CHAPTER

BIOCHEMICAL INVESTIGATION ON DIFFERENT CLONES OF TEA UNDER CONSIDERATION.

VI

INTRODUCTION

Tea, grown in about 30 countries is the most consumed beverage in the world (Stoner and Mukhtar 1995). Darjeeling tea is recognised for their delicate flavour, rich aroma and exquisite bouquet. Highly prized by connoisseurs, this tea is often known as the 'Champagne of teas'. There is no other area in the world which can produce the 'muscatel' flavour. Complex chemical nature of the flavour of tea draws attention of many tea scientists in the past (Kozai 1890; Groll 1897; Romburg 1898; Nannings 1900; Deuss 1915; Shaw 1934; Roberts 1958a,b, 1959a; Bhatia 1960, 1962; Stahl 1962). It was reported that tea aroma depends on a large number of different chemical compounds (Bokuchava and Skobeleva 1986).

There are now many different types of tea, each with its own individual characteristic flavour (Yamanishi 1995). There are several factors which are responsible for the difference in quality of tea (Yamanishi 1991). These include quantitative differences in the chemical composition of the fresh tea leaves which are determined by the genetical background of the clones of tea, the climate, soil and horticultural practices and secondly, the difference in the manufacturing process.

Biochemical characteristics of tea are of importance for the very basis of tea quality which is related to the interactions between biochemical components of the tea leaves during different phases of

processing. Aroma is caused by essential oils of tea, while the characteristics of taste are caused by catechins and some other amino acid like 'theanine', which is specifically contained in tea and contributes to its characteristic taste. Four forms of catechins such as epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG) have already been reported from tea (Wang 1991). More than 30 compounds as the major aroma constituents in green and black teas have been identified (Pokorny *et al.* 1995). But all the chemical constituents which are responsible for producing characteristic taste and flavour of tea are generally known as secondary metabolite and which are generally derived from the primary metabolites like sugar, amino acids, proteins etc. These metabolites again are dependent on the photosynthetic efficiency of a particular clone of tea. Besides, tea leaves contain a large range of enzymes which are of importance, as they influence and help in building tea quality during specified phases of commercial processing.

In this part of work an attempt has been made to estimate some important biochemical parameters with a view to understand their ability to produce quality tea.

MATERIALS AND METHODS

Materials

Young, mature and old leaves of selected five clones HV-39,

T-78, B-157, TV-19 and Thurboo-9.

Here two leaves and a bud is considered as young (pluckable height 6 cm.), seventh leaf from the terminal end as mature and tenth leaf from the terminal end designated as old one.

Methods

Quantitative estimation of tea leaf chlorophyll

Freshly collected foliage leaves of five selected clones were weighed and immersed in 5 ml. of methanols and kept in a deep-freeze (-5^0C) for 48 hours. Supernatant was collected and total volume was made upto 5 ml. with methanol and the intensity of the green colour was measured at 645 and 663 nm. in a spectrophotometer. The chlorophyll content was estimated following Arnon's principle (1949).

Quantitative estimation of soluble sugar

Sugar levels were determined following the methods of McCready *et al.* (1950) with minor modifications. Freshly collected clonal tea leaf sample was weighed and homogenised with 80 percent ethanol and centrifuged at 6000 r.p.m for 10 minutes. The supernatant was taken in a watch glass. The cooled supernatant was then evaporated to dryness. Trace of chlorophyll adhering to the surface of the watch glass was carefully removed using solvent ether. The remaining material in watch glass was carefully removed and taken in a test tube by washing it several times with 80 percent ethanol and the volume was made upto 5 ml. This was preserved as a source of soluble sugar.

The soluble sugar fractions were diluted suitably and 1 ml. of the diluted source samples were taken in a test tube and 4 ml. freshly prepared, pre-cooled 0.2 percent anthrone reagent was added. After 30 minutes, the intensity of the green colour was measured by spectrophotometer at 610 nm. Actual sugar contents were determined from the standard curve prepared by using different concentrations of dextrose solutions.

Quantitative estimation of free amino-acids

Freshly collected foliage tea leaves were weighed, crushed in 5 ml. 0.05M phosphate buffer (pH 7.5) solution and the homogenate was centrifuged at 5000 r.p.m. for 10 minutes. The supernatant was diluted suitably and taken as amino-acid source.

From the stock, amino-acid was estimated following the method of Moore and Stein (1948). Reaction mixture containing 1 ml. diluted extract and 4 ml. 0.3% ninhydrin solution was kept in a boiling water-bath for 15 minutes with glass marbel at the top of the hard-glass test tubes. When the reaction mixture turned to violet colour, the test tube was taken out, cooled and the volume was made upto 5 ml. with 80% ethanol. The absorption of the solution was measured at 580 nm. in a spectrophotometer. The quantitative estimation was made by comparing the Optical Density (O.D.) value of the standard curve prepared with L-valine as the reference amino-acid. For the preparation of ninhydrin solution, 300 mg. of ninhydrin powder was dissolved

in 100 ml of 80% ethanol.

Quantitative estimation of tea leaf proteins

Freshly collected tea leaf samples were weighed and crushed in 2.5 ml. 0.05 M phosphate buffer (pH 7.5) solution and 2.5 ml. 10 per cent TCA (Trichloro acetic acid) was added and kept for 30 minutes in a refrigerator (5^0C). The homogenate was centrifuged at 5000 r.p.m. for 10 minutes. The residue was taken and extracted in 5 ml. 2 (N) sodium hydroxide solution and kept in water-bath at 80^0C for 30 minutes, and then centrifuged at 5000 r.p.m for 30 minutes. The supernatant diluted suitably and taken as a protein source.

Protein content of freshly collected tea samples were estimated by reacting the protein solution with folin-phenol reagent and measuring the Optical Density (O.D.) values at 650 nm. in the spectrophotometer, according to method of Lowry *et al.* (1951). The quantitative determination was made after comparing the optical density (O.D.) values of standard curve previously prepared by using Bovine Serum Albumin (BSA).

Quantitative estimation of phenolic compounds

Extraction of phenolic compounds was done following the method of Bray and Thorpe (1954). Freshly collected leaf specimens were first weighed and crushed in 5 ml. ethanol and kept in water-bath at 70^0C for 15 minutes and then centrifuged at 4000 r.p.m. for 10 minutes. The supernatant was taken in a beaker and 5 ml. of distilled

water was added to it, then kept on a hot water-bath until the alcohol was completely evaporated. The aqueous part left over and 5 ml. of ether were taken in separating flask and shaken vigorously for 10 minutes and kept for 10 minutes. The upper ether layer was taken and 5 ml. of hot distilled water was added to it and it was kept in open air until the ether was completely evaporated. The volume of the aqueous solution was made upto 10 ml. with distilled water and diluted 50 times and taken as phenolic compound source.

For the estimation of phenolic compounds, 1 ml. of diluted source sample was taken in separate test tubes and 3 ml. of 5% sodium carbonate and 1 ml. prepared folin-phenol reagent added to each test tube. The reaction mixtures were then kept in boiling water-bath for 1 minute. The Optical Density was measured in the Spectrophotometer at 650 n.m. The quantitative determination was made after comparing the O.D. values of a standard curve prepared previously by using ferulic acid. The stock folin-phenol reagent was diluted in 1:10 proportion (1 ml. of stock folin-phenol plus 10 ml. distilled water just before use).

Quantitative estimation of Polyphenol oxidase activity

Freshly collected leaf samples were weighed and crushed in 5 ml. of 0.05M sodium phosphate buffer (Na_2HPO_4 / NaH_2PO_4) (pH 6.8) solution. The homogenate was centrifuged at 6000 r. p.m. for 10 minutes. The supernatant was diluted 5 times with same phosphate

buffer and was taken as enzyme source.

