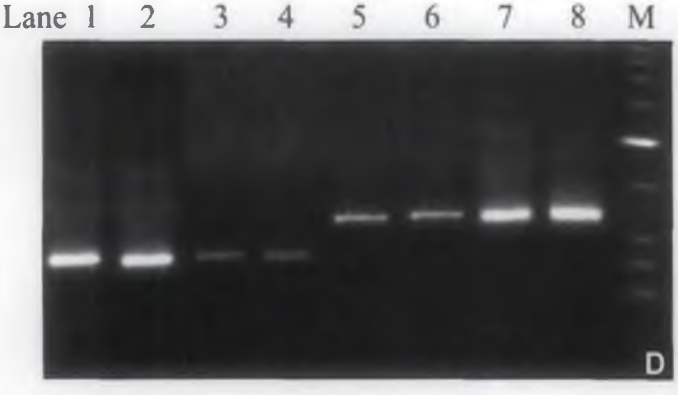


Fig.D. Purified RT-PCR products of 254 bp and 366 bp from control samples. Lane 1-4 and 5-8, young leaf, mature leaf, callus & shoot respectively.

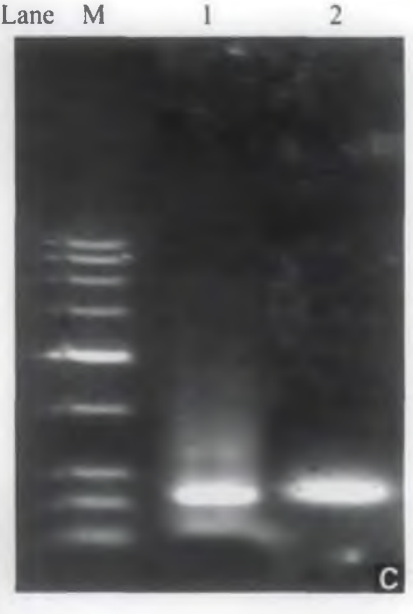


Fig.C. Caffeine synthase gene specific PCR product has been fractionated in 1% agarose gel. Lane M, low range DNA ruler marker; lane 1, 200 bp PCR product of T383 and lane 2, 202 bp PCR product of HV39 tea clone.

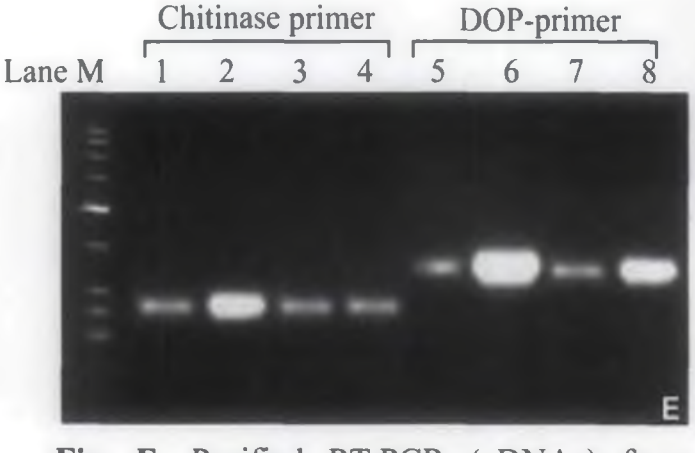


Fig. E. Purified RT-PCR (cDNAs) from treated samples (after 16 h induction with methyl jasmonate. Lane 1-4, YL,ML,callus, shoot respectively; Lane 5-8, callus, ML, shoot & YL, respectively.

Plate 13 [Figs. A-E]: Agarose gel electrophorasis for separation of RNA & DNA.

Table 44. Semi-quantification of RT-PCR products (μg amount of cDNAs/g FW) relating to chitinase gene specific transcript accumulation in young, mature leaf and *in vitro* grown tissues in different time courses after treated with inducing chemical, methyl jasmonate.

Time course (hr)	Tea plant (12 months old)				*In vitro* grown callus tissues		*In vitro* regenerated shoots of somatic embryos	
	Young Leaf (YL)		Mature leaf (ML)					
	Control (μg)	Induced (μg)	Control (μg)	Induced (μg)	Control (μg)	Induced (μg)	Control (μg)	Induced (μg)
0	134	134	143	143	121	121	124	124
0.3	134	138	143	165	121	124	124	130
2	134	145	143	172	121	131	124	144
6	134	167	143	187	121	142	124	153
12	134	183	143	223	121	149	124	172
16	134	214	143	253	121	154	124	183
24	134	187	143	231	121	140	124	170
36	134	169	143	198	121	134	124	159
48	134	154	143	165	121	127	124	137
72	134	127	143	149	121	123	124	127

The same results of induced systemic resistance (ISR) were obtained by RT-PCR method as revealed by cDNA quantification (Table 43 & 44). The result of RT-PCR amplification product was fractionated on 1% agarose gel for visual estimation (Plate 13, figs, B, D, & E). Plate 13, fig. d, clearly showing the differences in band thickness in different samples indicating the increased rate of specific gene transcript accumulation in different tissuee. Chitinase gene specific transcript has been accumulated in more quantity in the mature leaf, then young leaf but callus and shoot showed less amount of transcript accumulation. Treated samples in compare to control samples were accumulated more amount of mRNA transcript (*i.e.* cDNAs) (Plate 13, fig. E). Lanes 1-8 (treated samples) have the thick bands than those were the lanes 1-8 in plate 13, fig. D. The RT-PCR product was 366 bp and 254 bp in length. DOP-primer pair produced 366 bp cDNA product but chitinase gene specific primer pair produced 254 bp cDNA products. The chitinase gene specific transcript accumulated more in mature leaves (in relative band thickness) than in young leaves. The result was consistent with the increased quantity of total RNA concentration (μg/g FW) in the methyl jasmonate treated samples.

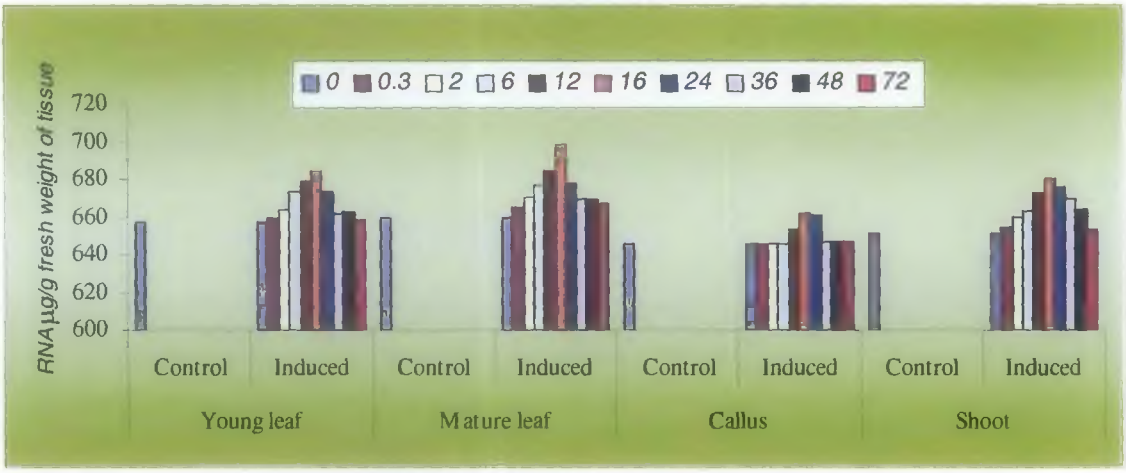


Fig. A : Quantitation of total transcript accumulation (RNAµg/g fresh weight of tissue) in young leaf (YL), mature leaf (ML), callus and shoot after 16 h induction with methyl jasmonate.

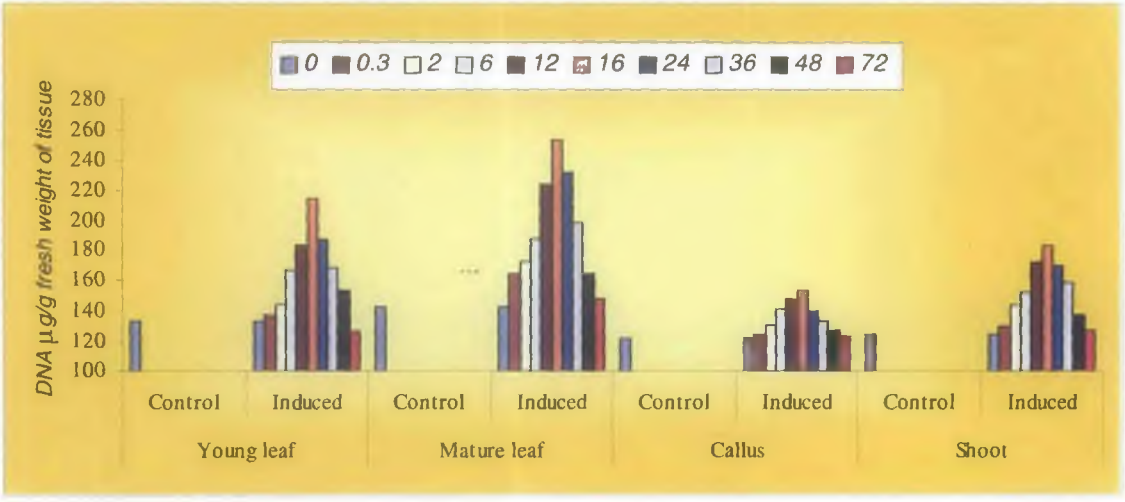


Fig. B: Quantification of RT-PCR products (cDNA µg/g fresh weight of tissue) after 16 h induction with methyl jasmonate in young leaf (YL), mature leaf (ML), callus and shoots.

Fig. 11 [A & B]: Quantification of transcript accumulation at the mRNA level (in total RNA) (Fig. A) and quantification of RT-PCR products cDNAs after 16 h induction with methyl jasmonat (Fig. B).

