S.L.J Tea Sci. 68(1), 12-19, 2003, *Printed in Sri Lanka*

A Comparative Assessment of Some Morphological and Anatomical Attributes to Identify Markers for Screening Polyploid Genotypes of Tea (*Camellia Sinensis* L.)

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The effect of polyploidy on morphological and anatomical attributes related to vegetative and reproductive characteristics were assessed in colchicine induced tetraploid cultivars of tea (*Camellia sinensis* L.) in comparison with their diploid progenitors. Attributes such as leaf area, fresh and dry weight of flush, pollen diameter, anther length, stomatal density, stomatal length and breadth, stomatal length to breadth ratio and number of chloroplasts per guard cell were studied in order to identify the most reliable ploidy indicator in tea. Of the attributes tested, number of chloroplasts per guard cell was found significantly different between the two ploidy levels for all the cultivars tested. In contrast, fresh weight and dry weight of flush, stomatal length and breadth and length to breadth ratio showed no such significant differences between ploidy levels in any of the cultivars. On the other hand, size of the pollen grain, anther length, leaf area and stomatal density showed significant differences between only in some of the diploids and their induced tetraploids. Results confirmed therefore, that number of chloroplasts per guard cell is a reliable indicator for level of ploidy in tea than the other criteria studied. This technique of chloroplast count could therefore, be used as a reliable indicator for rapid and initial screening tool to identify polyploid genotypes in tea.

INTRODUCTION

Tea (*Camellia sinensis* L.) which is one of the most popular beverage crops in the world is grown for its vegetative parts which is harvested periodically by plucking tender leaves. In crop improvement activities of asexually propagated perennial crops where vegetative parts are of economic use, polyploidy breeding appears to be better suited than other approaches. In tea, many desirable polyploids have been reported by several scientists from various part of the world (Bezbaruah, 1971; Amma, 1974; Sebastiampillai, 1976). It has been reported that triploid forms of tea were hardier and more resistant to the cold conditions than diploids (Simura & Inaba, 1952) and a cultivar which is widely recommended for planting in South India is reported to be a natural triploid (Jayasuriya & Govindarajulu, 1975).

Most *Camellia* species are diploids with a genome size of 30 chromosomes (Kondo, 1977) and tea (*Camellia sinensis* L.) in its cultivated forms are also diploids having chromosome number of 30 (2n=2x=30) (Wachira & Kiplangat 1991; Ahmed & Singh 1993).

The potential for screening of ployploids has not been widely exploited in tea due to difficulties in employing time consuming cytological methods. In the past, the ploidy level of tea has been confirmed by counting chromosome numbers in meristematic tissue, such as root tip cells and pollen mother cells. (Ye Dapeng, 1989a). However, these cytological techniques require time-consuming and tedious pre-treatments, fixation and staining. Alternatively, in some other crop species, less time consuming methods involving morphological and anatomical markers such as size of pollen grain (Tan & Dunn, 1973), size and frequency of stomata (Ciha & Brun, 1975; Mishra et al., 1991), periderm cell size (De Maine, 1998) and number of chloroplasts in the guard cells of stomata (Dudley, 1958; Chaudhari & Barrow, 1975) have been successfully used to identify polyploid cultivars. Hence, identification of markers related to morphological and anatomical characteristics is of great importance in screening polyploids whether they are artificially induced or naturally produced.

Few studies have attempted to analyse the effects of ploidy on stomatal density (Wachira, 1994), size of the stomatal guard cells (Amma, 1974; Chaudhuri & Bezbaruah, 1985), and number of chloroplasts in guard cells (Ye Dapeng, 1989b; Ahmed & Singh, 1993; Koskey & Wachira, 2000) in tea. In each of these studies only a single attribute has been considered in relation to the ploidy level. Though, there have been few attempts to evaluate the relationship between individual attributes either morphologically or cytologically using a single or few polyploid cultivars, no references have been made to compare these attributes between polyploid cultivars with their diploid counterparts. Therefore, to derive more meaningful results and to identify reliable ploidy markers, present study was undertaken to assess the variations in characteristics related to morphology and anatomy of induced polyploid cultivars in comparison with their diploid progenitors. This will help in devising a rapid technique for screening polyploids in tea using morphological, anatomical attributes rather than depending on tedious time consuming cytological procedures.