Polyphenol oxidase activity was assayed following the method of Kar and Mishra (1976) with slight modification. The reaction mixture for polyphenol oxidase (PPO) consisted of 2 ml. sodium phosphate buffer (pH 6.8), 1 ml. of 15 mM pyrogallol and 1 ml. of enzyme extract. It was then incubated together at 37^0C for 20 minutes. The reaction was stopped by the addition of 1 ml. of 5% sulphuric acid. The intensity of the colour was measured at 420 nm. in a spectrophotometer. The blank was prepared after inactivating the enzyme with the addition of 5% sulphuric acid, prior to the addition of pyrogallol (substrate).

Unit of enzyme activity was calculated as follows:

$$\frac{A \times Tv}{t \times v}$$ enzyme unit / minute / gm. fresh wt.

where, A = Absorption, Tv = Total volume of the extract, t = Time and v = Volume of enzyme used in each set.

Quantitative estimation of Catalase activity

Freshly collected leaf sample were weighed and homogenized with 5 ml. of chilled 0.1 M phosphate (Na_2HPO_4 / NaH_2PO_4) buffer (pH 6.8) solution. The homogenate was centrifuged at 5000 r.p.m. for 10 minutes. The volume of the supernatant was made up to 25 ml. with the same buffer and this was used as crude enzyme source.

The activity of the enzyme catalase was assayed following the

method of Snell and Snell (1971) modified by Biswas and Choudhury (1978). The reaction mixture for catalase composed of 2 ml. of the above extract and 2 ml. of 0.05 M hydrogen peroxide (H_2O_2), incubated together at 37^{0}C for 15 minutes. The reaction was stopped by the addition of 1 ml. of 1% titanium sulphate and the mixture was centrifuged at 6000 r.p.m. for 10 minutes. The intensity of yellow colour was measured at 420 n.m. in a spectrophotometer. The blank was prepared by inactivating the enzyme with addition of titanium sulphate prior to addition of H_2O_2.

Quantitative estimation of Peroxidase activity

For the assay of peroxidase, freshly collected tea leaf samples were weighed and homogenised in 10 ml. 0.05 M sodium phosphate (Na_2HPO_4/NaH_2PO_4) buffer (pH 6.5) solution. The homogenates were centrifuged at 6000 r.p.m. for 10 minutes. The supernatants were taken as enzyme source.

Activity of this enzyme was assayed following the method of Kar and Mishra (1976) with slight modifications. The reaction mixture for peroxidase consists of 1.5 ml. of phosphate buffer, 1 ml. of 15 mM pyrogallol, 1 ml. of 0.05M hydrogen peroxide (H_2O_2) and 1 ml. of above mentioned enzyme extract, alltogether incubated at 37^{0}C for 5 minutes. The reaction was stopped by adding 1 ml. of 5 percent of diluted sulphuric acid. Optical density was measured at 420 n.m. in a spectrophotometer. The blank was prepared by inactivating the

enzyme with the addition of 5 percent dilute sulphuric acid prior to the addition of pyrogallol and hydrogen peroxide.

In case of this enzyme assay, the activity of enzyme was expressed as (A x Tv) / t x V, where 'A' is the absorbance of the sample after incubation - the absorbance of the blank. 'TV' is the total volume of the supernatants, 't' is the time (minutes) of the incubation with substrate and 'V' is the volume of the supernatant taken for incubation (Fick and Qualset 1975).

Statistical Analysis

Statistical analysis has been done following the method of Bailey (1995).

RESULT AND DISCUSSION

The experimental result presented in the table –36 indicates that in all the selected clonal leaves, chlorophyll-b content is greater than the chlorophyll-a on unit (mg. per gm. fresh wt.) basis. HV-39 contains minimum amount of chlorophyll (both a and b), while maximum of chlorophyll amount is determined in T-78 considering average chlorophyll content of young, mature and old leaf. It has also been observed that amount of total chlorophyll obtained from young leaves of HV-39 and T-78, comparatively chlorophyll content of T-78 is 3.16 times higher than that of HV-39. It is also noteworthy to mention that in all the selected clonal leaves, both the chlorophyll content (a and b) higher amount is observed in old leaves, lowest amount found in young leaves and intermediate amount found in matured leaves. Chlorophyll-a and chlorophyll-b ratio shows some variations among the five selected clonal leaves. Thurboo-9 old leaf shows highest chlorophyll-a and chlorophyll-b ratio, whereas lowest chlorophyll ratio is observed in mature leaf of the same clone Thurboo-9.

Chlorophyll is probably the most abundant pigment in the biosphere, and an estimated one billion tons of chlorophylls are broken down every year (Matile *et al.* 1996). Chlorophyll is a cyclic, tetrapyrrolic, green pigment of plant which is capable of harvesting the solar energy and by the process of photosynthesis can prepare carbohydrates as plant food material. Tea leaves contain two essential

Table –36. *Chlorophyll content in different clonal tea leaves at different maturity.*

Clone	Maturity of leaves	Chlorophyll-a content (mg./gm. fresh weight)	Chlorophyll-b content (mg./gm. fresh weight)	Total Chlorophyll (a+b) (mg./gm. fresh weight)	Chlorophyll a:b ratio
HV-39	Young	0.42	0.57	0.99	0.74
	Mature	0.78	1.1	1.88	0.70
	Old	1.26	1.85	3.11	0.68
T-78	Young	1.23	1.90	3.13	0.65
	Mature	1.31	2.89	4.2	0.45
	Old	2.23	2.98	5.21	0.75
B-157	Young	1.40	1.74	3.14	0.80
	Mature	1.51	1.92	3.43	0.78
	Old	1.53	2.38	3.91	0.64
TV-19	Young	0.52	0.78	1.3	0.66
	Mature	1.47	2.3	3.77	0.64
	Old	1.81	3.08	4.89	0.58
Thurboo-9	Young	0.45	0.62	1.07	0.72
	Mature	0.53	1.33	1.86	0.40
	Old	2.23	1.78	4.01	1.25

pigments - chlorophyll-a and chlorophyll-b and four major carotenoids such as beta-carotene, lutein, violaxanthin and neoxanthin (Hazarika and Mahanta 1983,1984). They vary seasonally with plant types and changes in these pigments during tea processing to affect the quality of tea. The latter is dependent on the extent of enzymatic breakdown of products like chlorophyllides during tea processing (Banerjee 1996). The principal changes in chlorophyll during black tea processing involve the formation of 'pheophorbide' and 'pheophytin' (Wickremasinghe and Perera 1966a). These two compounds and chlorophyllides occur in fermented tea leaves but pheophytins are also formed on heating (Saijo and Takeo 1970). The decline in chlorophyll content during the processing of black tea contributes to its transformation of pheophytin (Sanderson 1972).

Although 14 carotenoid compounds in the tea leaves have been identified (Tirimanna and Wickremasinghe 1965), all the carotenoids are decreased during processing of black tea (Hazarika and Mahanta 1983), which shows that mature leaves contain a higher amount of carotenes and xanthophylls than young tea leaves, with a concomitant increase of beta-carotene and lutein plus zeaxanthin during tea leaf maturation (Venkatakrishna *et al.* 1977).

During tea leaf processing in commercial manufacture of tea, withering causes degradation of chlorophyll to the extent of about 15 percent (Nikolaishvili and Adeishvili 1966). Breakdown of chlorophyll

affects appearance of made tea, a part of the degraded chlorophyll produces chlorophyllide by the action of the enzyme chlorophyllase present in the tea shoots (Dev Choudhury and Bajaj 1980). Tea leaf chlorophyllase activity is inversely related to the chlorophyll contents and is partly responsible in determining the proportions of pheophytin and pheophorbide during commercial processing of tea leaves (Banerjee 1996).