Methyl jasmonate treatment rapidly induced accumulation of chitinase gene specific mRNA transcript. This treatment induced the expression of defense related gene which was observed after 0.3 h of treatment, but declined to near control levels by 72 h after treatment. The mRNA transcript accumulated 2 to 6 times higher levels in treated leaves and tissues than observed in control leaves and tissues. Induction of chitinase gene specific transcript accumulation was two times more than the control after 16 h of treatment (Figure 11, B).

4.8.4. RT-PCR product sequencing and analysis using BLAST algorithm

tcaaatggccactgggtgtagcagtccaagttatcttcactttcctgggacaaagtttgtagcataagcccatgcattgttgtta
acagggtcagcaaggtggtcagccaggttctccaatggtccctttcctgtcacaattgcctgaacaaagaacccaaacattg
agaacatggccagtcttccattcttgatctccttcaccttcagctctgcaaaagcctctggtcatcagctaggcccaatgggtc
gaagcttcccaccggggtagagcgggtcggtcacctccccgagtggtccaccagcaatgcggtagccctcaacagcacc
catcaagataattggactacacccagtggccatttg

The BLAST (Basic Local Alignment Search Tool) algorithm BLASTN gives the following significant alignment information about the 366 bp DNA sequence of tea plant. The DNA sequence matches with basic chitinase gene of *Nepenthes khasiana* (1717bp, Accession no. gb|AY61883.1|) complete cds of 171 bp, Score = 65.8 bits (35), Expect = 1e-07, Identities = 35/35 (100%), Gaps = 0/35 (0%), Strand=Plus/Minus, as follows-

```
Query  2     CAAATGGCCACTGGGTGTAGCAGTCCAAGTTATCT  36
             ||||||||||||||||||||||||||||||||||
Sbjct  1709  CAAATGGCCACTGGGTGTAGCAGTCCAAGTTATCT  1675
```

Predicted peptide sequence(s): 366bp

The GenScan algorithm has predicted (81 amino acid coding sequences) the following peptide sequence(s) from 366bp DNA sequence of tea plant: Predicted coding sequence(s): 04:15:39|GENSCAN predicted peptide 1|81 amino acids,

MGAVEGYRIAGGPLGEVTDPLYPELKVKEIKNGRLAMFSMFGFFVQAIVTGK
GPLENLADHLADPVNNNAWAYATNFVPGK.

GENSCAN_predicted_CDS_1|246_bp.
atgggtgctgttgagggctaccgcattgctggtggaccactcggggaggtgaccgacccgctctaccccgagctgaaggt
gaaggagatcaagaatggaagactggccatgttctcaatgtttgggttctttgttcaggcaattgtgacaggaaagggaccat
tggagaacctggctgaccaccttgctgaccctgttaacaacaatgcatgggcttatgctacaaactttgtcccaggaaagtga

The TBlastX has shown the significant alignments with chitinase protein, Accession number AY618881.1 *Nepenthes khasiana* basic chitinase 1-1 gene, complete cds, Length=1572

Score = 37.7 bits (76), Expect = 4.1, Identities = 14/15 (93%), Positives = 14/15 (93%), Gaps = 0/15 (0%), Frame = -2/+2

```
Query 46   STREITWTATPSGHL 2
           ST EITWTATPSGHL
Sbjct 1520 STTEITWTATPSGHL 1564
```

The sequence information of 366 bp was deposited into the GenBank of NCBI and approved an accession no.EU373553 as a hypothetical protein gene of *Camellia sinensis*.

DISCUSSION

In the present investigation, twenty one clonal tea genotypes were used for the analysis of their genetic diversity and phylogenetic relationships. Selection of polymorphic alleles was carried out very conservatively, and only clear and repeatable polymorphic bands were selected. The results indicated that the percentage of ISSR polymorphic bands (88.54%) was higher than that of RAPD (77.77%) marker. The results in this study suggested that the ISSR markers were superior to RAPD markers in the capacity of revealing more informative bands in a single amplification. The observed difference between ISSR and RAPD might be explained in terms of functional constraints since some RAPD bands may be associated with functionally important loci (Penner, 1996). The large number of shared bands among individuals was evidenced for the high degree of similarity among the twenty one tea genotypes tested.

Three main groups could be recognized from the result of cluster analysis based on RAPD and ISSR data. Dendrograms were constructed by means of UPGMA on the basis of band sharing and genetic distances showed three main clusters, which grouped the twenty one tea genotypes into three types, China, Assam and Cambod type. The China type consisted with the tea clones T383, T78, T135, HV39, TeenAli17, AV2 and BS/7A/76, Assam type included the following tea cultivars UPASI-3, UPASI-9, UPASI-26, TV20, TV21, TV27 and TV28 and lastly Cambod type consisted with the following tea clones TV18, TV19, TV22, TV23, TV25, TV26 and TV30. With RAPD data matrix, the highest genetic similarity value of 0.848 was obtained between T135 and T383. The lowest value obtained was 0.194 between TV22 and TV18. The genetic similarity between T383 and HV39 was 0.898 according to Nei and Li's coefficient when the ISSR markers were considered and lowest was 0.673 between TV18 and TV27. The value of the genetic similarity coefficient of the three tea populations varied between 0.47 and 0.67, with an average of 0.57. The result indicated that both RAPD and ISSR have the high resolution in the genotype identification of tea. These results support the taxonomic treatment in which the tea was grouped into three varieties namely China, Assam and Cambod variety.

The Cambod variety is morphologically and biochemically in between the China and Assam type tea. Although Cambod tea morphologically closes to Assam tea variety

yet it has been placed into separate variety, Cambod variety. The cluster analysis based on RAPD and ISSR data revealed that it is the most isolated group. Cambod type tea had the smallest within group diversity while the Assam tea had the moderate and China tea with largest diversity for both RAPD and ISSR markers. The result indicated that the China tea is highly variable and the germplasm of natural China tea might be useful in breeding programs. More than 70% of the total diversity occurred within populations. This is expected with highly outcrossing species characterized by low level of population differentiation. The highly significant differences ($P < 0.001$) among the tea populations revealed by an analysis of molecular variance could be due to the small size and discontinuity of the stands.

The Nei's genetic analysis showed highest similarity between Assam and Cambod type (67%), moderate between China and Assam type (59%) and least similarity between China and cambod type only 47%. This genetic similarity index indicated a wide range of genetic base among the three tea types *viz.* China, Assam and Cambod type. Therefore, the wide genetic base observed in the present study is an account of the wide range of outbreeding nature which has evolved as preventive mechanism for selfmg increases genetic exchange and diversity. Similar observation has been made in *Camellia sinensis* (Paul *et al.*, 1997), which is outcrossing/outbreeding in nature. Such information can be very useful in plant system where *a priori* knowledge on breeding habits is not available.

In this study it has also been found that there are considerable amount of genetic variation present among the twenty one tea genotypes on the basis of DNA polymorphism in RAPD and ISSR fingerprinting, which were very much consistent with the earlier report of Bera and Saikia, (1999); Lai *et al.* (2001) and Wachira *et al.* (1995). The degree of DNA polymorphism obtained from these studies will help in the detection of genetic variability among tea genotypes and their phylogenetic relationship. The cluster analysis in *Camellia sinensis* of RAPD and ISSR data revealed an almost three clusters of the 21 tea genotypes. The Nei's (1972) genetic measures showed on an average 59% genetic relationship between the China and Assam type tea and 47% genetic identities between China and Cambod type and 67% between Assam and Cambod type tea. The gene diversities (H_S) within the China, Assam and Cambod type were 0.285, 0.241 and 0.207, respectively for RAPD markers and 0.291, 0.233 and 0.193, respectively for ISSR markers.

The genetic diversity within populations calculated from the Nei's index averaged 0.27. This could be explained by the narrow genetic base of the tea samples which are considered for the present study. The highly significant differences were found among populations upon an analysis of molecular variance (AMOVA), a markedly higher proportion of diversity was observed within populations (71%) as compared with between populations (29%). The fact is that most diversity is within populations reflects the highly outcrossing nature of tea species. The population structure analyses further demonstrated the genetic difference of Cambod variety from the other two varieties and the closure relationships of *Camellia sinensis* ssp. *lasiocalyx* and *Camellia sinensis* var. *assamica*. The pairwise comparison of population of each variety against the other showed that whenever China type was compared with any of the other two tea varieties, the total heterozygosity within the population (H_t) and between subpopulations (D_{ST}) and the genetic differentiation coefficients (G_{ST}) were much higher than the same between other tea varieties. These values clearly suggest higher genetic divergence of *C. sinensis* var. *sinensis* from the other two tea types (Assam and Cambod type). This is further evidenced from the low gene flow (N_m) from *C. sinensis* var. *sinensis* to the other tea types. The exchange of genes between populations homogenizes allele frequencies between populations and determines the relative effect of selection and genetic drift. High gene flow between populations precludes local adaptation and will also impede the process of speciation (Barton and Hewitt, 1985). The very low N_m present in *C. sinensis* spp. *lasiocalyx* further reflects the little bit of reproductive isolation it holds from other varieties of tea.

According to the present study, interpopulation gene flow (*Nm*) was 0.76, $Nm < 1$, which indicates the limited genetic exchange (a value of gene flow (N_m) < 1.0; fewer than one migrant per generation into a population) between populations, suggesting that genetic drift and inbreeding may become the main factors influencing genetic structure of tea populations. High levels of gene flow could prevent genetic differentiation from genetic drift among populations, but low levels may result in population adaptation to an ecosystem, then genetic isolation among communities (Li *et al.*, 2004). A wide population distribution and barriers caused by habitat fragmentation due to mountains and others have prevented gene exchange among the tea populations. In addition, *Camellia sinensis* is insect pollinated, the spreading

distance of pollen is short, and there is little possibility of external pollen (genes) moving into the flowers. All these have limited genetic exchange to some degree and increased the genetic differentiation among individuals within the population, thus limiting gene flow between them. Nei's genetic diversity, Shannon's diversity index and AMOVA detected the almost similar genetic variation among three tea populations.

