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.

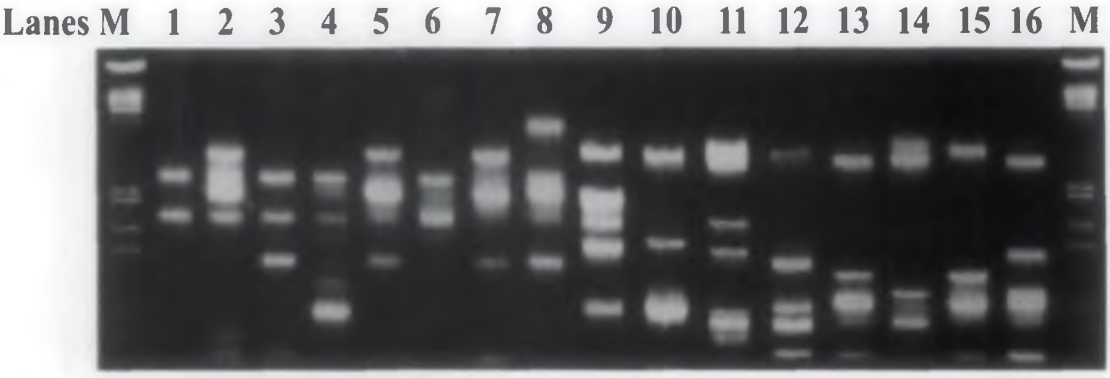


Fig. A: RAPD profile of the four selected tea genotypes generated by four different primers (Lanes 1-4 for L5; 5-8 for OPA-16; 9-12 for OPA-18 and 13-16 for OPA-19). Lanes 1,5,9 & 13 for clone HV39; 2,6,10 & 14 for clone T383; 3,7,11 & 15 for clone TeenAli17; 4,8,12 & 16 for clone T78. Lane M, Lambda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

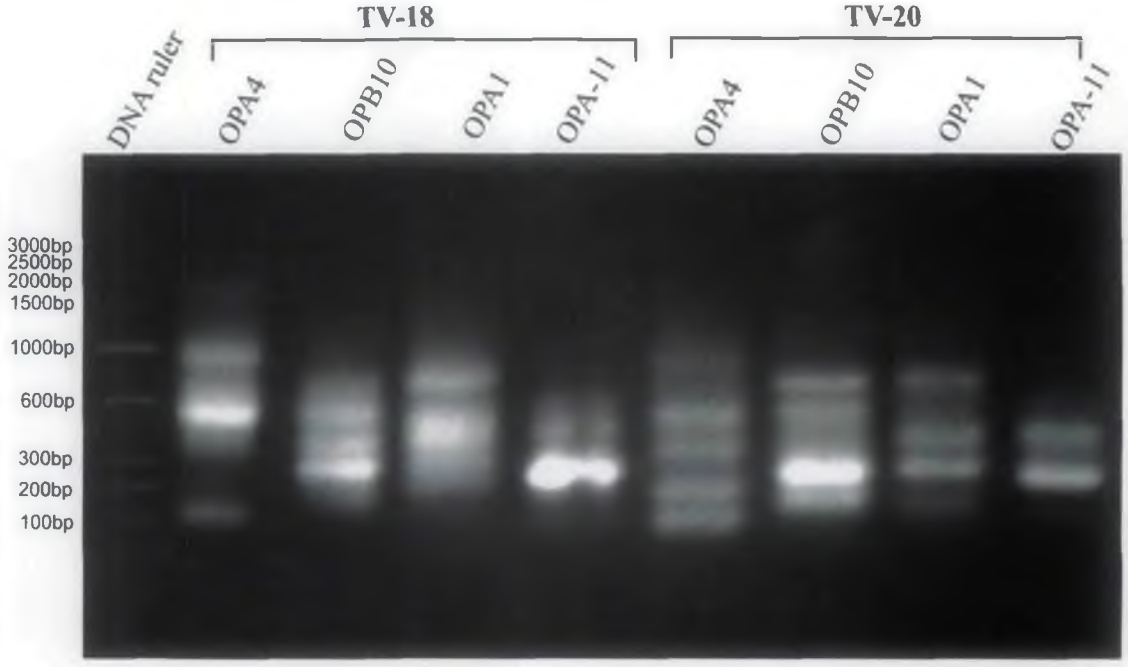


Fig. B. RAPD based DNA fingerprinting of two tea cultivars *viz.*TV18 and TV20 using four random primers of 10-mer.

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.

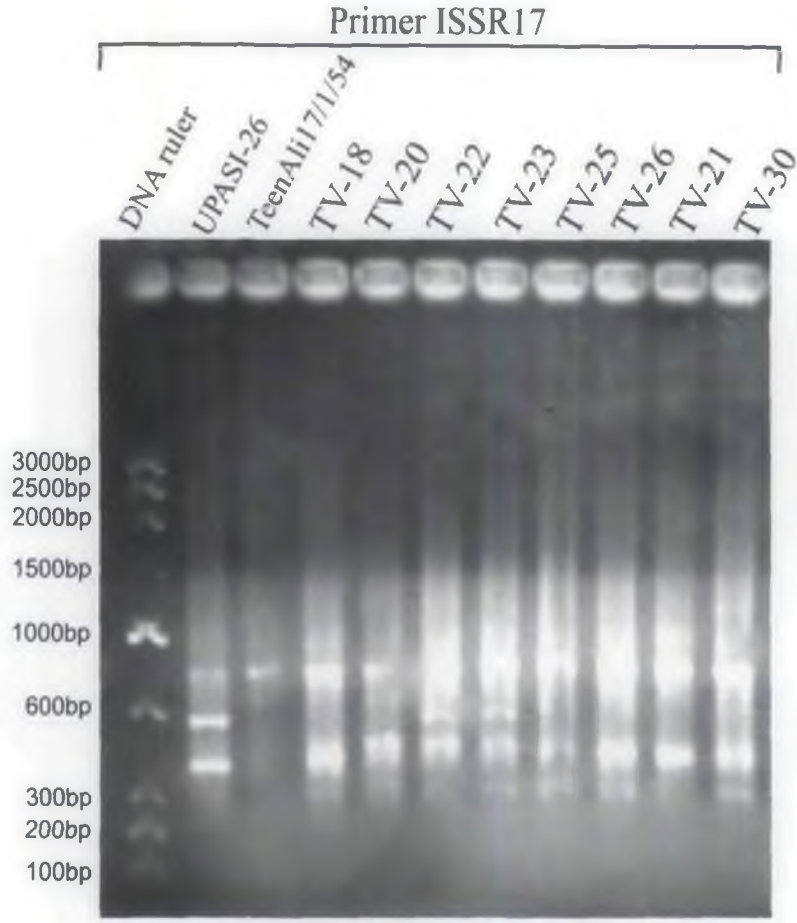


Fig. A: DNA fingerprinting of ten tea cultivars viz. UPASI-26, TeenAli17/1/54, TV-18,TV-20,TV-23,TV-25,TV-26, TV-21 and TV-30 using ISSR17 primer.

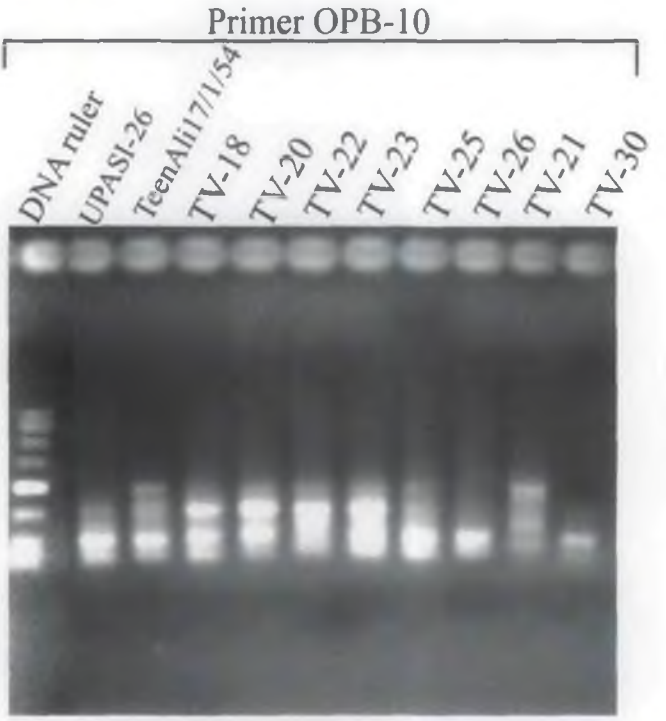


Fig. B: RAPD based DNA fingerprinting of ten tea cultivars *viz.* UPASI-26, TeenAli17/1/54, TV-18, TV-20, TV-22, TV-23, TV-25, TV-26, TV-21 and TV-30, using one random 10-mer primer OPB-10.

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).