It is reported that during leaf senescence, there is a fall in chlorophyll content (Tosh *et al.* 1979, Singh *et al.* 1987). But in the present investigation, the comparative study regarding chlorophyll content in young, matured and old leaves of five selected clones cultivated in Darjeeling hills clearly indicated that in these clonal tea leaves, the phenomenon is just the reverse case i.e. with the ageing of these selected clonal leaves, the chlorophyll content is found to be increased. In all the selected clonal leaves, the chlorophyll content steadily enhanced with the age of the tea leaves. For example, the total chlorophyll content after carefully compared between young and old leaves of Thurboo-9, it is found that the chlorophyll content of old leaf is 3.74 times higher in comparison to young leaf.

The ratio of chlorophyll-a and chlorophyll-b is also an interesting findings from this part of work. Kurahotta *et al.* (1987) reported that along with senescence of leaf, chlorophyll-a: chlorophyll-b ratio steadily decreased. Similar observation is also recorded in five selected

clonal leaves of Darjeeling hills. For example, in young leaves of TV-19, the chlorophyll-a and chlorophyll-b ratio is 0.66 and the same ratio value decreased to 0.58 in old leaves of the same clone. However, in T-78 and Thurboo-9, the above trend is not followed. In the young leaves of T-78, the chlorphyll a: b ratio is 0.65 whereas the ratio value enhanced to 0.75 in old leaves of the same clone T-78.

Furthermore, if the value of chlorophyll-a and chlorophyll-b content be compared in a particular selected clone during natural ageing of leaf, it is found that with the ageing, both chlorophyll-a and chlorophyll-b content enhanced but the rate of enhancement is generally higher in chlorophyll-b except Thurboo-9. At the advent of calculating the chlorophyll a:b ratio, the value of denominator becomes comparatively high and as a result, chlorophyll-a and chlorophyll-b ratio value steadily decreases during tea leaf ageing excepting T-78 and Thurboo-9 old leaves. Kumar *et al.*(1993) also found distinct clonal variation in chlorophyll-a and b content among three different cultivars of tea (Assam hybrids, China and Cambod types). This result has some agreement with the finding of Kumar *et al.* (1993).

Starch is the main reserve polysaccharide in tea plant. Starch in large quantities is stored in the root system. Gadd (1928) of Tea Research Institute of Sri Lanka first drew attention to the importance of starch reserves in the roots of tea bushes for their recovery following prunning. Growth of buds from pruned tea bushes takes place at the

expense of carbohydrate resources (Kandiah 1971). Relation of carbohydrates with early growth of tea shoots and bud break is also reported (Selvendran 1971, Manivel 1981). Presence of soluble sugars in tea leaves was reported (Cartright and Roberts 1954) but their exact role in tea processing is far from clear. It has been observed that lignin, hemicellulose and alpha-cellulose contents increase with the maturation of tea leaves (Selvendran *et al.* 1972). The tea made from fresh leaf plucked at different hours (morning and afternoon), three to four fold increase of soluble sugar was reported by Ullah (1967) during the manufactured tea derived from freshly plucked tea leaves at morning hour. Important role in tea aroma formation from aldehydes, resulting from the reaction of amino acids with sugars was also reported earlier by Kretovich and Tokareva (1948).

Fig. –36 represents soluble sugar content in mg./gm. of fresh weight basis. It has been observed that sugar content is minimum in the young leaves and maximum in the mature leaves excepting Thurboo-9. There is a definite tendency of decline in soluble sugar content when the leaves become old. Thus, after careful comparison of the sugar content of Thurboo-9 in different status of leaf, it has been revealed that in mature leaf, soluble sugar content is higher about 20 percent than in young leaf, again, in comparison to mature leaf, old leaf has about 33 percent less sugar content.

During withering process, loss of sugar present in the tea leaf

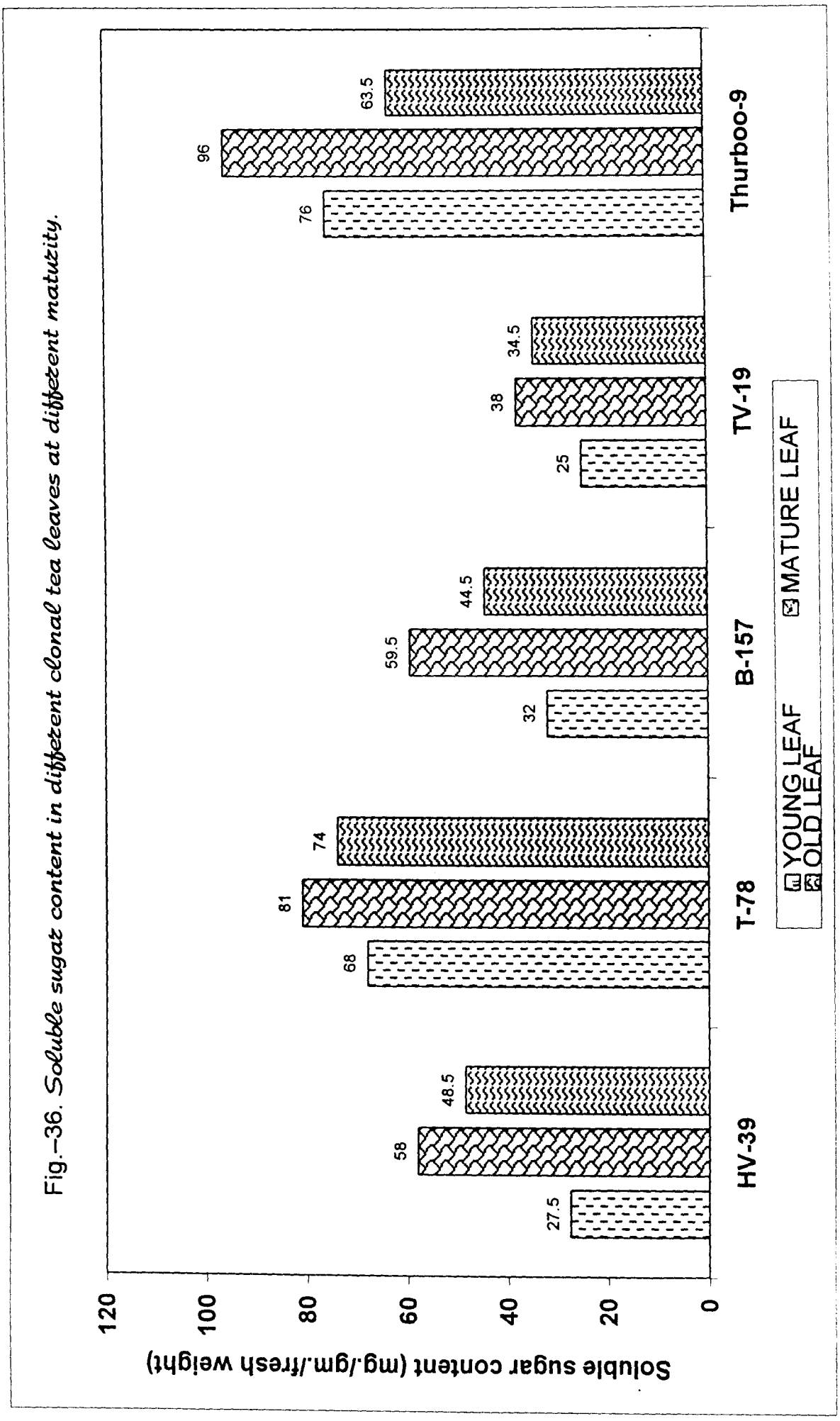


Fig.–36. *Soluble sugar content in different clonal tea leaves at different maturity.*