Wachira *et al.* (1995) studied the genetic diversity and relationship of 38 tea clones of China, Assam, and Cambod tea (*C. assamica* ssp. *lasiocalyx* Planchon ex Watt) using RAPD markers and showed that 30% of the total diversity resided among populations while 70% resided within populations based on Shannon's diversity index. Analyses based on band sharing separated the 38 tea clones into three distinct clusters in UPGMA analysis in a manner consistent with both the present taxonomy of tea and with the known pedigree of some clones. RAPD analysis also discriminate all of the 38 commercial clones, even those which cannot be distinguished on the basis of morphological and phenotypic traits. Similar results were also observed in the present investigation. Analysis of molecular variance (AMOVA) and Nei's total genetic diversity results indicated that 29% diversity resided among the populations and 71% genetic diversity resided within the populations. The result of cluster analysis was generally consistent with the taxonomic classification.

The present study is consistent with the observation of Wachira *et al.* (1995) showing three distinct clusters in the phonetic dendrogram. Wachira *et al.* (1997) studied the genetic diversity and relationship among the cultivated 28 tea genotypes and its wild relatives using RAPD markers and organelle-specific polymerase chain reaction. The measures of similarity obtained indicated that RAPDs were taxonomically informative in *Camellia*, and the species relationships revealed were generally consistent with those obtained using morphological, compatibility and terpenoid affinities.

Using RAPD markers Cheng-Wen *et al.* (2007) detected genetic diversity among the four tea population of China. The genetic diversity analysis indicated that Shannon's index was 0.43; 74.7% of which was within population genetic diversity while 25.3% was among population variation. Liang *et al.* (2007) investigated the genetic diversity and relationship among the 36 clonal tea cultivars of China using ISSR markers. The average polymorphism information content (PIC) was 0.90, Nei's

gene diversity (H) and Shannon's information index (I) were 0.23 and 0.38, respectively. The genetic similarity among all the tested clonal cultivars ranged from 0.58 to 0.84, averaging 0.69, which indicated that the genetic basis was relatively narrow. AMOVA showed that variance component among cultivars (94.4%) was far higher than that among regions (5.6%). Wei *et al.* (2005) showed the genetic diversity and relationship among four populations of *C. euphlebia* on the basis of ISSR markers. The results indicated a low level of genetic diversity in *C. euphlebia* at the species level (H_T = 0.153 and H_{SP} = 0.24) and at the population level (H_E = 0.11) and a relatively high degree of differentiation among populations (G_{ST} = 0.31, Shannon's index analysis = 37.5%, AMOVA analysis = 0.35, (88%). Gene flow among populations was 0.544. Paul *et al.* (1997) studied the diversity and genetic differentiation of India and Kenyan tea using AFLP markers. They showed that most of the diversity was within population, with 79% of the variation being within and 21% being between populations of India and Kenyan tea. The present results may serve as reference information for tea breeding and germplasm conservation purposes.

The AMOVA analysis revealed that the percentage of variances attributable to the differences among and within groups were 31.07% and 68.93% for RAPD and 28.87% and 71.13% for ISSR, respectively. These results indicated considerable genetic differentiation among the three groups studied. The Pearson's correlation coefficient between similarity matrices based on RAPD and ISSR was 0.678–0.849. The Mantel test revealed that the correlation was highly significant ($P < 0.001$). This implied that the results of RAPD and ISSR studies were highly congruent. However, ISSRs seemed to be more discriminative in cultivar identification then RAPDs because cultivars that could not be distinguished by RAPD markers were separable based on ISSR markers. This finding suggests that the evolution rate of the ISSRs might be faster than that of RAPDs in tea samples studied.

Therefore, ISSR markers may be considered suitable for the study of genetic diversity and determination of genetic relationships of closely related tea cultivars. Other studies also indicated that ISSR markers exhibit higher levels of polymorphism than RAPD markers (Parsons *et al.*, 1997; Esselman *et al.*, 1999). The observed difference between ISSR and RAPD might be explained in terms of functional constraints since some RAPD bands may be associated with functionally important

loci (Penner, 1996). The first evolutionary rate and hypervariability of ISSR may suggest that ISSR bands represent neutral markers (Esselman *et al.*, 1999).

Li *et al.* (2005) evaluated the genetic diversity of 69 tea cultivars of the pre-concentrated core germplasms of *Camellia sinensis* in China by RAPD markers (0.22-0.72, genetic diversity). Luo *et al.* (2004) investigated 31 *Camellia sinensis* resources in different ecosystem using RAPD markers (genetic diversity, 0.22-0.71). Data obtained in the present study were almost similar with the above observation (genetic diversity, 0.29-0.71). Several types of molecular markers have been employed previously to quantify the genetic diversity within tea germplasm collections in different countries (Matsumoto *et al.*, 1994; Tanaka *et al.*, 1996). These include RFLP, RAPD markers, which all revealed a very narrow genetic base of the different collections. Lai *et al.* (2001) studied the genetic relationships in cultivated tea clones and native wild tea in Taiwan using RAPD and ISSR markers. A few Tocklai variety (TV clones) has also been characterized on the basis of RAPD analyses by Bera and Saikia (1999). Matsumoto *et al.* (1994) characterized the genetic diversity in tea cultivars on the basis of PAL (phenylalanine ammonia lyase) cDNA probe analysis. Raina *et al.* (2001) evaluated the genetic integrity of micropropagated diploid and triploid elite tea clones on the bases of RAPD, ISSR and RFLP fingerprinting. Six Korean tea populations were analysed on the basis of RAPD-PCR to reveal their genetic diversity among the tea populations by Park *et al.* (2002).

Matsumoto *et al.* (2004) used PAL cDNA probe to evaluate the genetic diversity of the Korean tea plant (*Camellia sinensis* var. *sinensis*) in RFLP. They analyzed 297 plants collected from the Korean tea farm. In Japanese teas the PAL locus is composed of 3 multi-fragment alleles, but at least 10 fragment alleles were apparent in the Korean teas. RFLP analysis using PAL cDNA was very useful for the detection of genetic diversity in Korean teas, because the result of this analysis were similar to those of previous RAPD and morphological studies and were able to reveal the existence of the 2 tea groups. Kaundun *et al.* (2000) studied the diversity of 27 superior tea (*Camellia sinensis* var. *sinensis*) accessions from Korea, Japan and Taiwan using RAPD-PCR (Random Amplified Polymorphic DNA- Polymerase Chain Reaction) markers. The Shannon's index used to partition diversity into inter-and intra-group, revealed that 71 percent of variability resided within groups and 29 percent between groups. A dendrogram based on the UPGMA-link method using

Jaccard's distances and multivariate factorial correspondence analysis clustered the tea accessions into two main groups, regrouping the Taiwan cultivars on the one side and the Korean and Japanese accessions on the other side.

Mondal and Chand (2002) studied 25 diverse tea (*Camellia sinensis* (L.) O. Kuntze) using the simple sequence repeat anchored polymorearse chain reaction (SSR-anchored PCR) or Inter SSR-PCR (ISSR). A dendrogram was constructed using UPGMA method revealed three distinct clusters of Cambod, Assam and China type tea, which concur with the known taxonomical classification of tea. These results suggest that the ISSR-PCR method is potentially useful for genetic fingerprinting and molecular taxonomic classification of tea genotypes. Chen *et al.* (2005) used RAPD marker to evaluate the genetic diversity and relationship among 15 well known elite tea genetic resources of China. The 15 tea genetic resources were grouped into three groups by UPGMA cluster analysis based on RAPD data. The genetic distances among the genetic resources were from 0.16 to 0.62, with an average of 0.37.

RAPD markers have been earlier used to study taxonomic relationships (Demeke *et al.*, 1992) and shown to detect higher polymorphism than RFLP markers (Thormann *et al.*, 1994). The difference in the generation of bands per primer was probably due to the differences in the primer sequence and primer-template interaction (Weir *et al.*, 1997). According to Rafalski *et al.* (1992) variation in the number of bands in RAPD profiles is independent of the complexity of the genome. It is observed that the genetic variation at the DNA level is much more prominent than that at the protein level because of the codon degeneracy. Approximately 29% of mutations occurring at the nucleotide level cannot be detected by amino acid changes (Nei, 1987). An additional 70–75% of amino acid substitutions cannot be detected by ordinary protein electrophoresis because of maintenance of net protein charge. In total, the detected genetic variation *via* allozymes is expected to be at least five to six fold less than at the DNA level (Nei, 1987).

Detailed DNA characterization is also necessary for the protection of own tea cultivars. Usual DNA-based techniques such as RFLP through Southern hybridization and use of microsatellites are expensive; use of the latter is often hindered by lack of availability of DNA sequence information, though it has inherent advantage. PCR-based RAPD approach has been a handy and convenient alternative technique for investigations of genetic variation and genome mapping. Because of the nature of

primer sequences, RAPD analysis samples the genome more randomly than other methods and has been successfully employed in the construction of linkage maps. Being simple and non-radioactive, the technique is quite sensitive and used to detect genetic variation in many organisms. It has been extensively used for molecular fingerprinting, phylogenetic analysis, genetic mapping and population diversity analysis. The variation that can be accounted for, between and within populations through RAPD, appear to be unlimited. Yet, the dominance nature of these markers is a greater leveler and introduces subjectivity in understanding the structure of populations, where allelic frequencies of genes matter. The Nei and Li's coefficient has been used to study the gene structure among the tea population and genotypes where we can analyse the population structure and gene frequencies from the binary data matrix of RAPD and ISSR.

The present investigation showed that if assay conditions are carefully controlled, the RAPD and ISSR methodology may provide a cheap, rapid and effective means to evaluate the genetic diversity among a large number of tea populations and help devise sampling strategies to complement classical morpho-agronomic descriptors. PCR-based fingerprinting technique, RAPD and ISSR are informative for estimating the extent of genetic diversity as well as to determine the pattern of genetic relationships between different varieties of *Camellia sinensis*, with polymorphism levels sufficient to establish informative fingerprints with relatively fewer primer sets. The results may serve as reference information for tea breeding and germplasm characterization, and conservation purposes. However, other primers should also be tried to provide better understanding of the genetic relationship of tea cultivars. But an extensive screening of large number of primers with more number of cultivars is necessary before any concrete conclusion is drawn of the degree of diversity existing among the different genotypes of the three tea varieties.