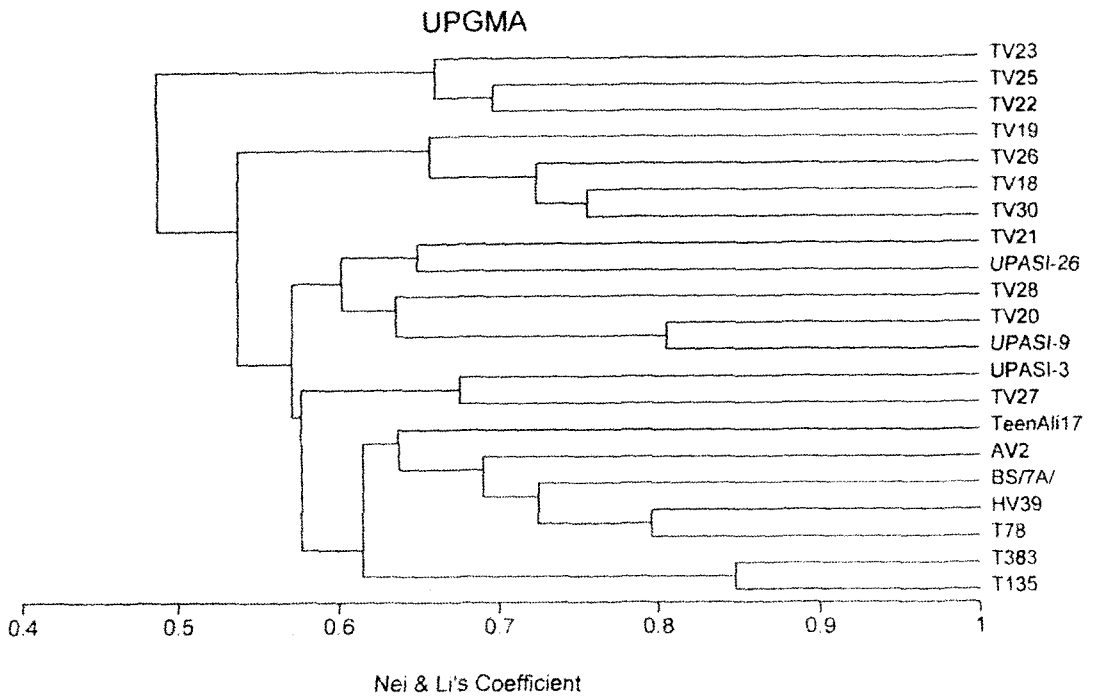


Figure 2. Dendrogram showing the genetic relationship among the twenty one tea cultivars based on RAPD markers using Nei & Li's (1979) coefficient.

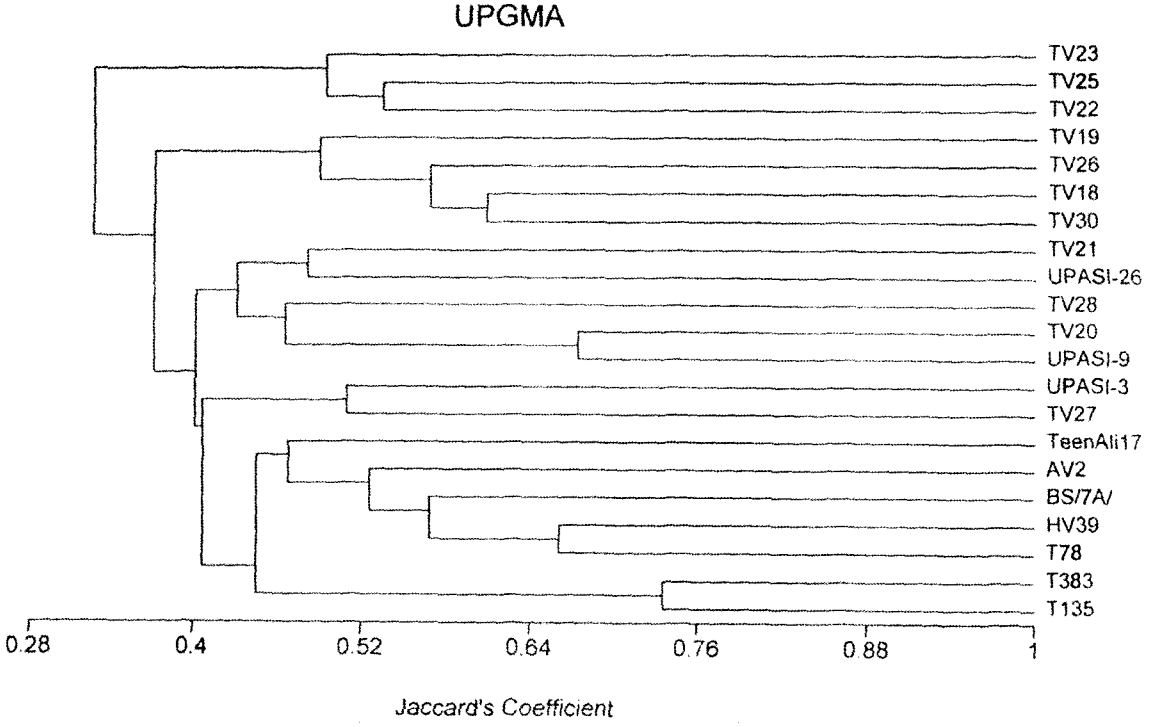


Figure 3. Dendrogram showing the genetic relationship among the twenty one tea cultivars based on RAPD markers using Jaccard's (1908) coefficient.

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.

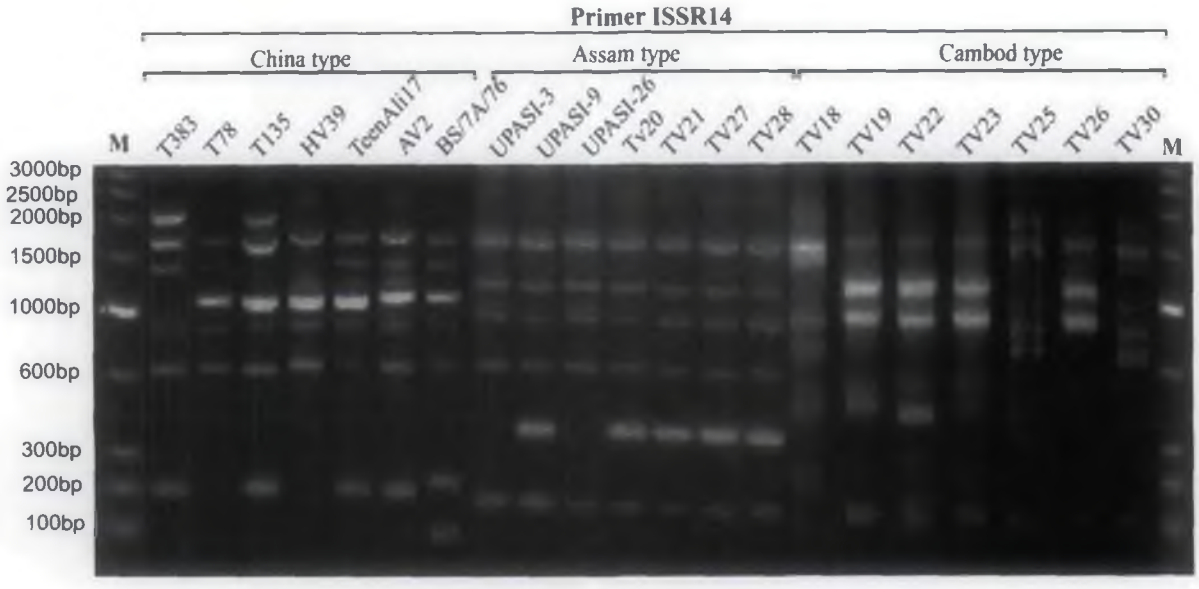


Fig.A: DNA fingerprinting of 21 tea genotypes based on ISSR markers. Lane, M for low range DNA ruler marker.

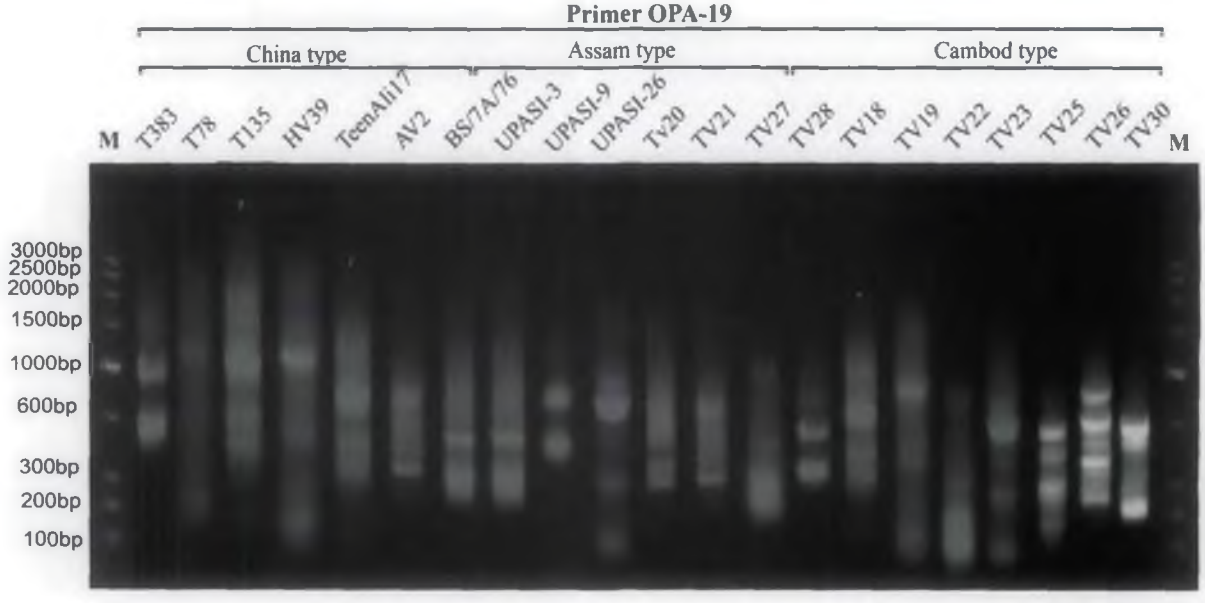


Fig.B:DNA fingerprinting of 21 tea genotypes based on RAPD markers. Lane, M for low range DNA ruler marker.