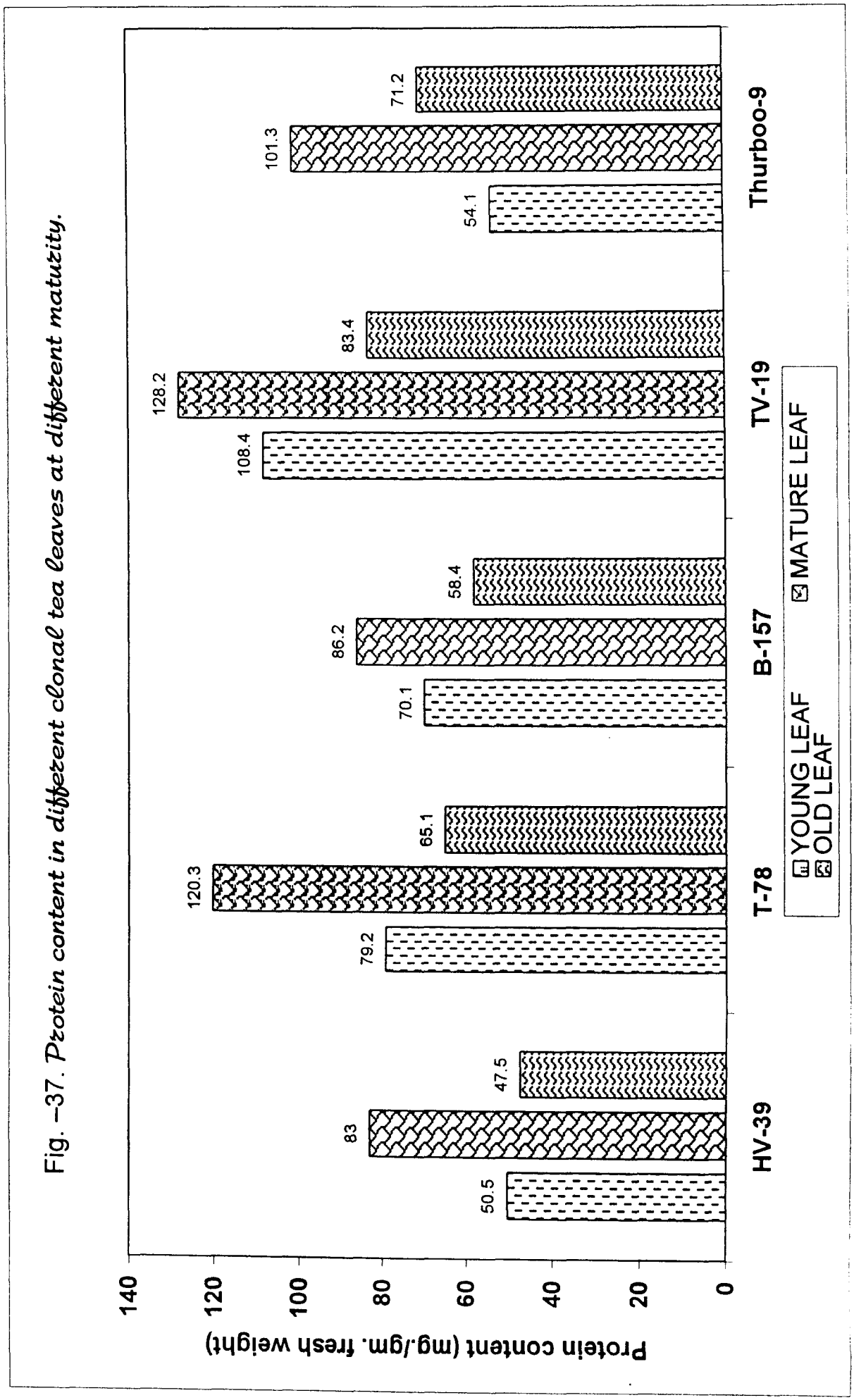
amounting to about four percent (Roberts 1958b). Those sugars are getting reduced and which include glucose-6-phosphate, fructose-6-phosphate, glucose-1-phosphate (Selvendran 1969). It was also reported that a part of sugar in tea leaves was also metabolised into amino-acids during withering process (Dev Choudhury and Bajaj 1980a). Takeda (1979) observed that when freshly collected tea shoots (pluckable) were stored at 2^0C by water culture under dark and light condition, the total available carbohydrate (TAC) was estimated to be declined during storage in dark condition and TAC content was observed to be positively correlated with the growth of bud. The soluble sugars present in the fresh tea leaves actually indicate the amount of sugar present in the leaf-cell cytoplasm and which is readily utilized for the purpose of metabolic activities mainly as a substrate for cellular respiration.

The sugar content is comparatively low in young leaves of the five selected clones of Darjeeling hills and the possible reason for this fact is that (i) chlorophyll content is less in this status of leaf and (ii) in bud condition, the photosynthetic machinery of the cell often remains in immature form, as in leaf buds the existence of proplastids are very common and such proplastids cannot perform the function of the plastids. In the old leaves, the decrease of sugar content may be due to gradual declines of photosynthesis and inactivation of photosynthetic enzymes (TES Ann. Rep. 1981-82). In this connection, the direction of

movement of photosynthates, that is, downward movement of photosynthates from lower leaves of the tea bush, causing two different states of production and utilisation of sugar content by tea bush was reported in Sri Lanka by Sanderson and Sivapalan (1966b).

The amino-acid content of fresh foliage leaves of five selected clones of Darjeeling hills shows a very interesting correlation with the protein content of the same tea leaves. There is an inverse relationship between the value of protein content and amino-acid content; for example, protein content is maximum in mature leaves (figure –37) while amino-acid content is generally minimum in the same leaves (figure –38). Maximum amino acid level is recorded in the young leaves of HV-39 which is, interestingly enough, has a minimum amount of protein level. In young leaves the protein content is low and it may be possible that machinery of protein synthesis is not well operative or rather bio-chemically incomplete in such leaves. Thus amino-acids of the cytoplasmic pool are not converted into protein and as a result young leaves generally show higher levels of amino-acids. There is a possibility that the mature foliage tea leaves produce more carbohydrates than what the young foliage tea leaves can consume. Again it has been reported that the shoot tips of tea producing growth promoting substances and in the matured tea leaves (older leaves) producing growth inhibitors (Banerjee 1996). Older tea leaves also intercept and utilise the growth-promoting substances originating in the

Fig. –37. *Protein content in different clonal tea leaves at different maturity.*