The genetic similarity among all the tested (twenty one genotypes) clonal cultivars ranged from 0.47 to 0.67, averaging 0.56, which indicates that the genetic base is relatively quite high. Thus, there is an urgent need to enhance the genetic base of the germplasm stock of the indigenous tea for future breeding and improvement programs by launching collection mission of wild and subspontaneous teas. The aim of conservation is to preserve existing genetic diversity and the potential of a species to evolve. Any conservation program should ensure the long term survival of a

species and maintain its ecological and evolutionary processes and both of these require the preservation of genetic variation.

In the light of these present results, it is wise to conserve the whole diverse populations of the tea germplasm rather than selected individuals from different populations or only some created elite tea clones. Conservation of the maximum amount of diversity would rationally be achieved by targeting whole diverse populations instead of collecting a few samples from each population. Individuals from populations containing rare alleles should nevertheless be secured so that important genotypes are saved. Genetic diversity was greatest within China type tea followed by Assam and Cambod type tea. In this respect, it is noteworthy that China type tea, the most diverse population should be conserved properly along with other varietal genotypes for the future improvement of commercial tea through breeding and genetic technological approaches.

Therefore, all the existing population of tea should be protected *in situ* free from anthropogenic and agricultural impacts in order to preserve as much genetic variation as possible. Furthermore, we should collect samples from all the natural populations and develop methods for *ex situ* conservation of the genetic resources for tea. Caffeine synthase gene specific DNA segment was amplified through gene specific PCR amplification using gene specific 20-mer primer. The caffeine synthase gene specific primer was designed on the basis of cDNA's sequence information of tea by using Primer3 program.

Two different sized fragments were amplified in gene specific PCR reaction while using different DNA template. The 2002 bp partial sequence was obtained from tea clone HV39 and 200 bp partial sequences was achieved in tea clone T383. Both the sequences were deposited to the GenBank of NCBI (Accession no. AY599069 for 202 bp sequence of tea clone HV39 and Accession no. AY601112 for 200 bp sequence of tea clone T383). According to the Blast results, the present study revealed that these two PCR amplified DNA sequence of 202bp and 200bp of tea clones HV39 and T383 respectively are only the partial sequence information of caffeine synthase gene of tea. So, this DNA sequence information of caffeine synthase gene can be utilized either to develop decaffeinated tea through homology dependent gene silencing or through RNA interference. Caffeine synthase gene can also be

differentially expressed during the induced systemic resistance and acts as a defense related protein.

Callus growth and development has been studied from stem explants using different media. A differential response of callus growth and morphogenesis was obtained from the stem explants in different media. In all the four tea clones (UPASI-26, T383, TV30 and HV39) minimal growth has been noted in B5 medium. The general performance of callus in B5 was good while maximal growth and development of callus has been observed in the MS medium. The nutritional requirements in callus culture have been examined in details by Gamborg and Shyluk (1981). The present observations with callus initiation from stem explants of different tea clones, however, necessitate changes in the current idea regarding regulatory mechanism of morphogenetic changes in the callus tissues grown *in vitro*. As many as two different media have been tried for support of callus growth in the tea clones investigated. A significant difference between the media used for supporting the growth and development of tea tissues was that the media tried in tea tissue culture contains varying proportion of growth factors like auxin and cytokinin. The major differences between the two high salt media tried lie not only in the amount and form of nitrogen, but also in the relative amounts of some of the micro-elements. In B5 medium manganese sulfate has been replaced by boric acid, zinc sulfate, cupric sulfate and molibdic acid but neither contains any organic constituents. The growth pattern of callus from each of the four tea clones has been recorded in details.

A differential growth response has been noted for different callus tissues with respect to medium and plant growth regulators. The growth patterns was varied significantly in the tea clones (T383, UPASI-26, HV39 and TV30) while cultured in two media MS and B5. The performance of all four tea clones was uniformly poor in B5 medium as compared to MS basal medium. Callus initiation was quite good in MS basal medium without any additives or supplements. It was clearly indicating in the present investigation that the optimal growth and development of callus requires vitamins supplement with high salt concentration in the medium. Growth indexes (GI) were 1.48, 1.41, 1.44 and 1.22 in T383, UPASI-26, HV39 and TV30, respectively after 8 weeks of culture in MS basal medium. Tea clone T383 has the good response in this medium among the four tea clones tested. In case of B5 medium growth indexes (GI) were 1.00, 1.35, 1.12 and 1.03 in T383, UPASI-26, HV39 and TV30,

respectively after 8 weeks of culture initiation. The results revealed that tea clone UPASI-26 given highest GI rate 1.35 in compares to T383 clone in MS medium.

It suggests that the *in vitro* grown tissue culture is not dependent in the medium used solely but to the genotypes too. The growth performances of the callus initiation and morphogenesis have also been studied in MS medium supplemented with different combinations of plant growth hormones, auxins (IAA, NAA, 2,4-D, IBA, and PBOA) and cytokinins (BAP, TDZ and Kin). The analyses of the growth pattern (increase in fresh weight) and growth index (GI) reveal that auxin and cytokinin in general promote growth of callus in all the four tea clones investigated. Growth indexes (GI) were 3.13, 2.94, 2.72 and 2.72 in T383, HV39, TV30 and UPASI-26, respectively in MS medium with 4 mg/l 2,4-D and 1 mg/l BAP, among the various combination of growth hormones tried in the present investigation. Tea clone T383 responding well in this combination out of four tea clones tested. Callus initiation and growth performance was observed in undefined organic supplement such as coconut milk (CM) in various concentrations in MS medium. The test showed that coconut milk was the capacity to enhanced callus growth and development. Growth indexes (GI) were 1.43, 1.91, 2.73, 2.94 and 2.32 in control, 5%, 10%, 15% and 20% concentration of CM, respectively in tea clone T383.

It suggests that coconut milk can enhance the in vitro tissue culture growth and morphogenesis as compare to plant growth hormones. The remarkable growth stimulating property of coconut milk had led to attempts to isolate and identify the active principles. The milk contains different kinds of amino acids (glutamine, glycine, lysine, methionine, tryptophan, arginine, aspartic acid, and histidine); Inorganic elements (such as potassium, sodium, phosphorus, and magnesium); organic acids (citric acid, succinic acid, malic acid, and shikimic acid); sugars (sucrose, glucose, fructose); sugar alcohols (mannitol, sorbitol, myo-inositol); vitamins (nicotinic acid, panthothenic acid, biotin, riboflavin, folic acid, thiamine, pyridoxine); growth substances (auxin, gibberellin, zeatin, unknown cytokinins). Coconut milk contains all the necessary organic additives required for callus growth and development.

Influence of NAA and IAA was spectacular in tea clone T383 where about one and half fold increase in growth has been noted. However, tea clone TV30 reveals relatively less growth in IAA supplemented in MS medium, while the same effect has

been noted in material UPASI-26 with NAA. With a fixed concentration level of BAP the optimal growth index has been noted at the concentration of 1mg/l IAA and 4mg/l NAA. In general the optimal callus growth was initiated at a low level of (2 mg/l) IAA. The callus showed varying growth rates in different levels of kinetin (Cytokinin). The optimal concentration range of Kinetin was 4 mg/l. The effect of 2,4-D on callus formation and development has been variable in different species.

Cytokinin tends to promote the formation of chlorophyll in callus and suspension cultures, auxins can be inhibitory. The induction of caulogenesis or rhizogenesis in callus cultures usually requires an adjustment of the levels of auxins and cytokinins that are necessary for callus initiation and growth. For caulogenesis, a high cytokinin to auxin ratio is generally required but for rhizogenesis, a high auxin to cytokinin ratio or only auxin is required. Auxin to cytokinin ratio or only auxin is required. Auxin induces the synthesis of polyamines during root formation.

Shoot formation through morphogenesis from the callus of T383 was obtained in various combinations of auxin and cytokinins in MS medium. A potential cytokinin, TDZ (n-phenyl-N-1,2,3-thiadiazol-5-Urea) showed the better performance in callus initiation and morphogenesis in shoot formation. Various combinations of the mixture of auxins (IAA, NAA) with cytokinins (BAP and TDZ) were tried in the morphogenesis for the shoot formation. The combinations of 4 mg/l BAP + 2 mg/l NAA and 3 mg/l TDZ + 2mg/l NAA were given the better shooting performance 65% and 74%, respectively in tea clone T383, after 8 weeks of incubation. Rooting was better while the ½ MS medium supplemented with 1 mg/l IBA. After the well developed roots and leaves the regenerated plantlets was transferred to hardening chamber and ultimately to the greenhouse for acclimatization.

Auxin promotes the growth of plant tissues *in vitro* by inducing the secretion of hydrogen ions (H^+) into and through the cell wall. Binding of auxin leads to lipid breakdown and acidification of the wall, increasing its extensibility. Potassium ions are taken into the cell to counteract the electrogenic export of H^+ ions (protons) and this has the effect of decreasing the water potential of the cell so that water enters and the cell expands (Rayle and Cleland, 1977; Bottger, 1986). RNA metabolism also effected by inducing the transcription of specific messenger RNA (mRNA) molecules (Bevan and Northcote, 1981). In tobacco cultures, auxins appear to stimulate the synthesis of a β-1,3-glucanase enzyme, which hydrolyses β-1,3-glucan

polysaccharides in the cell wall (Felix and Meins, 1985) thereby loosening the wall, and possibly also producing biologically active oligosaccharides. Ion exchange may occur because auxin indirectly stimulates the ATP-ase enzyme located in cell membrane which is responsible for the transport of H^+ and OH^- into and out of cells or increase the permeability of proteins and other ions.