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

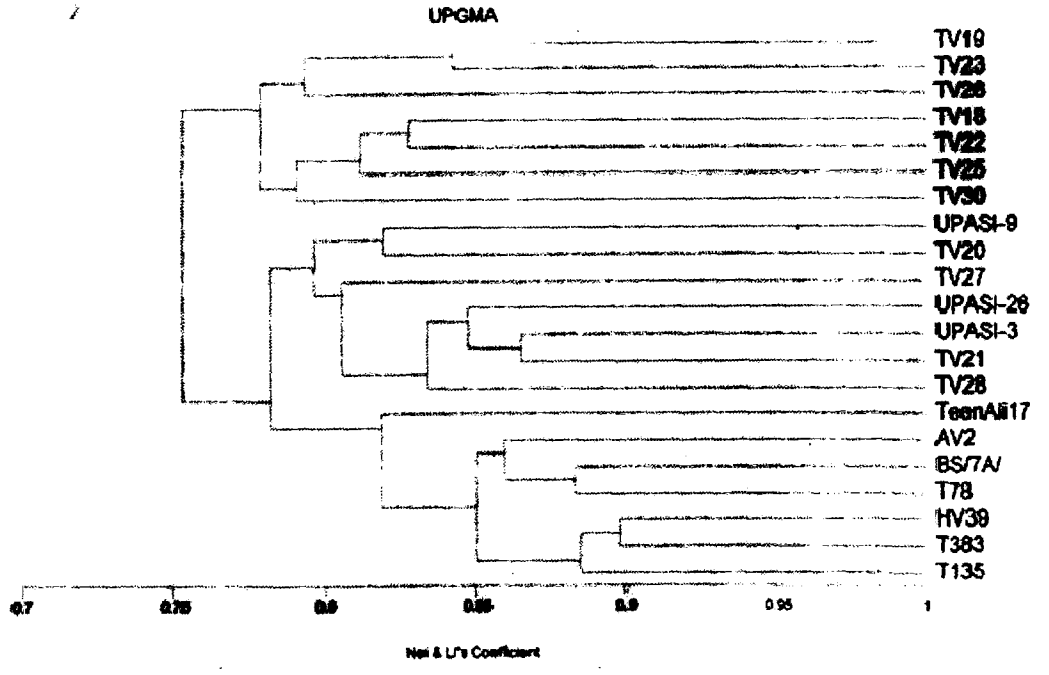


Figure 4. Dendrogram showing the genetic relationship among the twenty one tea cultivars based on ISSR markers using Nei & Li's coefficient.

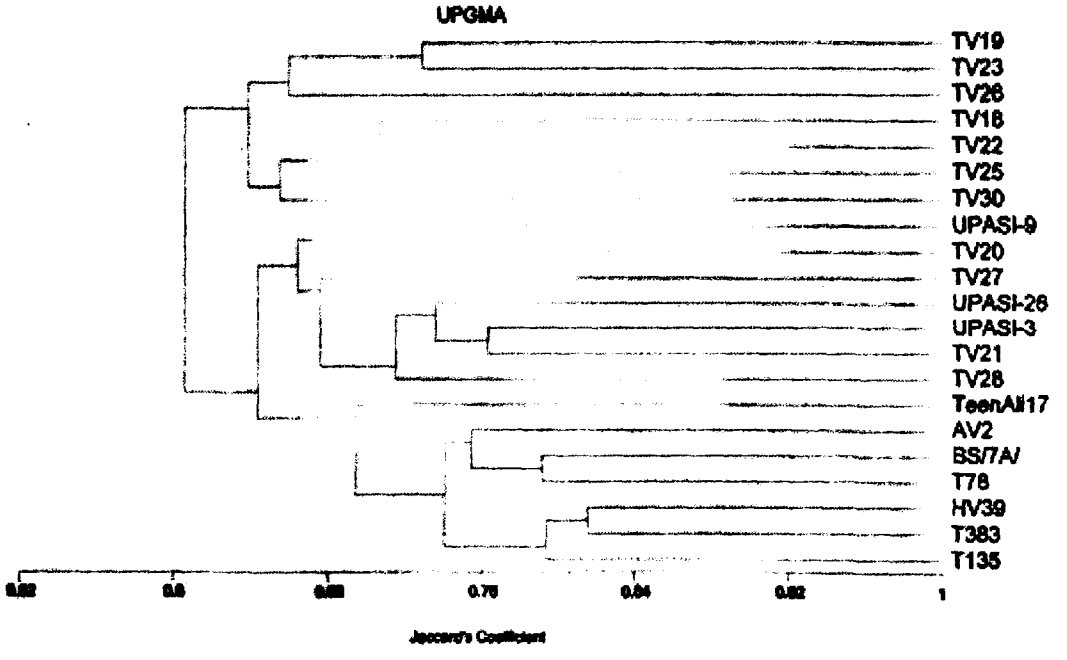


Figure 5. Dendrogram showing the genetic relationship among the twenty one tea cultivars based on ISSR markers using Jaccard's coefficient.

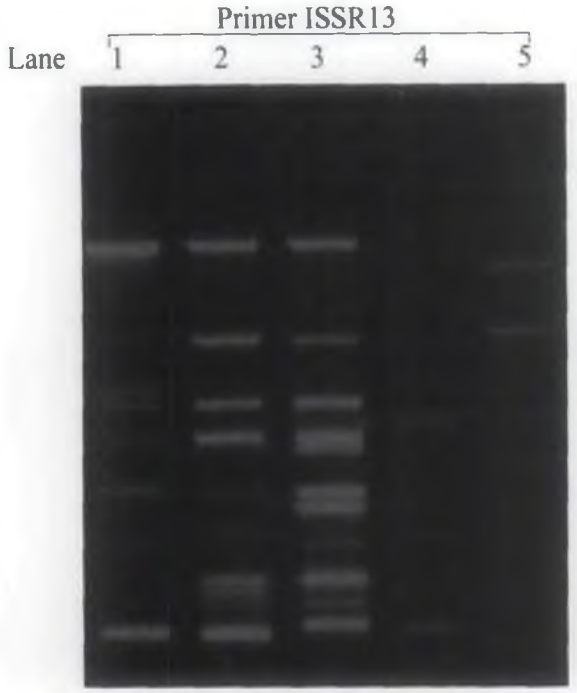


Fig. A. ISSR based DNA fingerprinting in five tea genotypes. Lanes 1-5, BS/7A/76, AV2, TeenAli/17/1/54, TV20 & HV39, respectively.

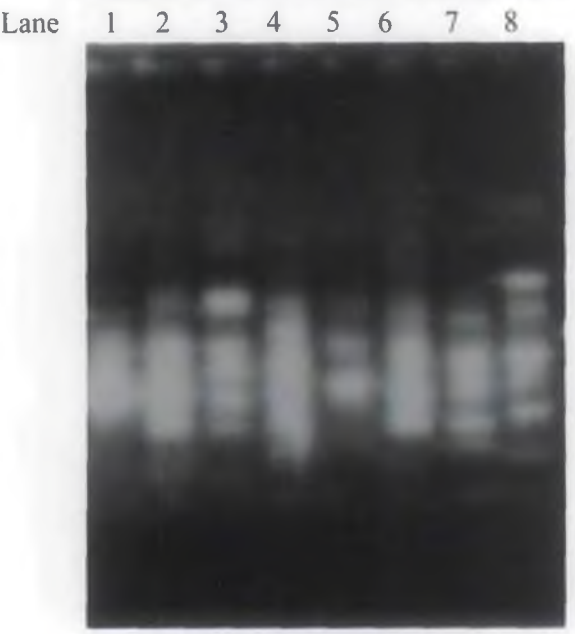


Fig. B. RAPD based DNA fingerprinting in eight tea genotypes using random 10-mer primers. Lanes 1-8; BS/7A/76, T135, T78, TV27, TV20, TV28, TV25 & TV18, respectively. Primers, lane 1-2, OPA-9; lane 3-4, OPA-12; lane 5-6, OPA-13 and lane 7-8, OPB-20.

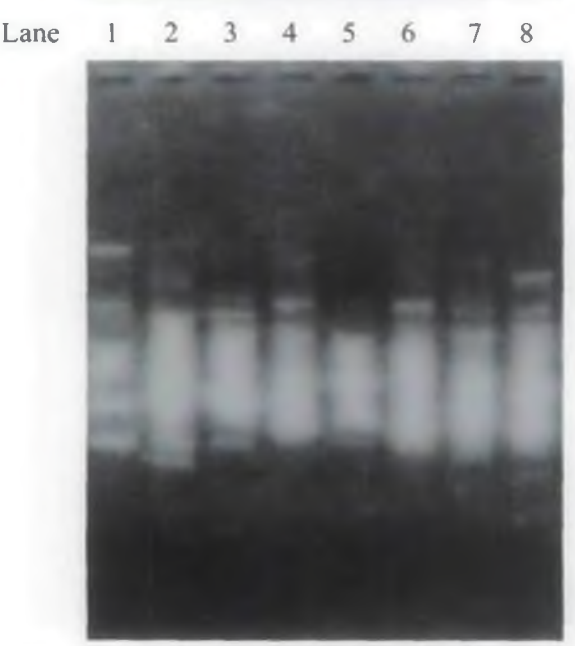


Fig. C. DNA fingerprinting of eight tea genotypes using ISSR primers. Lanes 1-8; UPASI-26, TeenAli17, T383, TV22, TV23, AV2, UPASI-9 and UPASI-3, respectively. Primers, lane 1-4, ISSR18 and lane 5-8, ISSR814.1.

