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.