Auxin is seems to be capable of erasing the genetically programmed physiology of whole plant tissues, which had previously determined their differentiated state. Cells which respond to auxin revert to a differentiated state and begin to divide. Lo Schiavo *et al.* (1989) observed that auxins cause DNA to become more methylated than usual and suggested that this might be necessary for the re-programming of differentiated cells. Tissue specific programmes specifically associated with differentiation would become eradicated by hypermethylation, with perhaps a small fraction of the cells reaching an ultimate state of differentiation in which they become capable of morphogenesis or embryogenesis.

The auxin most frequently employed to initiate callus culture is 2,4-D; but as cultures maintained on 2,4-D may be liable to become genetically variable, some investigators prefer to use NAA or IAA or to transfer callus to a medium containing one of these alternative compounds once it has been initiated by 2,4-D. To induce callus growth from explants of dicotyledonous plants, a cytokinin is almost always added to the medium in addition of an auxin. The presence of a cytokinin may not be necessary to obtain callus from explants of monocotyledons and in these plants a somewhat higher auxin concentration is usually used (2-10 mg/l).

The present results showed that somatic embryogenesis readily occurs *in vitro* on the both immature and mature cotyledonary tissues of *Camellia sinensis* cv. T383. Success depends on both the physiological maturity of the cotyledon and the kind of culture medium used. The fact is that the fully-grown but still immature September cotyledonary tissues had the highest rate of somatic embryogenesis than the mature cotyledonary tissues of November. The reduced embryogenic capacity of mature November cotyledon could be loss of competence associated with maturation. The embryogenic potential of the September cotyledon is of particular interest with a view to enable the efficient success of embryo conversion for molecular biological works. It is important to emphasize the positive response of the immature cotyledonary

tissues with relation to the kind of explants, especially with September seed cotyledons.

The present study improves the efficiency of the somatic embryogenesis system earlier described by Kato (1986) in *C. japonica* and *C. sinensis* and by Zhuang and Liang (1985) in *C. reticulata* and for *C. sinensis*, in which only cotyledon explants were used. It also enhanced the rate of embryogenesis from immature cotyledons than that of the results reported by Ponsamuel (1996) and by Jha *et al.* (1992) in *C. sinensis*, by using novel auxin PBOA (1 mg/l) (Roy, 2006a) alone or in combination with BAP (5 mg/l) or with Kinetin (5 mg/l). In a previous study it had been shown that 2,4-D, the auxin most widely used for embryogenesis culture by Evans (1981), induced callus but completely inhibited somatic embryogenesis, which is totally in contrary to the present findings. In the present study it has been observed that somatic embryogenesis was quite good in number (9-12%) in culture supplemented with 2,4-D (4 mg/l) in combination with IAA (1 mg/l) along with rhizogenesis, which was reverse result of the findings of Jha *et al.*(1992) in *C. sinensis* cv. T-78. Non-embryogenic friable callus development in some cases of mature cotyledon explants has been occurred but very rare in immature cotyledons.

Adventitious embryo formation from cotyledon culture has been reported in a few species of tea (*C. sinensis* L. cv. Yabukita) by Kato (1986), by Wachira (1995a) in *C. sinensis* and by Vieitez (1992) in *C. japonica*. It was obtained either in unsupplemented basal medium or in media supplemented with high cytokinin plus a low auxin or a cytokinin only (Nakamura, 1988; Kato, 1988, 1986). In the present study somatic embryogenesis was reported without growth regulators, which was not in accordance with the report of Jha *et al* .(1992). BAP (5 mg/l) or 10% CM can alone induce the somatic embryo development in 5% and 15% explants respectively. Somatic embryogenesis has been enhanced up to 40% by addition of novel auxin PBOA (1 mg/l) in combination with 5 mg/l BAP or Kinetin. Same type of results had reported earlier by Ponsamuel (1996) in *C. sinensis*.

BAP influenced the morphology of the somatic embryos produced in presence of novel auxin PBOA. Cytokinin effects of this kind on the embryogenesis. Highest rate of differentiation was reported in *C. sinensis* var. Yabukita by Nakamura (1988) in presence of 5 mg/l BAP in MS medium. The developing embryos passed through the typical globular, torpedo, and cotyledon stages in the course of their development

to reach maturity. Secondary somatic embryogenesis was noted in the present study when primary somatic embryo transferred to germination medium and maintained the secondary embryogenesis through successive subcultures.

Direct somatic embryo differentiation seems to ensure genetic stability. High rates of largely direct embryogenesis occurred on immature cotyledons of September in comparison to mature cotyledon of November in medium supplemented with BAP and PBOA; suggest that growth regulators increased the number of somatic embryos. Somatic embryo development in *Camellia* did not require two phases of culture procedure, initiation and maturation occurring on the primary medium (Tulecke, 1987). A different medium was needed for their germination, but sometimes-secondary embryo formation occurred in the germination medium too. The present results seem to indicate the general applicability of the methods described in the present work, to a wide range of tea genotypes, to obtain several cycles of subsequent embryogenesis to be used to produce artificial seeds or to produce genetically modified transgenic tea plants for the improvement of tea yield and quality. The present study reports the high rate of somatic embryogenesis and enhanced embryo conversion rate in elite tea clone, T383. Somatic embryo germination protocol has been finalized after a long trail in different combinations of media and hormones. Among the various combinations were tried in the somatic embryo conversion, the combinations MS + GA_3 1.5 mg/l + 0.5 mg/l IAA and MS + 2 mg/l Brassin was given 42.78% and 51.09% embryo conversion, respectively.

The duration of auxin requirement has been most clearly determined for the induction of somatic embryogenesis, where it is now well established that an initial application must be withdrawn to permit embryo development. In carrot 0.05 μM 2,4-D were required to be present for 6 days to induce competent single cells to form somatic embryos. Beyond this stage auxin was inhibitory. The process of somatic embryogenesis is often initiated in media containing high levels of auxins (especially 2,4-D), but embryos do not develop further until the auxin concentration is reduced. Lazzeri *et al.* (1988), in *Glycine max* observed a highly significant interaction between the concentration of auxin and sucrose in the medium. There was a high frequency of somatic embryo formation with 1-2% sucrose and 6.25-25 mg/l NAA or 4% sucrose and 50 mg/l NAA. Ranch *et al.* (1986) was used 5 mg/l 2,4-D with 6% sucrose to obtain maximum embryogenesis in soybean. Lippmann and Lippmann

(1984) have shown that 1 mg/l 2,4-D and 1% sucrose to be satisfactory in the efficient embryogenesis in soybean.

The growth (germination) of preformed somatic embryos of several different species can be stimulated by the incorporation of GA_3 (0.3-1 mg/l) into the second medium (post-initiation) (Lu and Vasil, 1982) in guinea grass. In some plants embryo root growth is especially promoted, in others (*Santalum album*) shoot regeneration is stimulated (Bapat and Rao, 1979; Shoyama *et al.*, 1988).

The acclimatization (hardening) and field performance of micropropagated plants is a particularly important aspect that needs utmost attention. Substantial number of micropropagated plants does not survive after transfer from controlled *in vitro* conditions to *ex vitro* environment of the greenhouse and later in the open field. The crucial part of any *in vitro* propagation system is mass and rapid production of plantlets which are phenotypically uniform and genetically similar to the mother plant, otherwise the advantage of desirable characters of elite/supreme clones will not be achieved. There are very few limited report has been carried out to assess the genetic fidelity of tissue culture raised tea plants. Phenotypic variation has been carried out by Vuylsteke *et al.* (1988) for the identification of variant plantlets among micropropagated plantlets. In the present investigation, 54% survival was achieved after hardening the plantlets in *ex vitro*.

The karyotypic analyses of *in vitro* regenerated tea plant were carried out by Jha *et al.* (1992) and the biochemical analysis by Damasco *et al.* (1996) in support of true-to-type of the genotypes. But these techniques are not suitable for assessment of the genetic variability in the tissue culture raised plants and under the influence of environmental conditions or developmental processes (Rani *et al.*, 1995). The kayrotype analysis cannot detect any DNA sequence polymorphisms in *in vitro* raised plants (Mondal *et al.*, 2004). The DNA based molecular markers such as RAPD, RFLP, ISSR, SSR, SNP, and CAPS, can be exploited regularly for the assessment of genetic fidelity of tissue culture raised plants (Isabel *et al.*, 1993; Rani *et al.*, 1995; Rani and Raina 2000; Mondal and Chand 2002; Devarumath *et al.*, 2002).

Thomas *et al.* (2006) studied the genetic fidelity of tissue culture regenerated plantlets from somatic embryo in tea using ISSR markers. Genetic variability of somaclones derived from single line cotyledonary culture ranged from 33.0 to 55.0%.

Out of 120 interactions attempted using Pearson's coefficient correlation, only 9.2% of somaclones exhibited significant similarity at genetic level. This strengthens the existence of wide genetic variation among the somaclones. The karyotype analysis has been done in the *in vitro* regenerated plantlets, T383 and showed true-to-type chromosome morphology, 2n=30, without any deviation. Although, DNA based molecular characterization is required to conclude that the regenerated plant is really without any somaclonal variation. Somaclonal variation is the inherent phenomenon of *in vitro* regenerated plants. The observation of same chromosome number in the tea clone *in vivo* as well as in the regenerated plantlets suggests chromosomal stability of the taxon.

