DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

MGAVEGYRIAGGPLGEVTDPLYPELKVKEIKNGRLAMFSMFGFFVQAIVTGKGPLEN LADHLADPVNNNAWAYATNFVPGK.

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

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

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

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

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