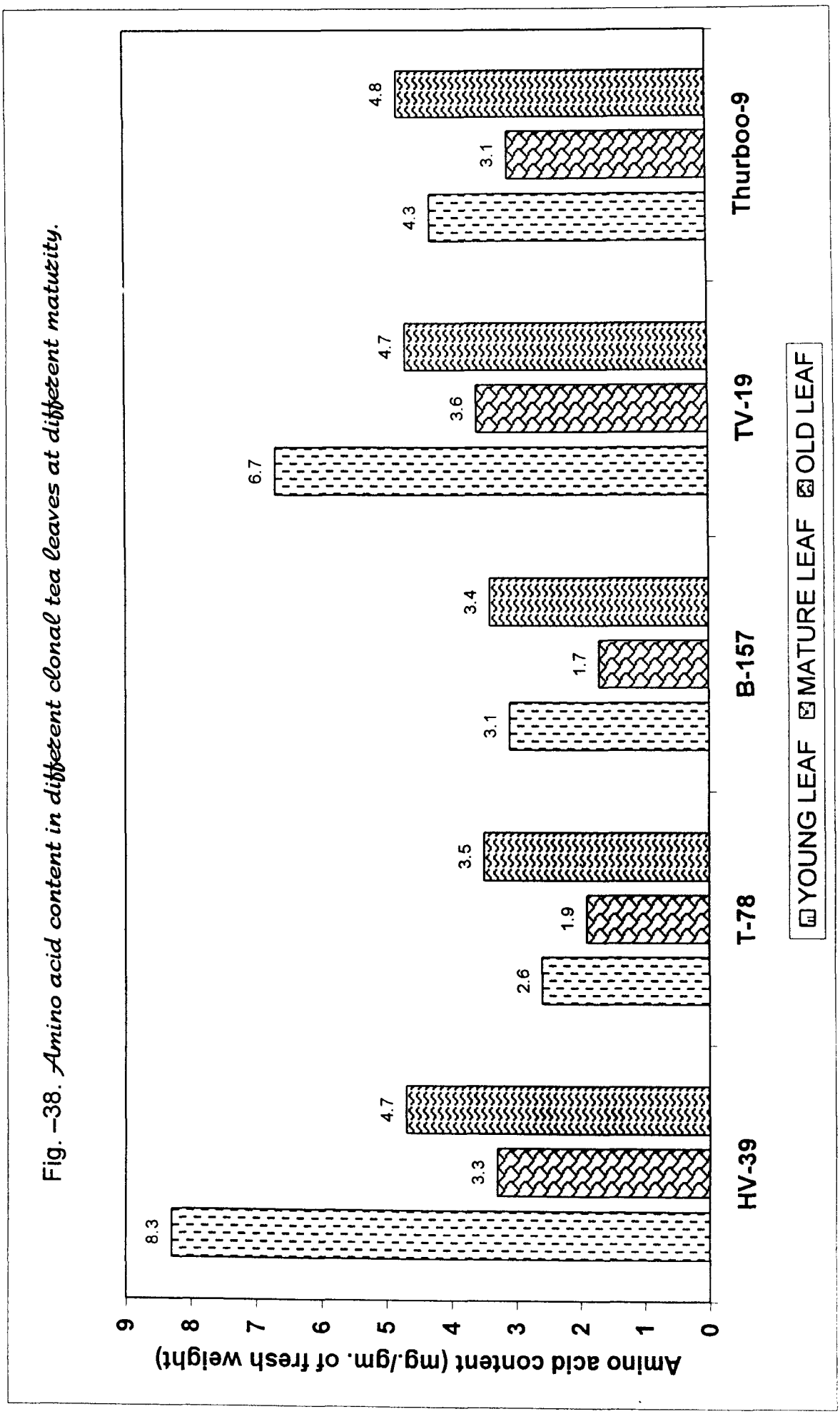


Fig. –38. *Amino acid content in different clonal tea leaves at different maturity.*

roots and thus discreminating protein synthesis is also evident due to inter-relationship of protein synthesis and the production of growth promoting and inhibiting substances (Huxley 1975).

It is also reported that decrease in protein content in old tea leaves might be due to hydrolysis of protein (Roberts and Wood 1951). Similar result was reported by various workers during leaf senescence (Strother and Vatta 1986, Barua and Jana 1986, Singh *et al.* 1987). If it is considered that the rise in protease activity with senescence is a basic biological principle, then it may be argued that in older leaves, protein level decreases due to higher 'protease' activity which hydrolyses protein and the rise of amino-acid in older leaves become the obvious phenomenon as observed in five selected clonal tea leaves cultivated in Darjeeling hills.

Free amino acids in tea leaves are important chemical constituents that considerably influence the quality of tea (Ruan *et al.* 1998).One of the amino acids 'theanine', is identified as 5-N-ethylglutamine, accounted for more than 50 percent of total amino-acid content and comprises about 1 percent of the total dry weight of tea leaves. Theanine has particular relevance in the manufacture of green tea as it protects enzymes from inactivation by polyphenolic products and primarily responsible for the quality of green tea (Wickremasinghe and Perera 1973). In general, green tea contain a greater proportion of theanine than black tea and constituting about 1.5

to 2.0 per cent of the dry matter of tea, is also an important constituent of the thearubigin complex of black tea liquors (Banerjee 1996). Theanine also contributes to its characteristic sweet taste in processed black tea (Chakraborty and Chakraborty 1998). One of the important reaction in the development of flavour in tea leaves are the formation of aldehydes from amino-acids (Eskin *et al.* 1965), but it's mechanism of action is still little understood (Motoda 1979). The relationship between amino-acid content and the quality of black tea was also reported (Chakraborty *et al.* 1978). Bokuchava and Popov (1954) proposed a scheme of oxidative deamination of amino acids in fresh tea leaves by quinones resulting from catechin oxidation. As a result of interaction between catechins and amino-acids, the end products of amino-acid oxidation (CO_2, NH_3 and aldehyde) and a monosubstituted quinone is capable of deaminating the next amino acid molecules which are formed through a number of intermediate reactions. The derived aldehydes or the products of their conversion, possess certain odours, some of which correspond well with tea aroma. Roberts and Rastidge (1962) postulated that amino-acids appear to be oxidized by the product of polyphenol oxidation to the corresponding aldehydes during the distillation process of black tea manufacture. Skobeleva and Popov (1962) observed the formation of carbonyl compounds on oxidation of amino-acids by catechins at elevated temperatures (70^0C-95^0C), 2-methylpropanol being formed from

valine and 3-methylbutanol from leucine. Popov (1966) also observed the formation of aldehydes in a mixture of amino acids with ascorbic acid in the presence of catechins under the influence of Cu++ or high temperatures (80^0-90^0C). Ascorbic acid was oxidized to dehydroascorbic acid which accomplished oxidative deamination of amino-acids resulting the formation of aldehydes. Investigations by Sanderson (1970) and Saijo and Takeo (1970) confirmed that carbonyl compounds can be produced by oxidation of amino-acids by o-quinone. Ohtsuki *et al.* (1987) determined free amino-acids and 5-methylmethionine (MMN) in green tea extracts, compared with the quality of commercial and the place of cultivation of the tea, concluded that their findings might have been the rapid and useful for determining the quality of green tea. It has been observed that concentration of amino-acids and amides in fresh and withered tea shoots vary significantly in clonal level (Bhatia 1962). Fifteen kinds of green tea manufactured from Korea, Taiwan and Japan were investigated in connection with physio-chemical characteristics and sensory scores by the expert panels. Total free amino-acid contents of Korean green tea was found to be higher than that of other countries and 16 amino-acids including theanine were also identified systematically. Theanine, glutamic acid and aspartic acid, which affect the 'brothy' taste of green tea infusion, were found to be highest in Korean tea infusion and lowest in Japanese tea infusion. In multiple regression analysis, the quality affecting factors of green tea were

elucidated as total amino-acid content including caffeine and chlorophyll-b content with the liquor value of green tea infusion (Oh *et al.* 1988). Some amino-acids produce aldehydes like phenyl-acetaldehyde methyl butanol and n-hexanol increase during withering process of black tea manufacture and during this process cis-2-pentanol, cis-3-hexanol, trans-2-hexanol, linalool oxide, nerol, geraniol, 2-phenyl ethanol and phenyl methanol significantly increased (Saijo and Takeo 1974, Takeo 1974), but carotenoids decreased (Hazarika and Mahanta 1983). It has also been reported that the oxidation of flavonols causes oxidative degradation of amino-acids including carotenes and linolenic acid. The amino-acids in turn are transformed to carbonyl compounds, particularly phenyl-acetaldehyde from phenylalanine during fermentation process of black tea manufacture (Saijo and Takeo 1970). The direct biosynthesis of volatile compounds during fermentation involves production of either leucine or acetate as precursors of volatile compounds depending on climatic stress was also reported (Wickremasinghe 1974).

Protein content (mg./gm. of fresh weight basis) was determined from different status of leaves obtained from five selected clones of Darjeeling hills (Fig. –37). The result clearly indicates that the protein content is highest in the mature leaves of all the selected clones. In Thurboo-9, the protein content of mature leaf is 1.87 and 1.42 times higher in comparison to young and old leaves respectively. The variation

in protein content at the clonal level is significant, (figure –37). It is evident that the amount of protein obtained from the young leaves of TV-19 is 2.14 times higher than that of HV-39.