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).

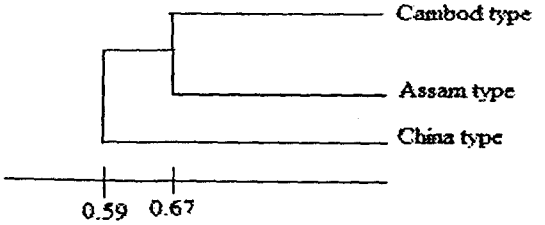


Figure 6. Phenetic dendogram constructed on the basis of genetic distance shown in table 23 based on ISSR markers among the three populations of tea varieties [China type, Assam type and Cambod type] to show their relationships.

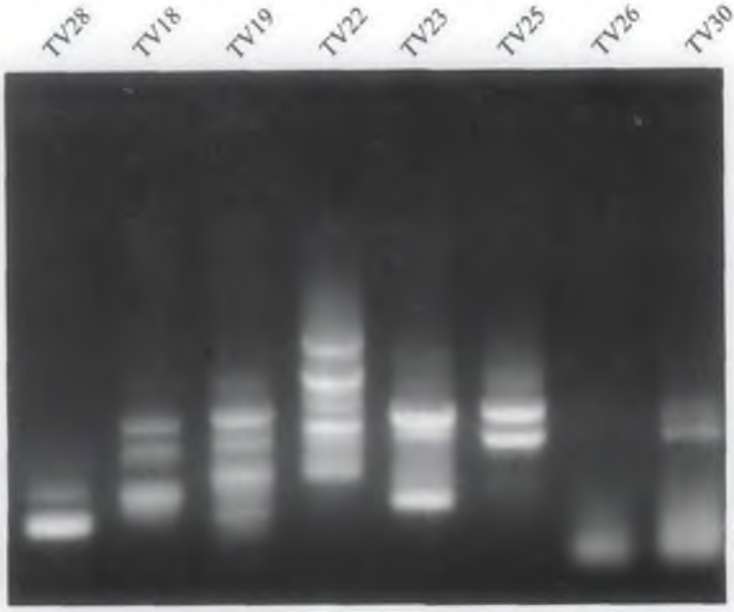


Fig.A: DNA fingerprinting of the eight tea clones were generated using ISSR primer UBC830.

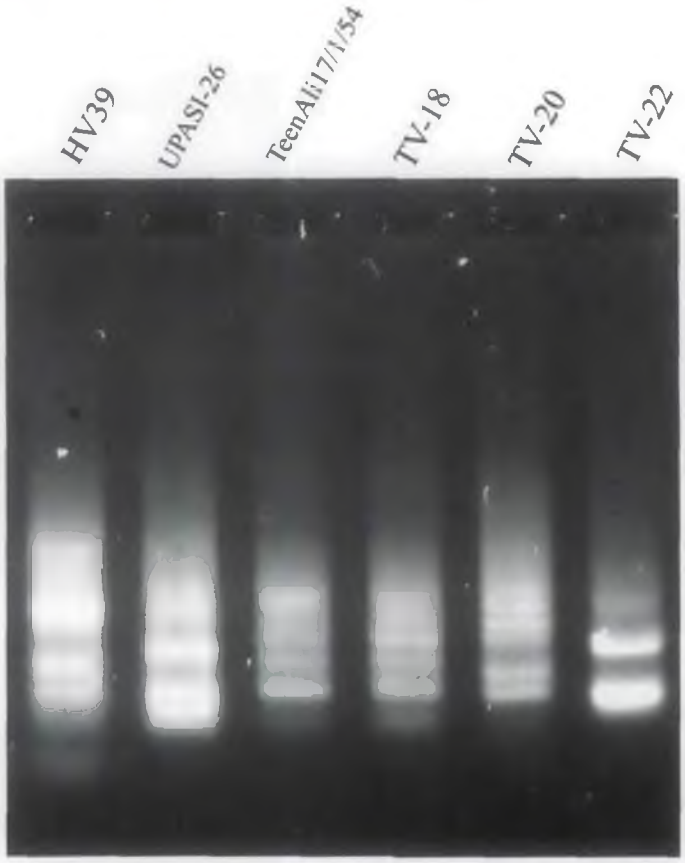


Fig. B: DNA fingerprinting of the six tea clones were generated using ISSR primer UBC825.

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

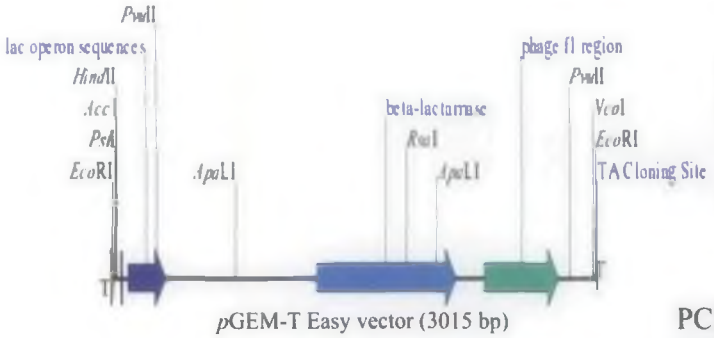


Fig. A. Structural features of *p*GEM-T Easy vector in linear state. PCR product with 3′ overhang of A-residue can easily be ligated into this vector in presence of T4 DNA ligase.

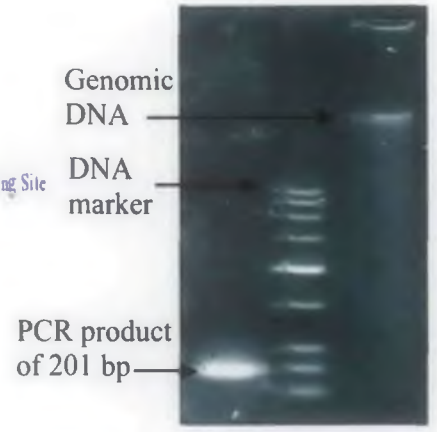


Fig.B. Chitinase gene specific 201 bp PCR product of tea clone T383 has been fractionated in 1% agarose gel electrophoresis.

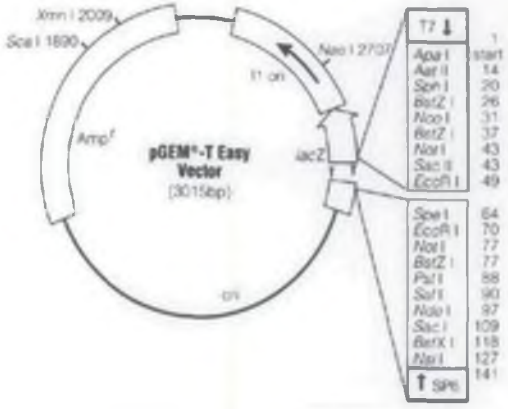


Fig. C. pGEM-T Easy vector circularized after ligation with 201 bp chitinase DNA of tea clone T383.

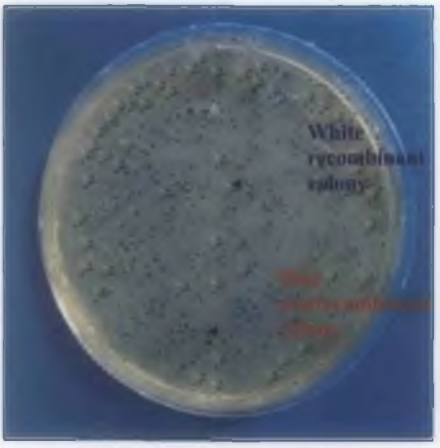


Fig. D. Transformed *E. coli* host JM109 with pGEM-T Easy vector was selected on X-gal and IPTG medium. White colonies were with recombinant vector.

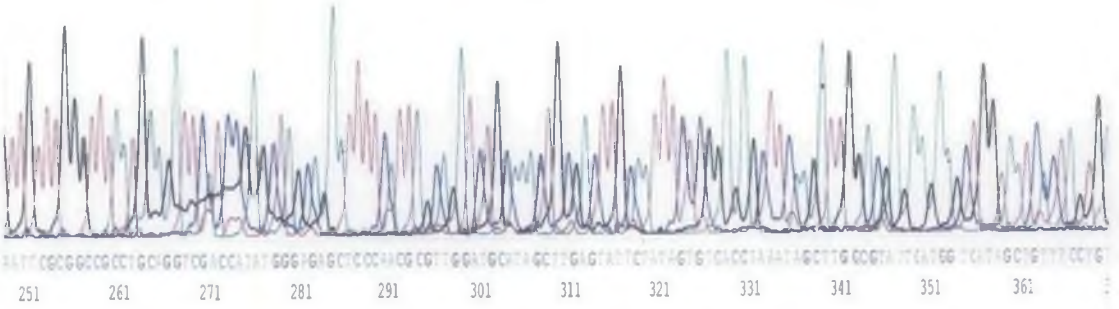


Fig. E. Electropherogram showing the partial DNA sequence of chitinase gene (201 bp) of tea clone T383.