MATERIALS AND METHODS

The plant material used was derived from a clonal plot, which was established in the field at Tea Research Institute of Sri Lanka. Five diploid cultivars (TRI 2023, TRI 2024, TRI 2025, TRI 2026 and DT 95) and their confirmed induced tetraploid clones were used in the study. These induced tetraploid cultivars were generated by Sebastiampillai, (1976) using colchicine treatment. Cytological examinations of root tip cells confirmed that these induced polyploid cultivars were tetraploids. Attributes such as leaf area, fresh and dry weight of flush, number of stomata per unit area, stomatal length and breadth and their ratio, pollen grain size, anther length, and number of chloroplasts per guard cell were assessed in tetraploid cultivars in comparison with their diploid counterparts.

Leaf area, fresh and dry weights of flush (fully opened two leaves and a terminal bud) were measured from randomly selected 3 bushes. Five samples from each bush were collected for measurements. Leaf area was measured in the laboratory using a leaf area meter (Model 121TM35, DELTA -7 DEVICE).

Stomatal density (number of stomata per unit area) was determined from preparations of imprints taken from the midsection of leaf abaxial. Imprinting of stomata was done with the aid of colorless nail varnish. Two microscopic slides from each of randomly selected five leaves taken from the 5th leaf position of the shoot were scored per clone. Number of stomata was counted on 5 random microscopic fields (0.19 mm^2) under 40x magnification. The same preparation was used for the measurements of stomatal length and breadth. Measurements of guard cell breadth and width were made in three stomata per sample using ocular micrometer.

Pollen grain size was measured under ocular micrometer microscope. Young floral buds (10 – 12 mm length, 8 – 10 mm width) were collected and fixed in 3:1 Ethanol : Acetic acid solution for 24 h and stored in 70% alcohol at 4°C. At the time of taking measurements, anthers were squashed in aceto-carmine and pollen diameter was measured. For anther length measurement, anthers were separated from mature flower buds (16 –14 mm length, 12 – 14 mm width) and was measured under magnification of 4x.

Counting of chloroplasts in guard cells of stomata was done after staining with potassium iodide. Specimens were prepared as described by Chaudhari and Barrow (1975) using 5th leaf from the apex. Counts were made from ten stomata (twenty guard cells) per plant and 30 stomata (sixty guard cells) per ploidy level under 1250x magnification.

All the above measurements and observations were repeated at least twice for more accuracy. Data are presented as mean $\pm$ S.E of means. T-test was performed to test the significant difference between means of the treatment.

RESULTS

Comparative morphological and anatomical attributes of five tetraploid cultivars and their diploid progenitors are summarized in Table 1.

Table 1: Variations in morphological and anatomical attributes of tetraploid (4x) tea cultivars and their diploid (2x) progenitors