One of the earliest report on involvement of proteins during tea leaf cemmercial processing was published by Stahl (1962). Saijo and Kuwabara (1967) separated fractions of proteins during tea leaf processing. Changes associated with the breakdown of tea leaf proteins and role of the resulting amino-acid during tea manufacture was studied by Bhatia (1962). It had been observed by Bhatia (1962) that concentration of amino acids and amides in fresh and withered tea shoots vary significantly at clonal level.

During leaf processing stage of commercial black tea manufacture, it has been observed that during withering stage of tea leaves, breakdown of protein into amino-acids take place. Peptidase present in tea leaves cause an increase in free amino-acid levels, particularly those of aspartic acid, glutamic acid, serine, glutamine, alanine, tyrosine, phenylalanine, valine, theonine and lysine (Bhatia and Deb, 1965; Roberts and Sanderson 1966). Protein constitute about 20 percent of the dry weight of black tea but less than 2 percent is in soluble form (Balasubramaniam 1995).

It has been observed from the Fig. –39 that the phenol content in five selected clones do not vary significantly. The obtained result indicates maximum phenol content in young leaves of B-157

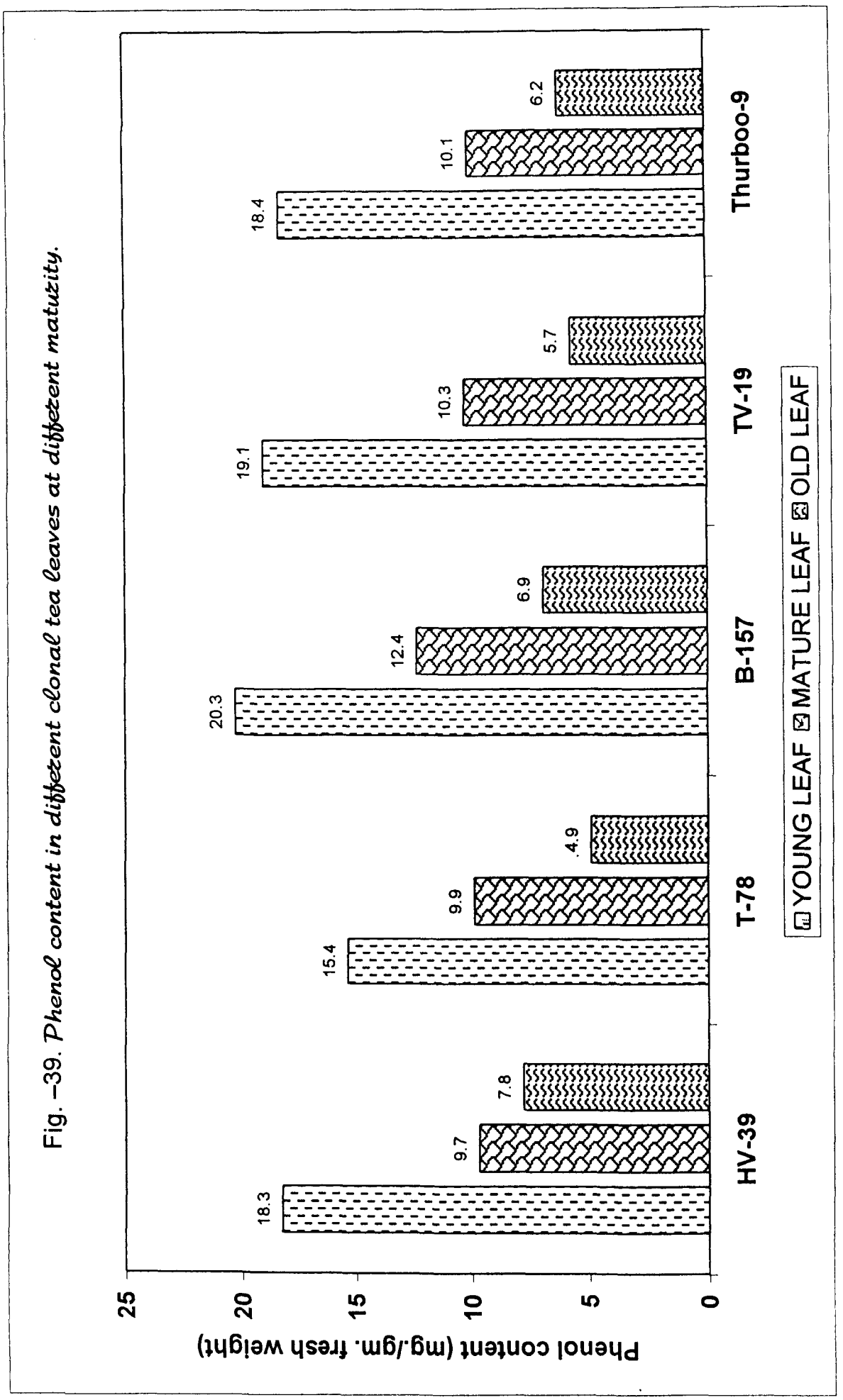


Fig. –39. *Phenol content in different clonal tea leaves at different matuzity.*

(20.3 mg./gm. of fresh weight), while in young leaves of T-78 it is minimum phenol content (15.4 mg./gm./of fresh weight). In mature leaves, B-157 also indicates maximum phenol content (12.4 mg./gm./of fresh weight), lowest value found in HV-39 (9.7 mg./gm. of fresh weight) and considering old leaves, highest phenol content found in HV-39 (7.8 mg./gm. fresh weight), while lowest phenol content found in T-78 (4.9 mg./gm. of fresh weight).

Another interesting feature is noted that during the course of leaf ageing, phenol content drastically decreases in Thurboo-9, the young leaves contain 18.4 mg./ gm. of fresh weight of phenol content which reduced to 10.1 mg./gm. of fresh weight in the mature leaves and the phenol content further declines to the level of 6.2 mg./ gm. of fresh weight in the old leaves. More or less same phenomenon has been observed in T-78, B-157 and TV-19 respectively.

This result is in good agreement with the findings of Banerjee (1996) where the phenolic activity was found to be the highest in the young tea leaves and gradual decline of phenolic activities was found in old tea leaves.

Phenolic compounds are water soluble and located in the vacuoles of the palisade cells of the tea leaves (Selvendran and King 1976). Phenolic components of tea leaves are most important, because they are mainly responsible for the unique character of processed tea (Roberts 1962). Sanderson (1972) identified different phenolic compounds in

fresh tea flush and considered all the phenolic components are equally responsible in consorted way for aromatization of tea in different steps of tea leaf processing in commercial tea manufacture. The polyphenolic composition of tea undergoes changes with leaf age and its activity is at its optimum in young leaves, hence the finest manufactured tea is obtained from the young flush. The main effect is the progressive decline in total phenolic materials along with an alteration in the proportion of flavonols, related to each other (Banerjee 1996). Flavonols and flavonol glycosides occur in small quantities in tea leaf (U1'yanova 1963) and the quantity of flavonols varies in different tea plant parts (U1' yanova and Erofeyeva 1966). Egorov (1975) revealed that phenolic compounds are very important for 'cognac' aroma of processed tea after organoleptic assessment of fresh tea leaves from Russia. Renold *et al.* (1974) have suggested that black tea aroma constituents such as 2-methylbenzaldehyde and 4-methoxy benzaldehyde may be the degradation products derived directly from tea leaf phenolic substances.