It is difficult and time consuming task to improve the quality and yield through conventional breeding method in such a highly heterogeneous long-lived and cross-pollinated crops. Traditionally the aim of most tea breeders was to increase plant yield without loss of quality. In the increasingly competitive market new plants must carry the traits increase in both production and quality to make replanting old estates economically viable. Plant tissue culture methods can be effectively used in improvement of tea by generating fundamental information on growth, development, physiology, biochemistry and cytogenetics of cells or organs in culture under defined conditions and correlations are likely to emerge which are applicable to whole plants. Manipulation at the cellular and sub-cellular level and subsequent regeneration of complete plants can result in variants with vigour, improved quality and yield and resistance to both biotic (pests and diseases) and abiotic stresses (frost, drought, water logging, herbicides). Tissue and cell culture technology along with advances in the field of genetic engineering are likely to make a major impact on the industry in the areas of rapid and mass clonal propagation, disease elimination and resistance, production of pure breeding lines (through haploid technology), germplasm storage and exchange, interspecific and intergeneric huybridization, development of polyploids and mutants and the improvements in quality and yield (Jain and Newton, 1990). Plants regenerated from somatic cells do not have the unpredictable mixture of characteristics found in plants derived through sexual reproduction.

Plant regenerated through callus culture may be with somaclonal variation whereas direct organogenesis normally results in "true-to-type" plants. *In vitro* culture techniques are useful for mass multiplication and creation of genetic variability as

desired. In order to achieve mass propagation, particularly by the formation of somatic embryos, the need and practicability of standardization and automation deserve to be investigated.

Micropropagation using somatic embryos (directly) offers some advantages over other method of clonal propagation. Since this route has potential for producing greatest number of plantlets of uniform genetic-make up and the production cost is cheap and efficient. Due to the bipolar nature of the somatic embryos, rooted plants can be achieved easily and they can be used for long-term storage. Kato (1986) reported successful regeneration of complete plants from cotyledon segments through somatic embryogenesis in *Camellia sinensis* and *C. japonica*. Since then a number of successful reports have come out all over the world from somatic embryos in a number of *Camellia* sp. including *C. sinensis*. Tissue culture technology can be used for boosting natural variation. Culture of immature zygotic embryos (Palni *et al.*, 2006), obtained from incompatible crosses and subsequent development of complete plants useful for wide hybridization. Tissue culture can be exposed to noxious substances (e.g. herbicides) or subjected to other abiotic or biotic stresses. Under these conditions most cells would perish except a few, if such cells can be regenerated into plants, then those plants and their progeny may also be tolerant or resistant. This method of mutant selection has already been successful with some plant species, and investigations in this direction on tea may yield useful results. In this method, a single gene controlled trait such as tolerance to other desirable traits such as high yield, time to flowering and flavour controlled by many genes may not be amenable in this selection system.

In the present investigation, cytological study was carried out in ten tea cultivars, T78, T383, TV30, HV39, TeenAli17/1/54, TV23, TV25, TV29, TV26, and UPASI-26, for their characterization at the chromosomal level. The chromosomal analysis in tea has been investigated with a view to gaining insight into the cytogenetic situation in these cultivars of *Camellia sinensis*. The taxon reveals 30 chromosomes in their somatic complements (Bezbaruah,1971). The present observation strengthens the concept of numerical uniformity in the chromosome complement 2n= 30 in cytological investigation. A distinct similarity was noted in general morphology of chromosome of the investigated ten tea cultivars. The homogeneity was represented not only in the numerical uniformity and gross

structural similarities of chromosomes, but also in the significant coincidence of total chromatin material between the members.

The taxa bearing such striking resemblance in cytological features, however, differ in details of karyotype features, especially with regard to the number of chromosomes with secondary constrictions. The presence of 3 pairs of nucleolar chromosomes is the characteristics of *C. sinensis* namely TV-23, and TV-25 and TV26 (Roy, 2006). Total chromosome length ranged from 1.24 μm to 4.20 μm in the investigated cultivars. Other minor differences in many karyotype involving the absence or variable number of a given chromosome type constitute chromosomal basis of further intervarietal differentiation in *C. sinensis*. The disparity index which is significantly high in all the investigated cultivars further indicates heterozygous constitution of the varieties which have probably arisen during long cultivation, selection, and maintenance through vegetative propagation. The significance of structural alternations of chromosomes in evolution and speciation had often been underestimated in the past due to over emphasizing the role of mutation in evolution. In the recent past with the aid of improved chromosome techniques, it has been possible to work out the chromosomal basis of intervarietal and even inter strain differences in a number of cruciferous taxa mentioned earlier. Very recently similar important role of chromosomal alternations in interspecific and intervarietal diversification of *Trichosanthes* have been emphasized by De Sarkar *et al.* (1987).

In the present investigation, partial genomic DNA sequence (201 bp) of chitinase gene has been amplified in PCR reaction. Chitinase gene specific 20-mer primer pair was constructed from the known chitinase gene sequence of plant using Primer3 software program. After the 35 cycles in a thermal-cycler, the PCR product was separated in 1% agarose gel and visualized on UV-transilluminator. It was observed that a distinct band was present at the DNA marker level of approximately 200 bp size on the gel. After purification of the chitinase gene specific PCR product, the DNA fragment was ligated into the T/A cloning vector pGEM-T *Easy* vector for multiplication of the insert. The PCR product with 3′ overhanging of single A-residue was cloned into the T/A cloning vector because it is easily ligated into the vector due to the presence of 3′ overhanging of single T-residue in the pGEM-T Easy vector.

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Ligated vector (pGEM-T Easy vector) was reintroduced through transformation into the host cell *E. coli* JM109, in a transformation reaction mixture. Transformed and non-transformed host cells as well as recombinant and non-recombinant vectors were discriminated in a single selection (Blue-white colour selection) medium (LB-ampicillin agar plate) with chromogenic substrate X-gal and inducer, IPTG. IPTG induces the synthesis of β-galactosidase enzyme in the host cell through α-complementation. The α-complementation is only possible if there is no insertion of the foreign DNA into the pGEM-T Easy vector and produces blue colour in the selection medium due to the production of functional β-galactosidase enzyme which acts on X-gal. The recombinant white colony with PCR insert was picked through sterile toothpick and transferred to a fresh liquid LB-ampicillin medium for multiplication of the desired insert (Chitinase gene fragment) by keeping at 37°C overnight at 200 rpm agitation.

The recombinant plasmid vector was isolated and purified for sequencing the DNA insert. DNA sequencing was done bidirectionally by using BigDye terminator technology (Applied-Biosystem, at Bangalore Genei, Bangalore, India). The 201 bp chitinase gene specific DNA sequence was deposited to the GenBank database of NCBI for public use (Accession no. EF763751). The efficiency of transformation was 60-80% clones per plate in this procedure.

The present study has been conducted to look inside into the chitinase gene of tea plant. The deduced partial nucleotide sequence (201 bp) of chitinase gene will be used as a probe to clone the full length chitinase gene from the cDNA as well as genomic DNA library in future. That will help the researcher to study the chitinase gene of tea plant in more details with respect to *exon-intron* boundary, promoter sequence and others. This will utilized in the improvement of tea production and quality by manipulating the chitinase gene in future in order to improve the plant immunity against the fungal pathogens to manage the disease manifestation without applying any health hazardous chemical such as pesticides/fungicides.

Members of the chitinase gene family are found in all plants, which express inducibly as PR-3 proteins and constitutively in tissues vulnerable to pathogen attack (Samac *et al.*, 1990; Collinge *et al.*, 1993). Several lines of evidence indicate that chitinases play a direct role in plant defense by attacking chitin, a β-1,4-linked polymer of N-acetyl-D-glucosamine, a major component of fungal cell walls. Purified

chitinases can inhibit hyphal growth *in vitro* (Broglie *et al.*, 1991) and chitinolytic breakdown products induce the production of defense compounds (phytoalexins) and systemic acquired resistance. These antifungal properties are greatly enhanced in the presence of β-1,3-endoglucanase, another PR-protein that attacks the glucan matrix in which chitin is embedded (Collinge *et al.*, 1993; Collinge., 2003). The acidic and basic chitinase genes have isolated and characterized fully in *Arabidopsis thaliana* (Samac *et al.*, 1990). Genes for chitinases have been analyzed at the molecular evolutionary level in maize and family poaceae (Wu *et al.*, 1994; Peter Tiffin, 2004). Plant-pathogen co-evolution is analyzed in *Arabis* sp, in relation to class-I chitinases (Bishop *et al.*, 2000). Sekeli *et al.* (2003) have isolated and cloned chitinase-I gene from winged bean seed and characterized its structure in relation to defense system. Chitinase gene fragments were isolated from a winged bean seed cDNA library using two sets of degenerate primers corresponding to the conserved regions of chitinase class I and IV proteins. Isolation and characterization of chitinase genes from pitchers of the carnivorous plant *Nepenthes khasiana* has been conducted by Eilenberg *et al.* (2006). Metraux and John, (1989) have studied in details the chitinase gene isolation and cloning. Roby *et al.* (1991) studied the regulation of chitinase *in vitro* in protoplast culture inducing the defense system with ethylene and other elicitors. Samac *et al.* (1990) have critically demonstrated the isolation and cloning of chitinase gene in model plant *Arabidopsis thaliana.* Datta *et al.* (1999) has shown that transgenic rice plant over expressing PR-5 gene can be more resistance against sheath blight disease pathogen *Rhizoctonia solani.* Plants over-expressing chitinases under the control of a strong constitutive promoter have been engineered and have shown improved resistance against fungal pathogens under laboratory conditions (Broglie *et al.*, 1986; Broglie *et al.*, 1991). Sharma and Kumar (2005) have studied the PR-5 (Pathogenesis related protein) induction in the drought conditions in tea plant. These enzymes can inhibit the growth of fungal hyphae *in vitro* (Schlumbaum *et al.*, 1986; Huynh *et al.*, 1992). Some chitinases are induced following pathogen infection (Wu *et al.*, 1994), and the overexpression of at least some chitinases in transgenic plants causes significant reductions in pathogen damage (Broglie *et al.*, 1991). Taken together, these observations support the notion that a primary function of plant chitinases is in defending plants against attack by fungal pathogens, although there is also evidence that chitinases may function as lysozymes degrading bacterial cell walls and may play a role in developmental processes (Passrinho and De Vries, 2002). The

production of transgenic plants with enhanced resistance to attack by the fungal pathogen *Rhizoctonia solani* has been developed (Broglie *et al.*, 1991).