Clone	Leaf area (cm^2)		Fresh wt. of 10 flush* (g)		Dry wt. of 10 flush* (g)		Pollen diameter (µm)		Anther length (mm)		Number of stomata /mm^2		Stomatal length (µ)		Stomatal breadth (µ)		Stomatal l length/breadth ratio		Number of chloroplast / guardcell	
	2x	4x	2x	4x	2x	4x	2x	4x	2x	4x	2x	4x	2x	4x	2x	4x	2x	4x	2x	4x
TRI 2023																				
	40.933	40.333	8.045	10.33	1.815	2.2	39.438	49.9	1.3038	1.6121	201.43	204	20.61	20.33	10.39	10.167	1.99	2.0	16.9	22.9
SE	±2.293	±4.516	±0.155	±1.27	±0.145	±0.03	±1.872	±0.740	±0.078	±0.043	±8.964	±8.796	±0.485	±0.753	±0.455	±0.25	±0.138	±0.02	±0.781	±1.041
P value	0.9144		0.216		0.1215		0.0008		0.0148		0.843		0.7729		0.6901		0.9643		0.0017	
cv %	15.27		13.92		7.37		6.08		9.92		9.79		5.36		6.21		8.59		10.34	
TRI 2024																				
	37.0	24.933	5.99	6.89	1.225	1.505	43.813	51.833	1.4133	1.75	191.9	226.32	20.166	19.934	10.598	10.698	1.92	1.88	18.6	22.3
SE	±2.835	±3.48	±1.53	±2.11	±0.105	±0.305	±0.624	±2.093	±0.050	±0.029	±10.42	±5.488	±0.624	±0.692	±0.660	±0.58	±0.072	±0.08	±0.484	±1.008
P value	0.0548		0.7628		0.4769		0.0083		0.004		0.0192		0.8097		0.9119		0.7234		0.0107	
cv %	17.75		40.47		23.63		5.27		4.39		8.9		7.35		13.0		9.08		8.64	
TRI 2025																				
	36.733	38.933	7.885	9.315	1.63	1.975	39.67	40.65	1.3933	1.4895	163.26	159.16	20.443	19.987	10.11	9.9467	2.03	2.0	21.33	29.667
SE	±4.42	±4.806	±0.915	±0.065	±0.09	±0.175	±0.46	±0.322	±0.023	±0.072	±12.98	±8.913	±0.483	±1.49	±0.28	±0.36	±0.075	±0.2	±0.6667	±0.667
P value	0.7531		0.2594		0.2217		0.1224		0.3233		0.801		0.7404		0.7405		0.9297		0.0009	
cv %	21.13		10.67		10.91		1.86		7.94		15.44		9.51		5.61		12.93		4.53	
TRI 2026																				
	35.267	31.867	8.025	8.81	1.715	1.94	63.125	41.75	1.3361	1.2797	184.29	175.29	21.75	21.057	10.367	9.99	2.1	2.11	18.33	23.2
SE	±0.636	±0.176	±1.395	±1.09	±0.095	±0.07	±1.875	±0.144	±0.065	±0.038	±8.138	±11.53	±0.4	±0.802	±0.434	±0.35	±0.057	±0.08	0.6009	1.1023
P value	0.0067		0.7008		0.1968		0.0006		0.4768		0.5416		0.4823		0.5369		0.9489		0.0192	
cv %	2.4		21.03		6.45		3.07		5.86		12.41		5.13		6.72		5.68		11.47	
DT 95																				
	44.8	49.267	4.905	5.48	1.11	1.235	43.83	43.688	1.7023	1.78	74.104	92.946	19.89	24.61	10.33	12.78	1.93	1.91	18.7	29.7
SE	±2.485	±1.235	±1.155	±0.47	±0.17	±0.035	±0.546	±0.373	±0.031	±0.078	±2.028	±6.405	±0.675	±3.038	±0.411	±0.96	±0.022	±0.09	±1.2104	±1.281
P value	0.1827		0.6815		0.5462		0.8276		0.3468		0.023		0.2037		0.0823		0.8911		0.0002	
cv %	7.23		23.3		14.8		1.9		5.65		12.71		17.12		11.24		5.83		11.51	

* flush - fully opened two leaves and a terminal bud

significant at $p \leq 0.05$

There was no significant variations observed between diploid and tetraploid cultivars tested in terms of fresh and dry weights of flush, stomatal length, breadth and their ratio. In relation to leaf area, significant difference could only be found between diploid cultivar of TRI 2026 and its tetraploid cultivar. Higher leaf area was recorded in TRI 2026 diploid cultivar than its tetraploid counterpart. Although there was no significant differences observed in fresh and dry weights of flush between any of the diploid and tetraploid cultivars tested, there was a consistent increase in fresh and dry weights of tetraploid cultivars than that of diploids.

Length, breadth and length to breadth ratio of stomata remained more or less constant irrespective of their ploidy level. Stomatal density was also not significant between two ploidy levels, except for cultivars, TRI 2024 and DT 95. There was size variation in pollen grains of tetraploids. The size of pollen grains was significantly larger in tetraploids of TRI 2023 and TRI 2024 than their diploid counterpart with exception of TRI 2026, where the pollen grains were larger in diploid than in the tetraploid cultivar. The same trend was found in relation to anther length too.

Interestingly, statistically significant variations were found in mean number of chloroplasts per guard cell between all diploid and their tetraploid cultivars studied (Fig. 1). In all tetraploid cultivars, mean number of chloroplasts per guard cell was higher than that of their diploid progenitors, which showed a consistent trend over the different cultivars tested.