It has also been reported that various phenols like flavonols and catechins during the rolling process of commercial tea manufacture are oxidised by polyphenol oxidase to yield two important flavones of tea, that is, theaflavins (TF) and thearubigins (TR) which gives brightness, briskness, and characteristic taste to the tea liquor (Ullah *et al.* 1984). Flavour of tea liquor is also due to the presence of free flavons or

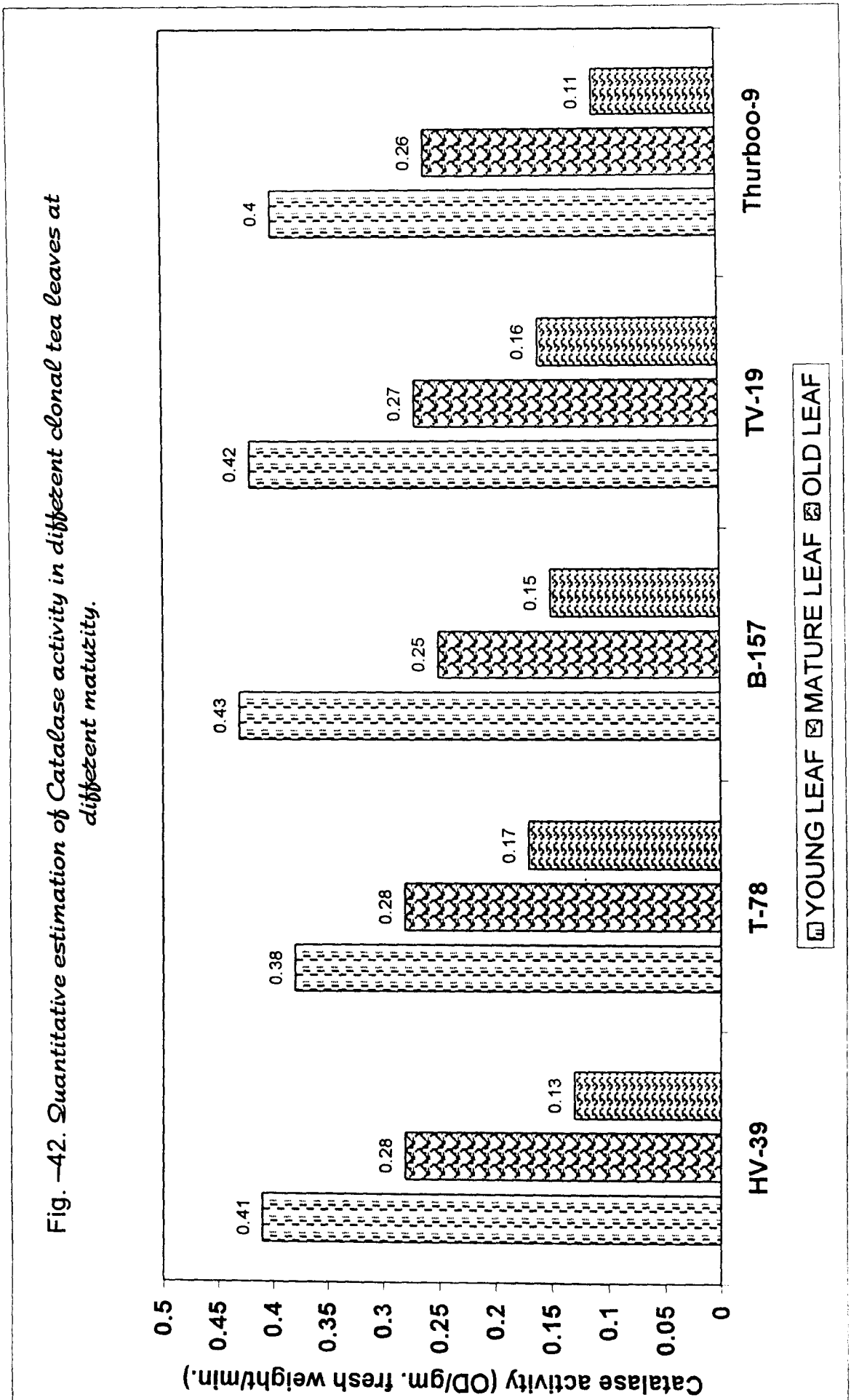
bond flavon compounds like 3-hexane-1-ol, benzyl alcohol, 3-phenylethanol etc. which are present in glycosidic bonds (Fischer *et al.* 1987). It is also suggested that phenolic compounds particularly chlorogenic acid inhibits the germination of fungal spores and thus renders biochemical resistance to the tea plants (Sarma 1960).

It has been observed from the Figure –42 that catalase activity is maximum in the young tea leaves and minimum in old leaves. An intermediate activities of this enzyme has also been observed in matured leaves in comparison to young and old ones in all the five selected clones of Darjeeling hills. In young leaf, highest catalase activity was found in B-157 (0.43) and lowest activity in T-78 (0.38); in matured leaf HV-39 and T-78 found to be highest (0.28) and lowest was recorded in B-157 (0.25); in old leaf, catalase activity found to be highest in T-78 (0.17) and lowest was recorded in Thurboo-9 (0.11). However, marginal difference were noted concerning to catalase activity in all the leaf status.

Catalase is an iron porphyrin enzyme which catalyzes very rapid decomposition of hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). Catalase removes highly toxic H_2O_2 from cell and by breaking H_2O_2, it supplies oxygen for cellular oxidation.

The catalase crude preparation had been obtained from fresh tea leaves and some kinetic characteristics of this enzyme had been studied by Tsagarelli *et al.* (1988). It had been observed by them that this

Fig. –42. *Quantitative estimation of Catalase activity in different clonal tea leaves at different maturity.*



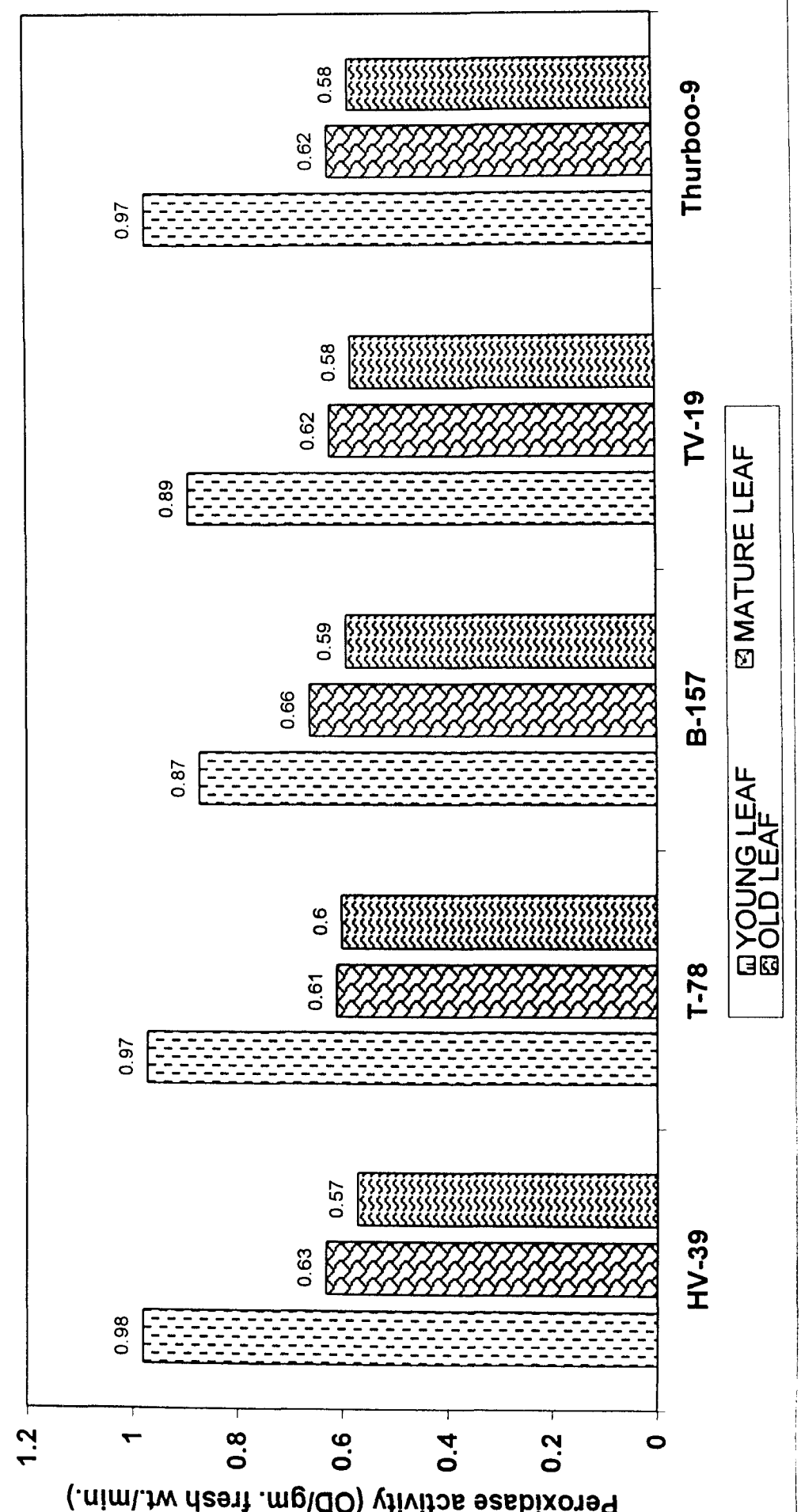
enzyme has shown the maximum activity at pH 5.6 to 6.5 at temperature 40^0C to 45^0C. It has also been observed by Tsagarelli and Pruidze (1990a, 1990b) that the dependence between the rate of catalase reaction and the concentration of hydrogen peroxide obeyed the Michaelis-Menton's classical kinetics. Tsagarelli *et al.* (1988) also isolated, purified and characterized the enzyme catalase from fresh tea leaves.