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

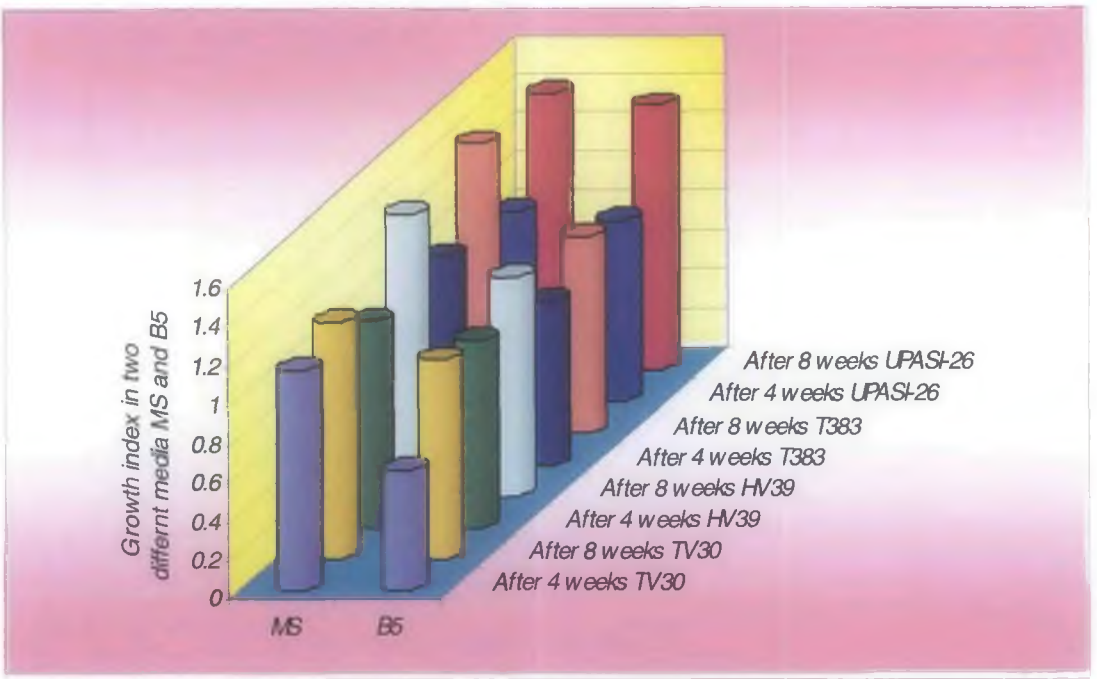


Fig. A: Growth index (GI) of material T383, HV39, TV30 and UPASI-26 on different media

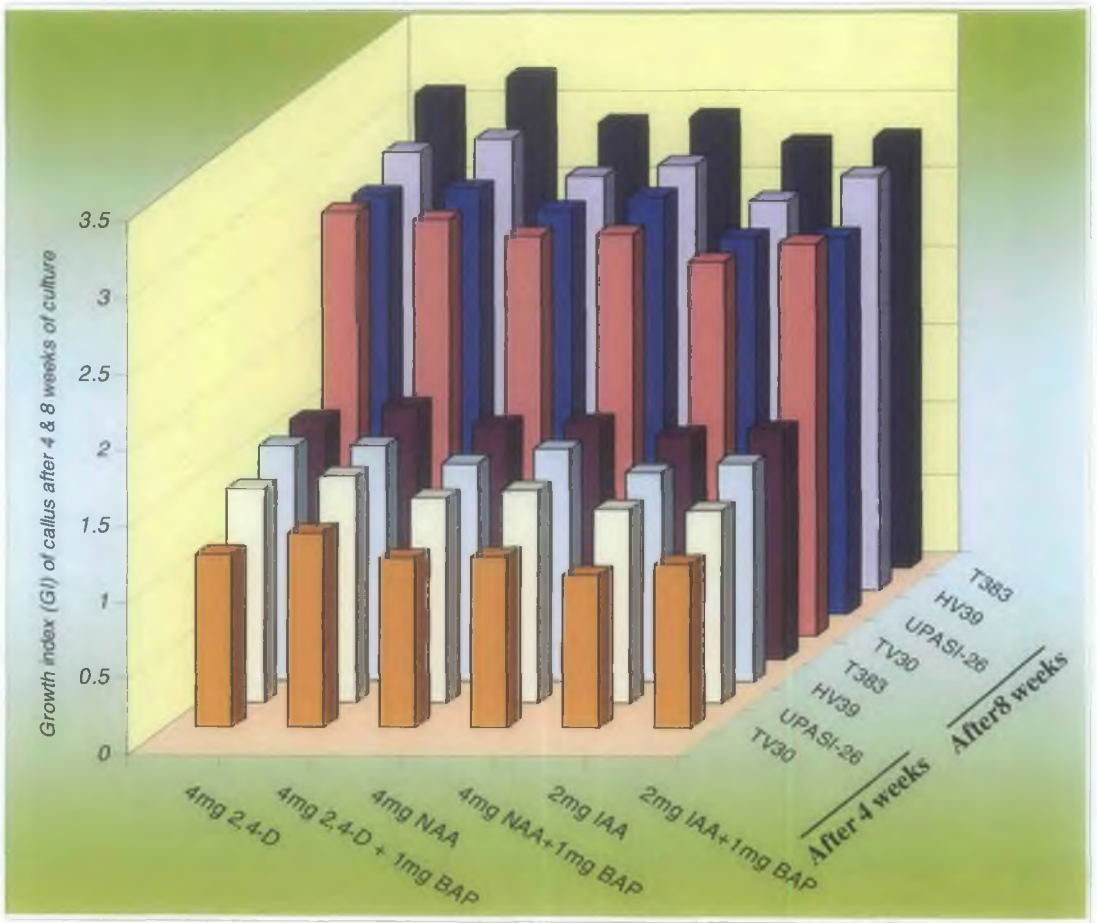


Fig. B: Growth index (GI) of 4 materials on different hormonal composition in MS medium after 4 and 8 weeks of initiation.

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).

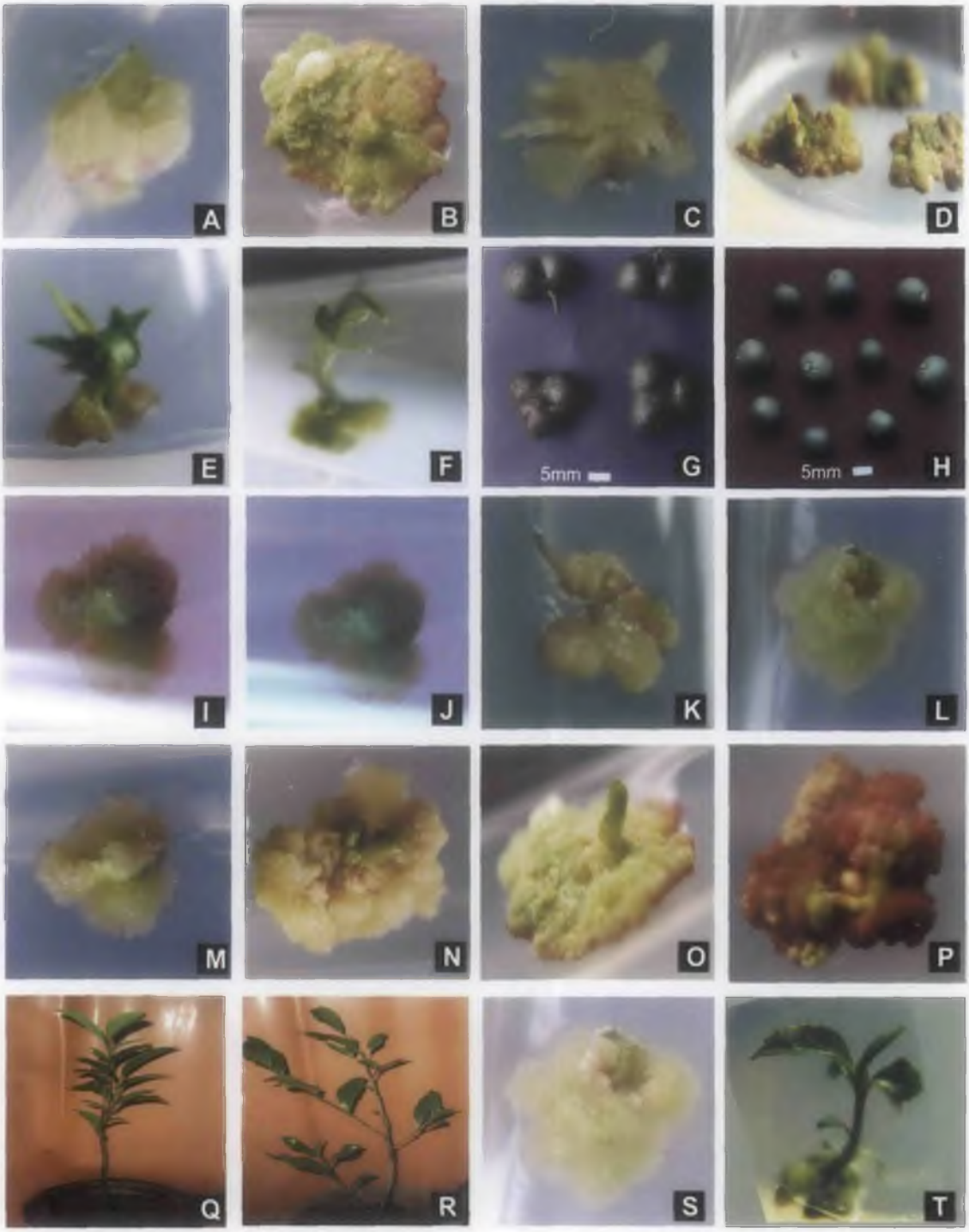


Plate 8 [Figs. A-T]: (A-F) Plantlet regenerated from callus culture of T383; (G) Mature tea fruits collected in November; (H) Tea seeds; (I-L) Four weeks old callus in T383, TV30, UPASI-26 and HV39 respectively was initiated from stem explants; (M-P) Callus growth after eight weeks of culture initiation 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