In the present study, effect of methyl jasmonate on gene expression was studied in tea (clone T383) at four different developmental stages-young leaf, mature leaf, *in vitro* grown callus tissues and *in vitro* regenerated shoots of somatic embryos. Defense related differential gene expression was induced by the treatment of methyl jasmonate in leaves, callus and regenerated shoots in tea where constitutive expression was low. Chitinases, as a PR-3 protein family, are well characterized for their function as pathogenesis-related proteins in many plant species (Bishop *et al.*, 2000). Chitinase may function in plant defense as inducible and /or constitutively expressed genes in many cases (Heil and Bostock, 2002), and have been shown in several cases to be inducible by wounding, ethylene, and methyl jasmonate (Zhao and Chye, 1999; Wu and Bradford, 2003).

In the present study, results hint at the complexity of the interaction between methyl jasmonate and tea leaves and tissues. The response of tea leaves and tissues to methyl jasmonate was strongly influenced by the stage of leaf development at different time courses (0, 0.3, 2, 6, 12, 16, 24, 36, 48, and 72 h). It has found that transcript accumulation was induced by methyl jasmonate in mature leaves where the quantities of accumulation were more than in the callus tissues. In callus tissues, transcript accumulation was very low. As described here, accounting for tissue ontogeny is critical when studying the molecular responses of tea in defense induction to methyl jasmonate. The constitutive and inducible defense strategies used by tea are dependent on the developmental stage of the tissues involved. With this understanding, differences in constitutive and inducible expression of tea defense genes in divergent tea germplasm source can be more accurately characterized. Chitinase gene specific mRNA accumulation was high in treated mature leaves in tea and least amount of transcript was accumulated in callus. Total RNA quantity in each control sample was (µg/g fresh weight) 657, 660, 645 and 651 in YL (young leaf), ML (mature leaf), callus and shoots, respectively. After 16 h of treatment with MeJa the total transcription levels were increased in each treated samples. The RNA quantities (µg/g fresh weight) were 684, 698, 662 and 680 in YL, ML, callus and shoot, respectively. The results indicated the sharp enhancement of defense protein gene expression in tea by MeJa including the chitinase gene. The peak accumulation

was observed after 16 h of spray of MeJa. If, it would not have been induced by the MeJa induction, total RNA quantity would not be increased as much. The total RNA consisting of mRNAs, rRNAs, tRNAs,and other special RNAs. Within the total mRNAs, chitinase gene specific mRNA molecules are present at its own concentration. Subsequently, this chitinase gene specific mRNAs are amplified through reverse transcriptase-PCR (RT-PCR) to quantify the mRNA transcript accumulation during ISR.

In the present investigation, chitinase gene specific mRNA has been converted to cDNAs and amplified *via* normal PCR amplification by using gene specific primer in One-step RT-PCR system. The RT-PCR produced 254 bp PCR product in all the samples tested but the quantity was different in tissues harvested after induction with Meja in different time courses (0, 0.3, 2, 6, 12, 16, 24, 36, 48, and 72 h). Chitinase gene specific RT-PCR product of 254 bp cDNAs concentration was more in induced material in compare to control material. RT-PCR was produced more number of cDNA copies of an mRNA whose copy number is more in the stating material and amplify accordingly. mRNA copy number can be determined in the competitive qRT-PCR using mimic template or cRNA as known copy number.

In the present study, we have done semiquantitative RT-PCR without mimic or cRNA template. In the present RT-PCR, 15µg RNA (total, including the chitinase gene transcript, mRNAs) was taken from each of the treated sample in different time course as a starting material. After the amplification in RT-PCR, the cDNA product was extracted, purified and quantitated with spectrophotometer. It was observed that amount of cDNA production has been increased variously in different treated samples, indicating the induction of chitinase gene specific transcription. Quantity of cDNA was 203, 253, 154 and 183 µg (in respect of starting RNA material, 15µg) in YL, ML, callus and shoot, respectively, because the mRNA has been amplified so many times. It was chitinase gene specific transcript accumulation because, primer pair was chitinase gene specific (20-mer), and given the product of 254 bp in size.

In the present investigation, a second cDNA product of 366 bp size was observed while RT-PCR reaction was done by using DOP-primer (34-mer) that was constructed on the basis of chitinase gene specific conserved protein sequences. The RT-PCR product of 366 bp was sequenced bidirectionally through the BigDye terminator technology (Applied-Biosystem, at Bangalore Genei, Bangalore, India). The

sequence information was then analyzed through BLASTN program which indicated that the 366 bp sequence contains the genetic information of basic chitinase gene of *Nepenthes khasiana* (1717bp, Accession no. gb|AY61883.1|). The result of GENSCAN showed that it can encode protein peptide containing 81 amino acids. The sequence of 81 amino acids was as follows,-

MGAVEGYRIAGGPLGEVTDPLYPELKVKEIKNGRLAMFSMFGFFVQAIVTGKGPLEN LADHLADPVNNNAWAYATNFVPGK.

When the protein-protein blast was done in TBlastX, the result has shown the significant alignments with chitinase protein, Accession number AY618881.1 of *Nepenthes khasiana* basic chitinase gene (complete cds, length=1572 bp). The sequence information of 366 bp was submitted to the GenBank of NCBI and after complete annotation of the data an accession no.EU373553 was approved and it was considered as a hypothetical protein gene of *Camellia sinensis*. The presence of this hypothetical protein encoding 81 amino acids confirming the earlier observation of Bailey *et al.* (2005) that during induced systemic resistance, induction with specific inducer can enhances the differential gene expression of some of the proteins that may be DNA binding protein, cell division regulatory protein, peroxidases, glucanases, chitinases and caffeine or light harvesting proteins.

In the present investigation, after induction with Meja, it induces some of the defense related genes involved during the induced systemic resistance and stimulating to express any one of the above mentioned defense related proteins through mRNA transcription, that has been amplified in this RT-PCR reaction, giving 366 bp cDNA fragment in case of DOP-primer and 254 bp in case of chitinase specific primer. In response to the induced systemic resistance plant induces to express various genes to protect themselves against pathogen attack through defense signaling pathways. The amplification of 366 bp cDNA in the RT-PCR reaction strengthening the observation that during induction plant may synthesize various proteins including pathogenesis related proteins to combate the disease invasion.

Elevated mRNA levels were detected in mature and young leaves after treatment with methyl jasmonate. The present result was consistent with observation of Bailey *et al.* (2005). Enhanced level of mRNA transcription was observed in *Theobroma cacao,* in response to mechanical wounding, ethylene and methyl jasmonate treatment. The level of induction exceeded 7 times control levels in both

young and mature leaves in *T. cacao.* The wounding induced expression of a protein which is a DNA binding protein was observed after 0.25 h, but declined to near control levels by 4 h after wounding. Differential expression was observed for genes putatively encoding a protein regulating cell division, a type III peroxidase, an endo-1,4-β-glucanase, a class VII chitinase, a caffeine synthase and a light-harvesting complex protein (Bailey *et al.*, 2005). Methyl jasmonate spray induced polyphenol oxidase (PPO) expression in hybrid poplar (Constabel *et al.*, 2000). PPO mRNA transcription level was increased after the treatment of 8 h and peak accumulation was observed at 16 h. Jasmonate and related octadecanoid metabolites are known inducers of defense responses against pests and pathogens in many species (Weiler, 1997). It has been observed that jasmonate-mediated response is essential for defense (Doares *et al.*, 1995; Howe *et al.*, 1996). Wounded poplar hybrid systemically accumulates mRNA encoding chitinase, β-glucanase and a bark storage protein like polypeptide (Davis *et al.*, 1993). *In vitro* studies have demonstrated further that plant chitinases are capable of hydrolyzing the cell walls of plant pathogenic fungi (Wargo, 1975) and releasing elicitors of defense reactions (Hadwiger and Beckman, 1980). Chitinase gene expression has been shown to be transcriptionally regulated by a number of inducers, including ethylene, elicitors, and pathogen attack (Broglie *et al.*, 1991). Recent studies indicate that the modulation of chitinase levels in plants involves the activation of gene transcription (Hedrick *et al.*, 1988). *In vitro* grown protoplast can be an excellent model system for such defense mechanism induction system that is responsive to ethylene and elicitor treatment (Brogleie *et al.*, 1986, 1999; Howard *et al.*, 1987; Dron *et al.*, 1988; Dangl *et al.*, 1987; Abeles *et al.*, 1971; Roby *et al.*, 1987 and Roby and Esquerre-Tugaye, 1987).

The knowledge gained concerning the chitinase gene, as methyl jasmonate induced plant defense genes, can be exploited in studying the response of tea to diverse plant pathogens and insect pests. In addition, the newly gained understanding of the responses of tea to methyl jasmonate will be used to identify beneficial organisms and abiotic treatments capable of inducing resistance to diseases through pathways involving inducible mechanisms.

SUMMARY

In the present investigation, genetic diversity and phylogenetic relationship has been revealed in twenty one tea (*Camellia sinensis*) genotypes on the basis of RAPD and ISSR based DNA fingerprinting. These DNA based markers have offered a possibility to study the genome directly and are least affected by environmental factors. Thus eliminate the shortcomings inherent in a phenotype observation and are almost numerous in number. Genomic fingerprinting was carried out using 7 ISSR and 12 RAPD primers. The DNA fingerprinting results indicated that the percentage of ISSR based polymorphism was 88.54% which was higher that of the RAPD based polymorphism, 77.77%. The number of alleles ranged from 4 to 9 per primer, with an average of 6.5 per primer in case of RAPD based fingerprinting. The number of scorable alleles per ISSR primer was in between 9 to 11 with an average of 10 alleles per primer. The seven ISSR and twelve RAPD primers were used in the present study. The size of amplification products in both the marker systems ranged between 250-2500 bp.