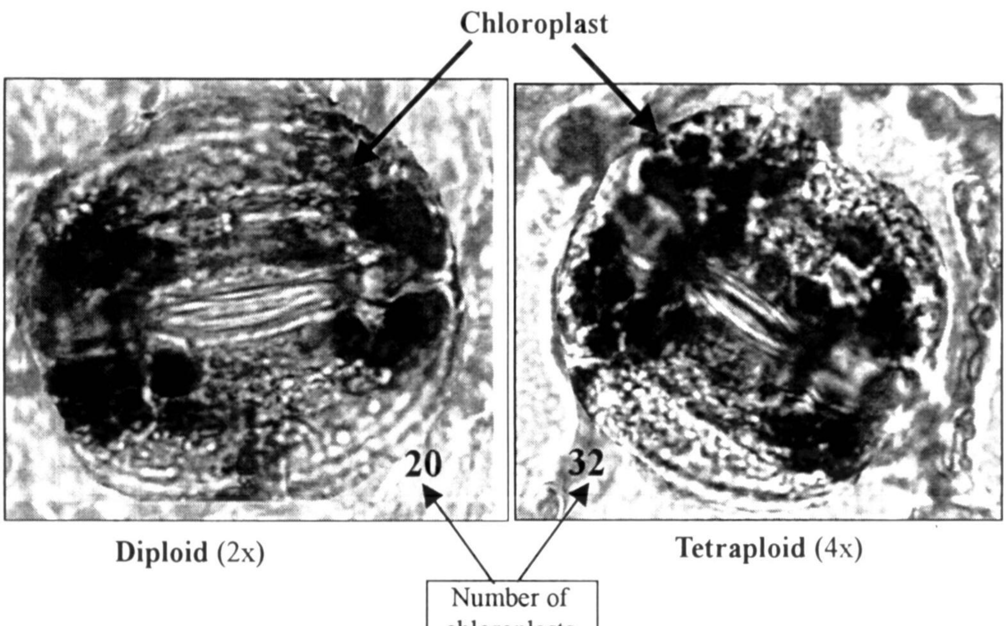

Figure 1: Chloroplasts in stomatal guard cells of tea at diploid (2x) and tetraploid (4x) levels

DISCUSSION

Any technique, which is to be used for the screening of material to determine ploidy level, must be accurate as well as more rapid than chromosome counting. In the past, most commonly used criteria for identification of ploidy level in tea other than counting of chromosomes, have been the size of the pollen grain (Thirukkumaren & Gunasekare, 2001), stomatal density (Wachira, 1994) and number of chloroplasts per guard cell (Ye Dapeng, 1989b; Ahmed & Singh, 1993; Koskey & Wachira, 2000). In the present study, however, only some of the diploid and tetraploid cultivars showed significant differences in relation to stomatal density, pollen grain size and anther length. Extensive checks with chromosome counts have shown that the size of pollen grains is an adequate criterion whereby a tetraploid can be distinguished from a diploid in *Datura spp.* (Blakeslee & Avery, 1937). However, in sugar beet Artschwager, (1992) reported that the size of pollen grain is not always a reliable criterion in identification of polyploids. These observations suggest that although attributes such as stomatal density and pollen grain size have been confirmed as reliable ploidy markers in tea in earlier studies, these characteristics may not truly reflect the level of ploidy. This was further evident by the present study where the pollen grains of the diploid cultivar, TRI 2026 were found to be larger than its tetraploid counterpart. Chaudhuri & Bezbaruah, (1985) reported that in tea genotypes studied by them there was no correlation between ploidy level and stomatal density. Wachira, (1994) also reported that there is a lack of consistency in stomatal density between diploid and triploid tea cultivars. Therefore, the results of the present study on stomatal density, pollen grain size and anther length are in agreement with the findings of other scientists.

Rashid et al., (1985) found a clear relationship between ploidy level and morphogenetic variations in tea in relation to leaf area. However, results of the present study did not show such relationship. Significantly higher leaf area was found in diploid TRI 2026 compared to its tetraploid cultivar. This is in agreement with Ng'etich & Wachira, (1992) who found the same trend in some Kenyan tea clones. This implies that polyploid clones may not necessarily possess larger leaves than diploids. Therefore, this criterion cannot be used for efficient screening of polyploid genotypes in tea. One possible reason for the above observation may be that attributes like leaf area is more affected by the environmental factors than the genotype.

In contrast, all the tetraploid cultivars studied consistently had higher chloroplast number per guard cell than their diploid counterparts. Number of chloroplasts in guard cells has been used to identify ploidy levels in several plant species such as *Gossypium* spp (Chaudhari & Barrow, 1975), *Trifolium* spp (Najcerska & Speckmann, 1968), sugarbeet (Mochizuki & Sueoka, 1955) including tea (Ye Dapeng, 1989b; Ahmed & Singh, 1993; Koskey & Wachira, 2000). This criterion could be used at any stage of plant growth. Present study provides conclusive evidence that, tetraploid and diploid plants of the tea cultivars studied could easily be differentiated based on the number of chloroplasts per guard cell. This ploidy marker is therefore, useful in rapid screening of large seedling populations for ploidy levels to save time and minimize labor needed for verification of selected materials using cytological methods. This may help in assembling a collection of polyploid genotypes for use in tea breeding and other research areas.