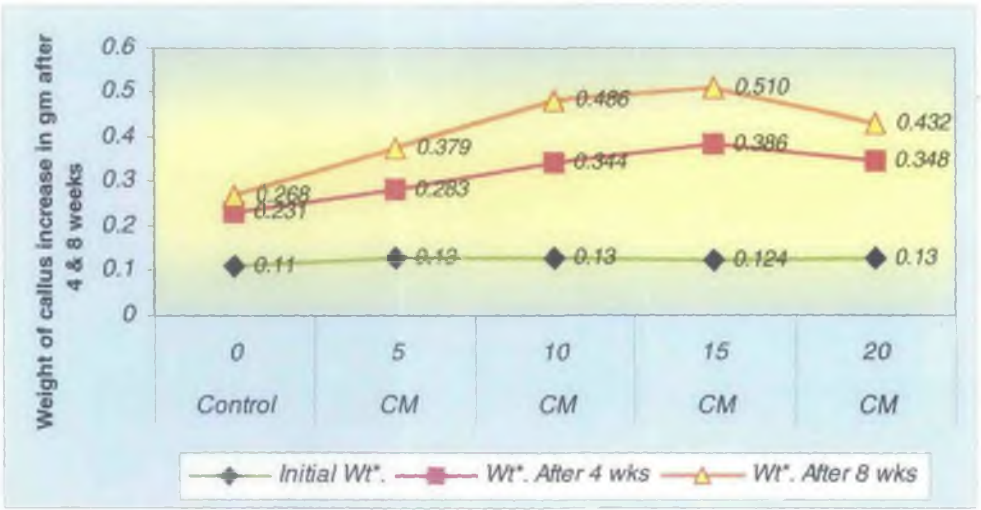


Fig. A: Effect of coconut milk (CM) as organic additives on growth and development of callus in *C. sinensis* clone T383 on MS medium.

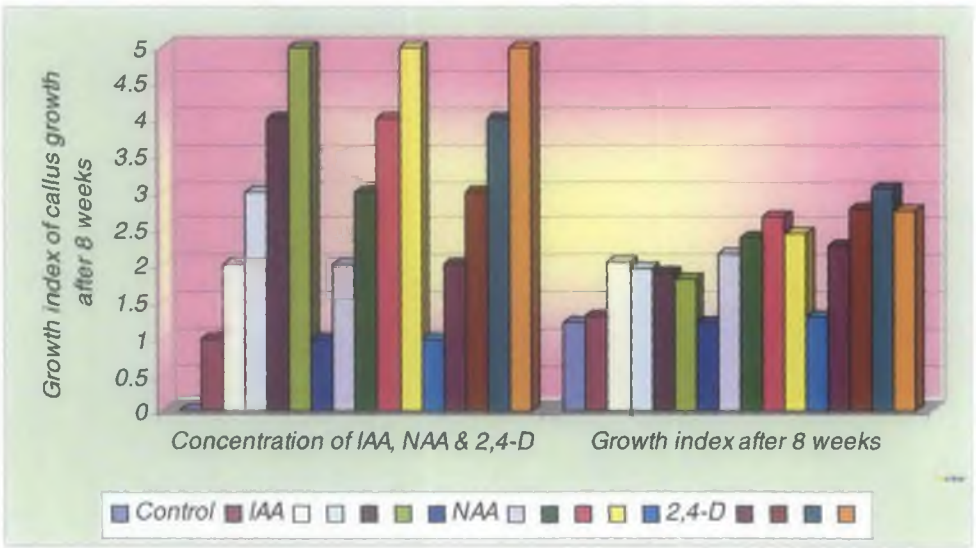


Fig. B: Effect of different concentration of growth regulators (IAA, NAA and 2,4-D) on callus growth and development of tea clone T383 on MS.

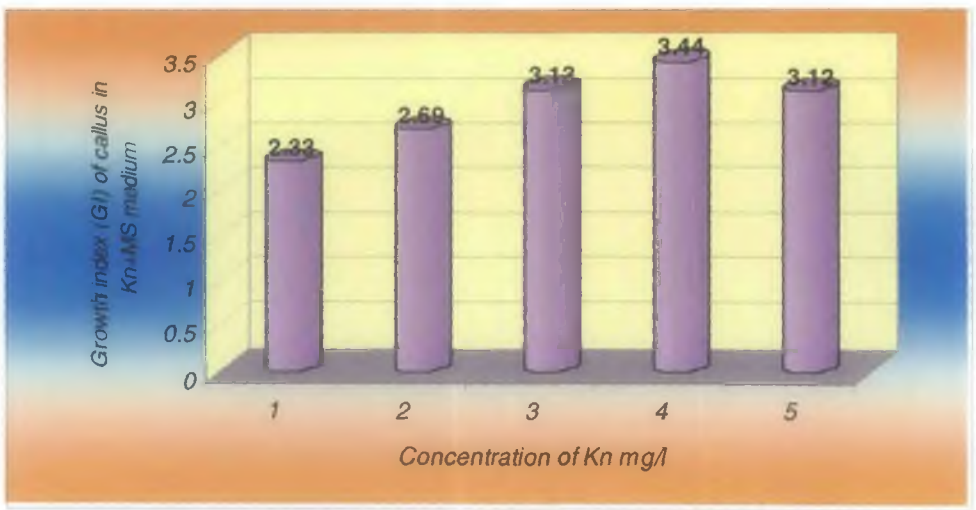


Fig. C: Effect of Kinetin on callus growth in tea clone T383.

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).

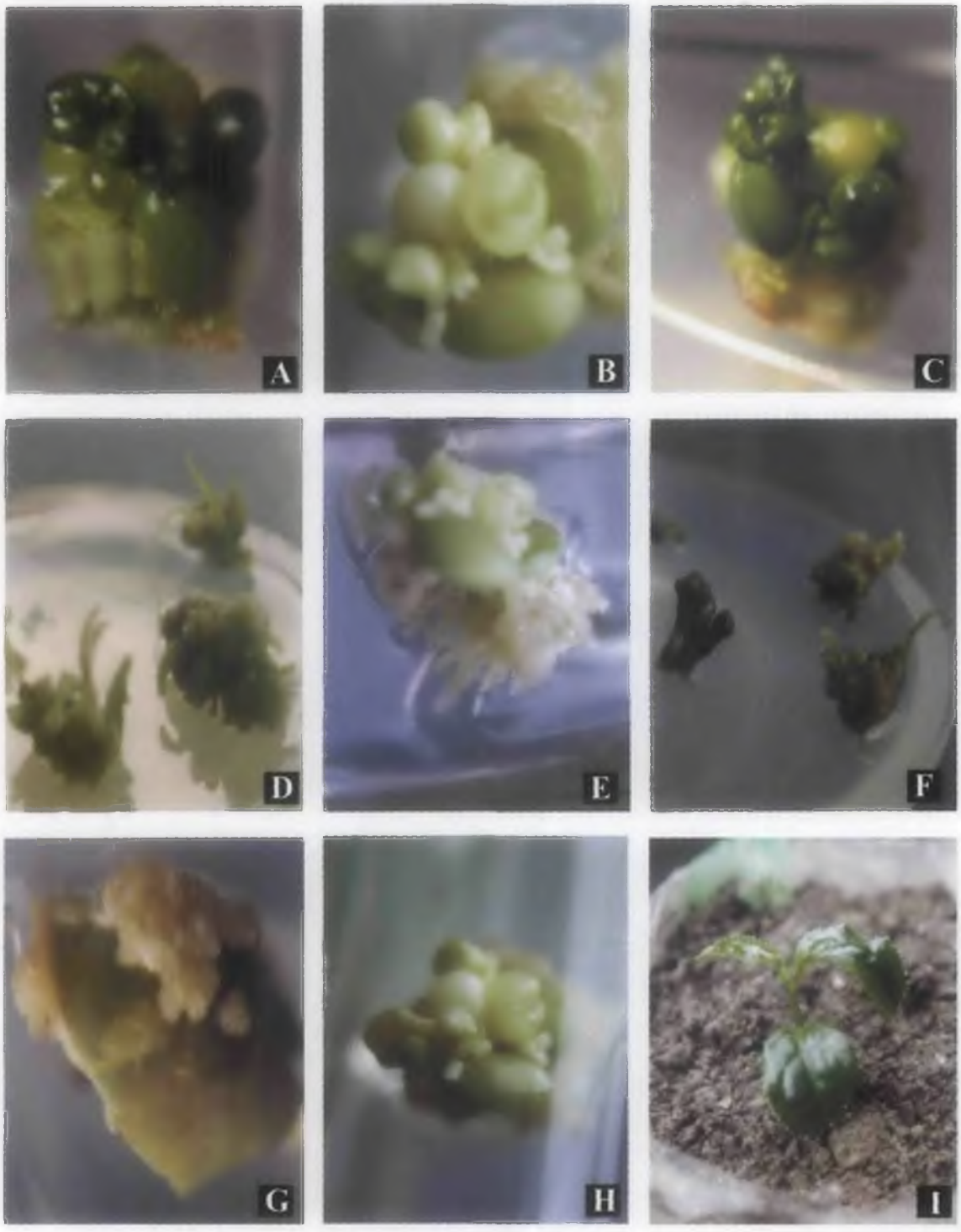


Plate 9 [Figs. A-I]: Somatic embryogenesis and plantlet regenerated from tea clone T383 using immature cotyledon as explants. (A) Bud-like somatic embryo which was green in colour; (B) Cup shaped somatic embryo which was pale yellow to creamy greenish in colour; (C) Seed-like somatic embryo with large 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