Binary data matrix was prepared on the basis of polymorphic bands. The binary data was used to calculate genetic similarity matrix among the twenty one tea genotypes. According to Nei and Li's method genetic similarity coefficient was varied from 0.898 to 0.673 with an average of 0.785 in case of ISSR markers and 0.848 to 0.194 with an average of 0.521 in case of RAPD markers. The Jaccard's similarity coefficient values were varied from 0.821-0.643, 0.733-0.346 and 0.777-0.424 in ISSR, RAPD and in pooled data of ISSR and RAPD markers, respectively. Dendrograms were constructed on the basis of genetic similarity matrix using the UPGMA algorithm in SAHN clustering module from NTSYS-pc software (version 1.5) by using the two different methods of coefficient such as Jaccard's coefficient (1908) and Nei and Li's (1979) coefficient.

Both the dendrograms showed three clusters dividing the twenty one tea genotypes into three groups-China type, Assam type and Cambod type group. China type is comprised of tea genotypes T383, T78, T135, HV39, TeenAli17, AV2 and BS/7A/76; Assam type comprised of UPASI-3, UPASI-9, UPASI-26, TV20, TV21, TV27 and TV28, and Cambod type consisting of tea genotypes TV18, TV19, TV22,

TV23, TV25, TV26 and TV30. Three clusters in the dendrogram were congruent with the taxonomic position of the tea species, *Camellia sinensis*. In nature, there are three tea populations comprising of population of *Camellia sinensis* var. *sinensis* (China type), *C. sinensis* var. *assamica* (Assam type) and *C. sienesis* subspecies *lasiocalyx* (Cambod type). The Pearson's correlation coefficients between different matrices analyzed by the Mantel test (Mantel 1967) were found to be highly significant (r = 0.678–0.849, $p < 0.001$).

Genetic diversity and heterozygosity was measured using the Nei's genetic diversity index and analyzed in POPGENE software to partition genetic diversity within and between populations. Genetic heterozygosity was ranged from $H = 0.129$ to $H = 0.435$, among the populations. Accordingly average genetic diversity within tea population was 0.25, 0.30 and 0.33 in Cambod, Assam and China tea, respectively. The results were indicating that China variety displayed highest level of variability (0.33), intermediate diversity showed by Assam variety (0.30) and least diversity was showed by Cambod variety (0.25). The Shannon's information index (I) was 0.31, 0.23 and 0.20 for China, Assam and Cambod variety on the basis of RAPD and ISSR data. The genetic diversity over all groups (H_T) on an average was 0.38, diversity within populations (H_S) was 0.27, and genetic differentiation (G_{ST}) between populations over all loci was 0.25. This indicated that only a low (25%) proportion of diversity is observed between populations as compared with diversity within populations (75%).

The China variety had shown the largest within group diversity (Hs = 0.285 - 0.291), the Cambod tea had the least diversity (Hs = 0.193 - 0.207) while moderate diversity was existed in Assam tea (Hs = 0.223 – 0.241). Interpopulation gene flow [$N_m = 0.5(1 - G_{ST})/G_{ST}$] was 0.76, $N_m < 1.0$ showing the limited genetic exchange among populations. Highest gene flow occurred between Assam and Cambod variety (0.878), while lowest (0.673) was between China and Cambod. AMOVA results (analyzed by WINAMOVA software ver.1.55) revealed that the variance components among groups and among individual samples within groups were 29% (28.87) and 71% (71.13), respectively, which was almost in concomitant with the results of G_{ST}. With this view of genetic similarity, it is noteworthy that China type tea, the most diverse population should be conserved properly along with other varietal genotypes to enhance the genetic base of the germplasm stock of the indigenous tea for the

future improvement of commercial tea through breeding and genetic technological approaches.

One of the PR genes, chitinase was amplified through PCR reaction using genomic DNA of tea clone T383. Chitinase gene specific primer pair was used in the PCR amplification system, which produced 201 bp PCR product of chitinase gene specific. Gene specific 201 bp DNA fragment was cloned into T/A cloning vector (pGEM-T Easy vector) for multiplication of the insert and to facilitate the DNA sequencing. The *E. coli* host cell JM109 was transformed with the recombinant plasmid vector pGEM-T *Easy* and then the recombinant colonies were selected from the blue-white colour screening plate. White colonies with recombinant vector were picked and multiplied in LB-ampicillin medium for overnight. pGEM-T *Easy* vector with 201 bp PCR insert was isolated and purified for sequencing the fragment.

Sequencing was done bi-directionally by the Big-Dye Terminator method of Applied-Biosystem. The 201 bp chitinase gene specific sequence was analyzed and deposited to the GenBank of NCBI (Accession no.EF673751). Caffeien synthase gene specific genomic DNA (from two tea clones, HV39 and T383) was also amplified by PCR reaction using gene specific primer pair. PCR products of 200 bp from T383 and 202 bp from HV39 were purified and sequenced by the same method of chitinase gene. After the analysis both the caffeine synthase gene specific partial DNA sequence was deposited into the GenBank database of NCBI (Accession no. AY599069 for 202 bp of HV39 and Accession no. AY601112 for 200 bp of T383).

In vitro callus culture was initiated from four tea clones (T383, TV30, HV39 and UPASI-26) using stem explants in MS and B5 basal medium and various combination of plant growth regulators. The growth index (GI) was highest 1.48 in MS medium and 1.18 in B5 medium in tea clone T383 after 8 weeks of culture initiation. Plant growth hormones have the potentiality to enhance the callus growth and development. Growth index (GI) was 3.18 when callus was transferred to MS medium containing 4 mg/l 2,4-D and 1 mg/l BAP and 2.94 in MS medium supplemented with 4 mg/l NAA and 1 mg/l BAP after 8 weeks of culture. Growth rate was increased when 4 mg/l Kn added to the MS medium. The remarkable growth stimulating property of coconut milk had been observed in the present study. Growth index was 2.94 in MS medium with 15% coconut milk, which is comparable to hormonal effects on callus growth. Shooting was better (74%) while the callus was

cultured in MS medium with 3 mg/l TDZ and 2 mg/l NAA. Root formation was initiated in ½ MS medium with 1 mg/l IBA and transferred to only ½ MS for the normal growth of the shoots and roots. Somatic embryogenesis was carried out using immature and mature cotyledonary tissues of tea clone T383. Somatic embryo induction rate was high when immature cotyledon was used as explants in MS medium with 1 mg/l PBOA and 5 mg/l BAP. The 45 (in number) somatic embryos were formed per explant after 14 weeks of culture.

Morphologically three distinct types of somatic embryos were developed in immature cotyledons these were 'Seed-like' embryos, 'Cup-shaped' embryos, and 'Bud-like' embryos. Secondary embryogenesis was observed during the maintenance of somatic embryos. Among the three types of embryos, 'Bud-like' embryos had a highest germination rate 42.78% with subsequent root and shoot development in MS-medium with GA_3 (1.5 mg/l) + IAA (1 mg/l). Somatic embryo germination rate was increased to 51% in MS medium containing only Brassin 2 mg/l. The success rate in the acclimatization process of the somatic embryo regenerated plantlets was 54% in 95% relative humidity.

Somatic chromosome complement was studied in ten tea cultivars, T78, T383, TV30, HV39, TeenAli17/1/54, TV23, TV25, TV29, TV26, and UPASI-26, for their characterization. Karyotypes of the chromosomes ($2n = 30$) were grouped arbitrarily on the basis of their length and position of the centromere into four types (A-D). Centromeric index (F %), total centromeric index (TF %), disparity index (DI) and total haploid chromosome length (TCL) were calculated. Chromosomes were found to be short to medium in size varied in length from 1.24 µm to 4.20 µm. Karyotypes were gradate and asymmetric in nature. On the basis of karyotype analysis, varietal distinction can be marked to some extent.

Mature leaf, young leaf from 12 month old plant (*in vivo*) and Callus (2 month old) and Shoot (6 month old) from somatic embryo (*in vitro*) of the tea clone (T383) were used for the study of induced systemic resistance after induction with methyl jasmonate in different time intervals (0. 0.3, 2, 6, 12, 18, 24, 36, 48, and 72 h). During time course accumulation of chitinase (PR-3) enhanced which triggered the defense in tea plants. As a result, PR genes have been expressed first as a mRNA transcripts and then translated to proteins. The transcripts accumulation during various time courses has been quantified by measuring the total RNA from the four treated and four control

samples. Total RNA concentration at control level was 657, 660, 645 and 651 µg/g fresh weights and after 16 h of induction were 684, 698, 662 and 680 µg/g fresh weights in mature leaves, young leaves, callus, and shoot, respectively. Total RNA transcript accumulation was enhanced highest 2-6 times after 16 h of induction. For chitinase gene specific transcript accumulation after induction, RT-PCR reaction was carried out to amplify the single mRNA transcript expression during induced systemic resistance. Same quantity of RNA (15µg) was taken from each control and treated samples of four tissue samples. RT-PCR products (cDNAs) were quantitated by spectrophotometry. After 16 h induction the transcript accumulation was highest in mature leaf 253µg cDNAs/ 15µg RNA samples and least was in callus tissue 154 µg cDNAs/ 15µg RNA samples.

The results indicated that induction of defense mechanism was highest in mature leaf followed by young leaf (214µg cDNAs/ 15µg RNA samples). Chitinase gene specific RT-PCR produced (cDNAs) size was 254 bp. It was chitinase gene specific transcript accumulation because, primer pair was chitinase gene specific (20-mer primer), and given the product of 254 bp. RT-PCR product quantity was more in induced material in compare to control material. DOP-primer was given the RT-PCR product of 366 bp cDNA, which was sequenced for analysis and after data analysis it was deposited to the GenBank database of NCBI (Accession no. EU373553). The amplification of 366 bp cDNA in the RT-PCR reaction strengthening the observation that during induction plant may synthesize various proteins including pathogenesis related proteins to combate the disease invasion. Elevated mRNA levels were detected in mature and young leaves after treatment with methyl jasmonate. The understanding about the chitinase gene specific transcript accumulation during induce systemic resistance after induction with methyl jasmonate, it can be exploited in studying the response of tea to diverse plant pathogens and insect pests.

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