REFERENCES

Ahmed N and Singh I D 1993 A technique for rapid identification of ploidy levels in tea. Two and a Bud 40, 31-33

Amma S 1974 Characteristics of tetraploid tea induced from gamma irradiated Yabukita variety. Study of Tea 46, 1-6.

Artschwager 1992 Colchicine induced tetraploidy in sugar beets; Morphological effects shown in progenies of a number of selections *In* Proc. Amer. Soc. Sugar Beet Tech., 1992, pp 296-303. San Antonia, TX

Bezbaruah H P 1971 Cytological investigation in the family Theaceae - 1. Chromosome numbers in some *Camellia* species and allied genera. Caryolgia 24, 421-426.

Blakeslee A F and Avery A G 1937 Methods of inducing doubling of chromosomes in plants. Journal of Heredity 28, 393-411

Chaudhuri T C and Bezbaruah H P 1985 Morphology and anatomy of aneuploid and polyploid tea (*Camellia sinensis* (L.) O. Kuntze). Journal of Plantation Crops 13, 22-30.

Chaudhari H K and Barrow J R 1975 Identification of cotton haploids by stomatal chloroplast count Technique. Crop Science 15, 760-763

Ciha A J and Brun W A 1975 Stomatal size and frequency in Soy Bean. Crop Science 15, 309-313

De Main M J 1998 Periderm cell size as a ploidy indicator in potato (*Solanum tuberosum* L. subspecies *tuberosum*) Annals of Applied Biology 133, 307-312

Dudley J W 1958 Number of chloroplasts in the guard cells of inbred lines of tetraploid and diploid sugar beets. Agronomy Journal 50, 169-170.

Jayasuriya P and Govindarajulu V 1975 Chromosome numbers in some tea clones. Planters Chronicle, 70 (13) 185-186.

Kondo K 1977 Chromosome number in the genus Camellia. Biotropica 9, 86-94.

Koskey J K and Wachira F N 2000 The use of plastid chloroplast count technique to determine ploidy levels of tea. Tea 21, 15-18.

Mishra M K, Prakash N S and Sreenivasan M S 1991 Relationship of stomatal length and frequency to ploidy level in Coffee. Coffee Research 21 (1) 32-41

Mochizuki A and Sueoka N 1955 Genetic studies on the number of plastid in stomata I Effects of Autopolyploidy in sugarbeets. Cytologia (Tokyo) 20, 358-366.

Najcerska C M and Speckmann G J 1968 Number of chloroplasts and pollen grain pores in diploid and tetraploid varieties of some *Trifolium* spp. Euphytica 17,357-362

Ng'etich W K and Wachira F N 1992 Use of non-destructive method of leaf area estimation in triploid and diploid tea plant (*Camellia sinensis* L.). Tea 13, 11-17.

A.K.N Rashid A, Chowdhry M and Badrul Alam A F M 1985 Studies on progenies of a cross between diploid and tetraploid tea. Sri Lanka Journal of Tea Science 54, 54-61

Sebastiampillai A R 1976 A simple technique for the induction of polyploids in tea. Tea Quarterly. 46, 12-15.

Simura T and Inaba T 1952 Studied on polyploidy of the tea plant. Tokai-kinki National Agricultural Experimental Station. Research Progress Report. 1,1-14.

Tan G Y and Dunn G M 1973 Relationship of stoamtal length and frequency and pollen grain diameter to ploidy level in *Bromus inermis* leyss. Crop science 113,332-334

Thirukkumaren G and Gunasekare M T K 2001 Use of pollen morphology and physiology to differentiate ploidy level of tea (*Camellia sinensis* L.) clones. Journal of Jaffna Science Association 9, 6-7.

Ye Dapeng S C 1989a . Cytology studies on the polyploid tea. Journal of Tea Science 9, 117-126.

Ye Dapeng S C 1989b Relation between the chloroplast number in each guard cell and the ploidy of tea. Journal of Tea Science. 9, 127-132

Wachira F N 1994 Triploidy in tea (*C. sinensis*) :Effect of yield and yield attributes Journal of Horticultural Science. 69, 53-60.

Wachira F N and Kiplangat J K 1991 Newely identified Kenyan polyploid tea strains. Tea 12, 10-13.