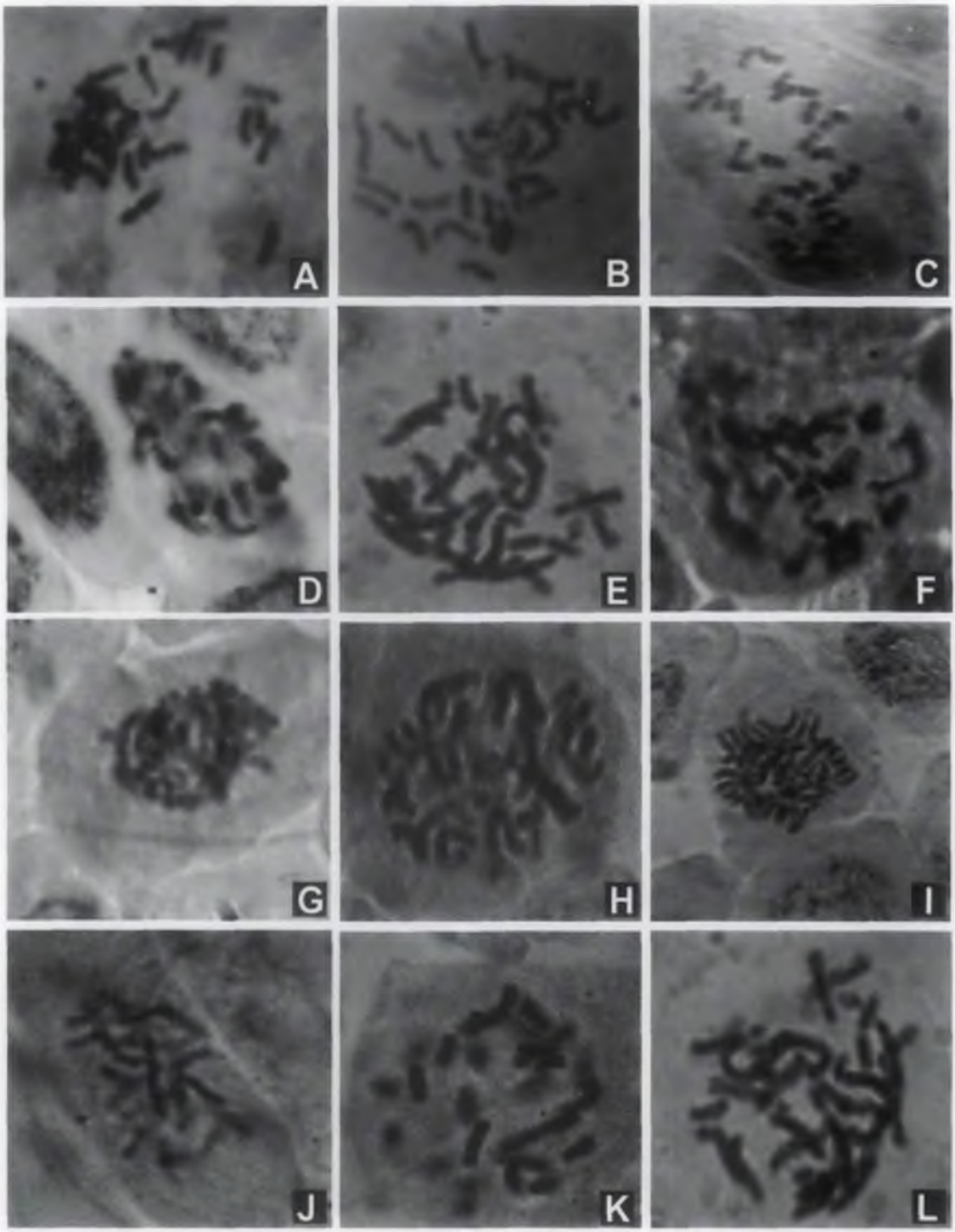


Plate 10 [A-L]: Photomicrographs showing 2n=30 chromosomes in the somatic complement in different cultivars of *Camellia sinensis*. (A) Tv26; (B) TV23; (C) TV25; (D) TV29; (E) T78; (F) T383; (G) HV39; (H) TV30; (I) UPASI-26; (J) TeenAli17/1/54; (K & L) *In vitro* regenerated plantlet showing true-to-type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

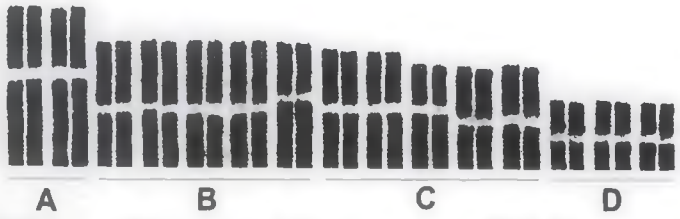
Karyotype formula: 2n = 30 = A4 + B5 + C4 + D2

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

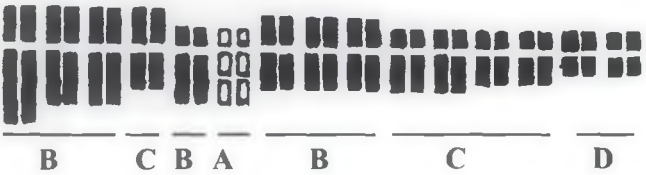
Idiogram of *C. sinensis* cv. UPASI-26.

C

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

Idiogram of *C. sinensis* cv.TeenAli17

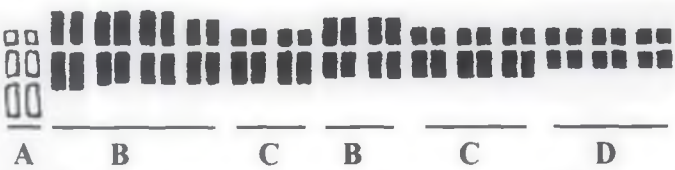
D



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

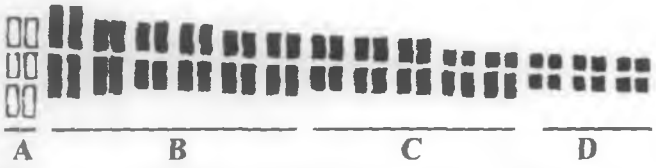
Karyotype formula: 2n = 30 = A4 + B6 + C3 + D2

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

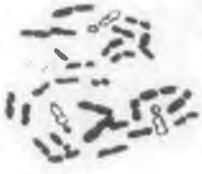
Type	No. of Chromosome Pair	Chromosome length (μm)			F%	Special features.
		Long arm (μm)	Short arm (μm)	Total length (μm)		
A	4	1.25-1.87	1.25-1.25	2.25-3.12	40.06	nSM^{Sat+}
B	6	1.25-1.83	0.62-1.25	1.87-2.50	48-50	M
C	3	0.93-0.93	0.62-0.93	1.55-1.87	33.81	nSM
D	2	1.25-1.25	0.62-0.62	1.87-1.87	33.15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

F

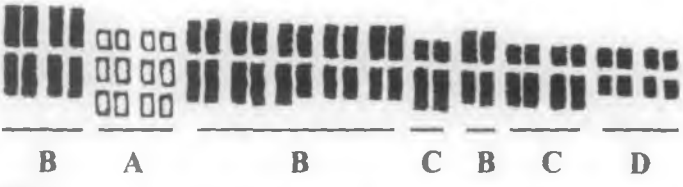


Karyotype of *C. sinensis* cv. T78. 2n=30

Idiogram of *C. sinensis* cv. T78.



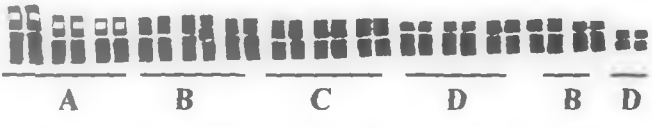
G



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

Idiogram of *C. sinensis* cv. T383.

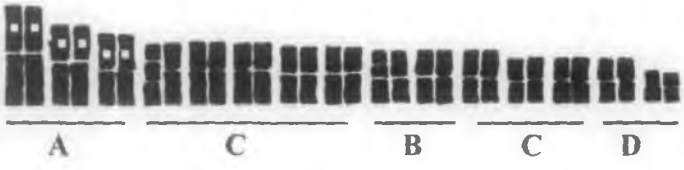
H



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

Idiogram of *C. sinensis* cv. TV23.

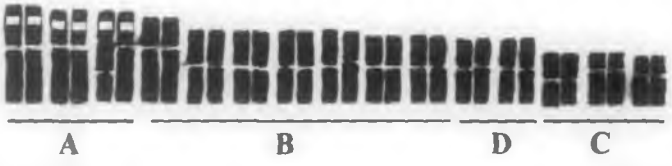
I



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

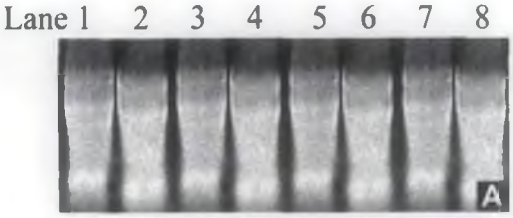


Fig.A. Equal RNA (15µg) from each sample was loaded and fractionated in 1.2% denaturing agarose gel. Lane 1-4 (control) and 5-8 (induced), mature leaf, young leaf, callus tissue, shoot respectively.

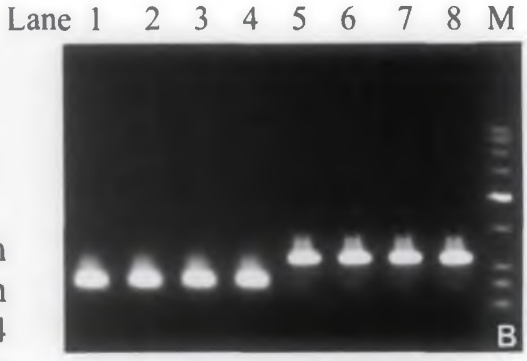


Fig.B. Chitinase gene specific transcript accumulation in RT-PCR. Lane 1-4, RT-PCR product of 254 bp using chitinase gene primer; lanes 5-8, RT-PCR product of 366 bp using DOP-primer in tea clone T383.

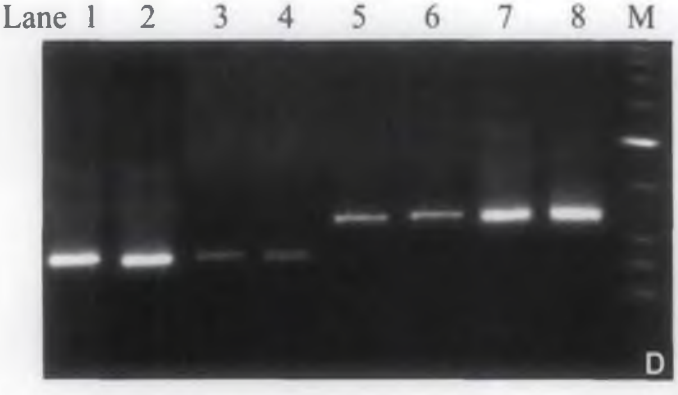


Fig.D. Purified RT-PCR products of 254 bp and 366 bp from control samples. Lane 1-4 and 5-8, young leaf, mature leaf, callus & shoot respectively.

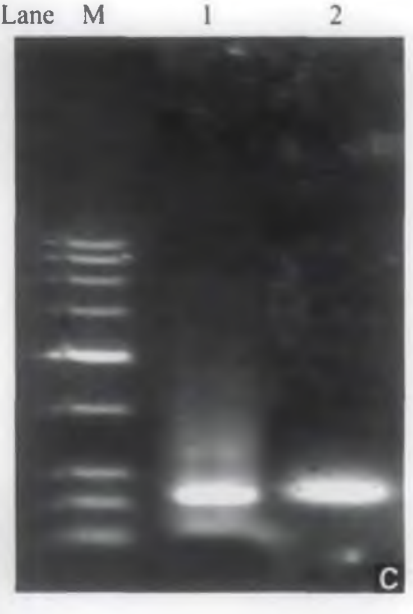


Fig.C. Caffeine synthase gene specific PCR product has been fractionated in 1% agarose gel. Lane M, low range DNA ruler marker; lane 1, 200 bp PCR product of T383 and lane 2, 202 bp PCR product of HV39 tea clone.

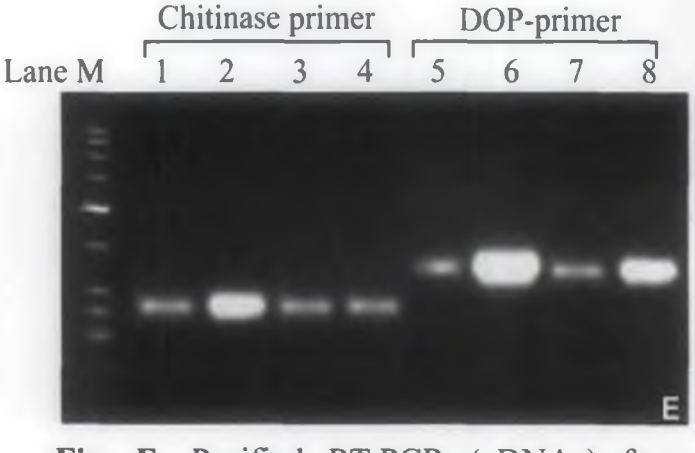


Fig. E. Purified RT-PCR (cDNAs) from treated samples (after 16 h induction with methyl jasmonate. Lane 1-4, YL,ML,callus, shoot respectively; Lane 5-8, callus, ML, shoot & YL, respectively.

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.

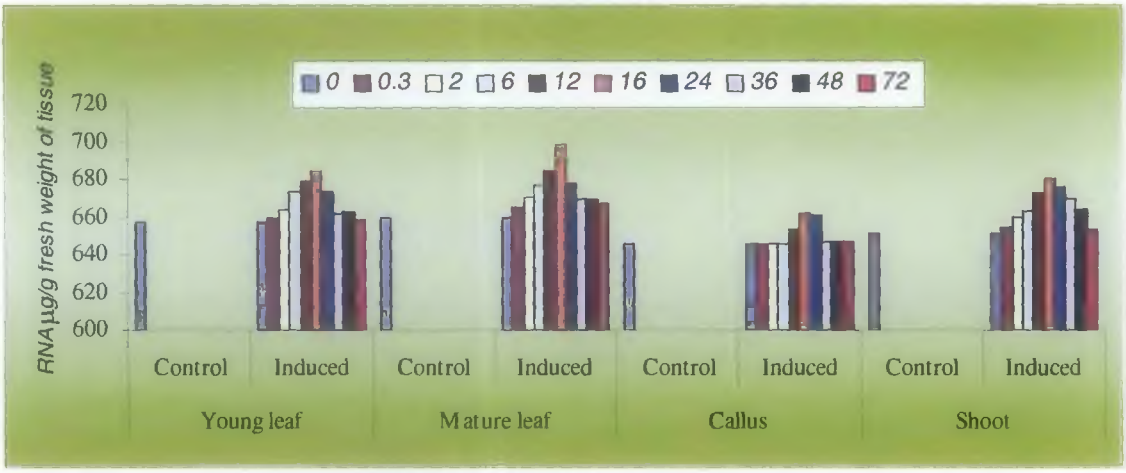


Fig. A : Quantitation of total transcript accumulation (RNAµg/g fresh weight of tissue) in young leaf (YL), mature leaf (ML), callus and shoot after 16 h induction with methyl jasmonate.

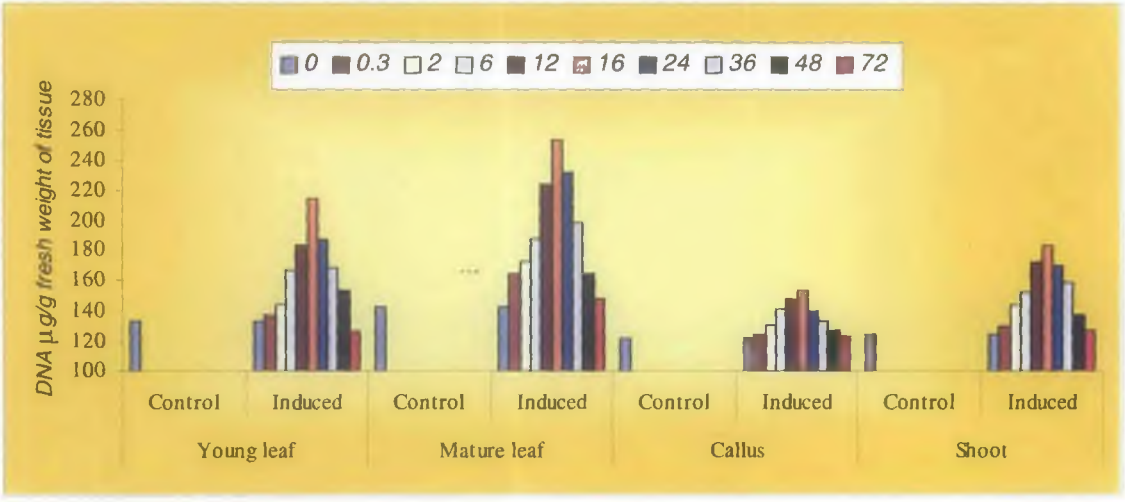


Fig. B: Quantification of RT-PCR products (cDNA µg/g fresh weight of tissue) after 16 h induction with methyl jasmonate in young leaf (YL), mature leaf (ML), callus and shoots.

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*.