The result obtained from the Fig. –41 regarding peroxidase activity, which shows maximum activity in the young leaves and the decreased activity has been observed with ageing of the leaves evidenced from five selected clones of Darjeeling hills. Similar observation was reported by Shah *et al.* (1976).

The quantitative estimation of peroxidase activity has been reported first time in tea of Darjeeling hills at the clonal level. Since peroxidase has been regarded as one of the principal enzyme and attributed diverse functions, including oxidation of phenolic compound of tea leaf, which ultimately produces theaflavins and tearubigins (regarded as a 'tea cream'). Tea leaf peroxidase activity can be exploited in the tea manufacturing process, specially during fermentation process (Roberts 1962, Wood and Roberts 1964, Dix *et al.* 1981).

The result presented in the Fig. –40 shows the value in connection with polyphenol oxidase activity which greatly differs from

Fig. –41. *Quantitative estimation of Peroxidase activity in different clonal tea leaves at different maturity.*



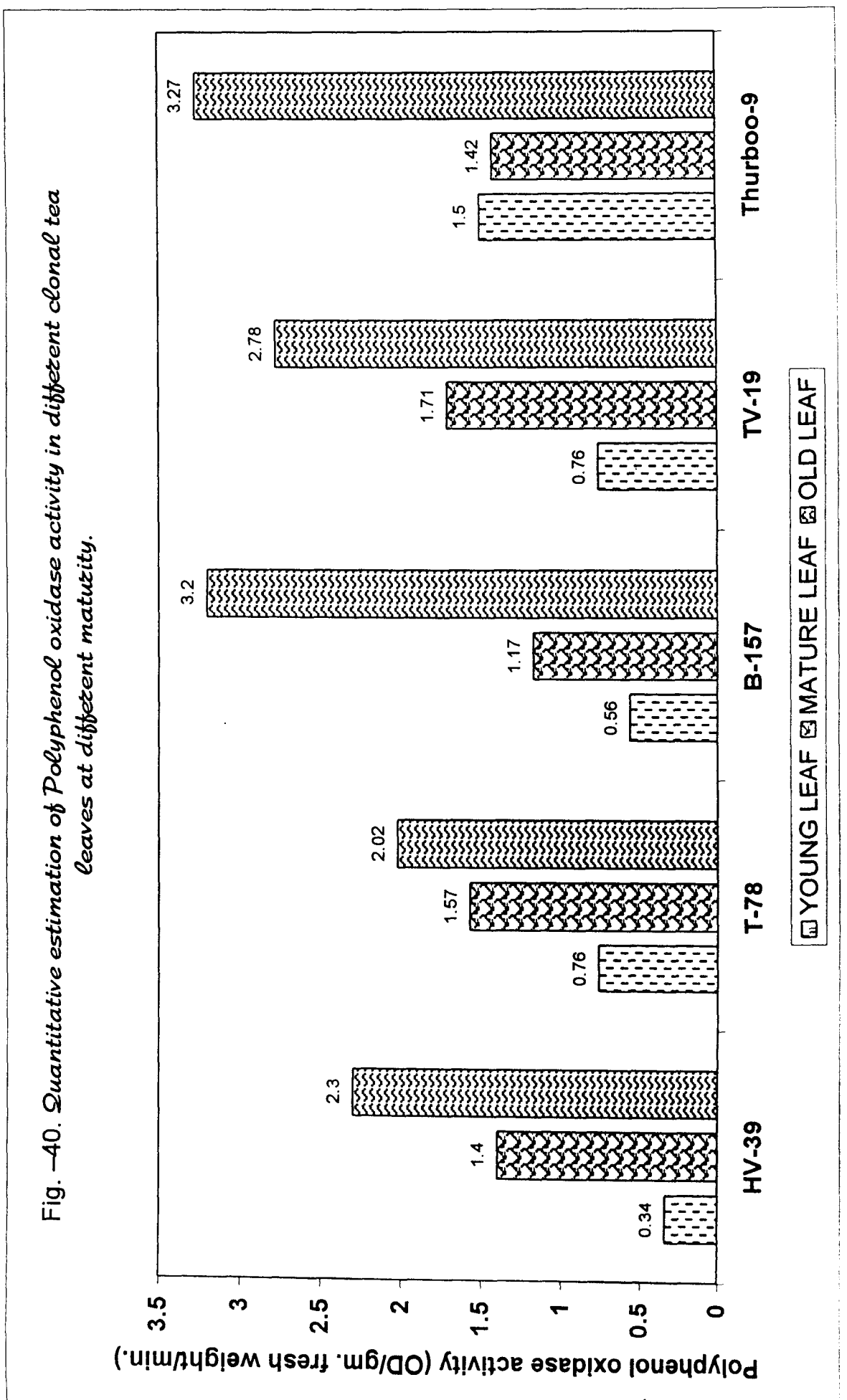


Fig. –40. *Quantitative estimation of Polyphenol oxidase activity in different clonal tea leaves at different maturity.*

clone to clone. Polyphenol oxidase activity obtained from young leaves of TV-19 and T-78 show the same activity (0.76 unit in both the cases), highest activity found in Thurboo-9 (1.5 unit) and lowest value obtained from HV-39 (0.34). Considering mature leaf, highest activity is observed in TV-19 (1.71), whereas lowest activity in B-157 (1.17), considering old leaf, higher PPO activity is noted in Thurboo-9 (3.27 unit) and lowest PPO activity is observed in T-78 (2.02 unit).

Tea leaf polyphenol oxidase (o-diphenol : O_2 oxido-reductase 1.10.3.1) is of crucial importance during the manufacturing of black tea. It's activity increases with the increase in copper concentrations and it is believed to be a copper-containing protein (Sreerangachar 1943a, b), consisting of at least four isoenzymes (Bendall and Gregory 1963; Gregory and Bendall 1966; Takeo and Uritani 1966); the major component has a molecular weight of 144000 $\pm$ 16,000 and contains 0.32 percent (w/w) of copper. This enzyme was considered to have inosoluble components but by an improved method of extraction (Coggon *et al.* 1973, Takeo 1965), soluble polyphenol oxidase preparation was obtained and this was purified over 200-fold using iso-electric focussing (Banerjee 1996).

Polyphenol oxidase is generally found in association with chloroplast (Oparin and Shubert 1950; Tolbert 1973); it may also be present in mitochondria (Bokuchava *et al.* 1970). According to Takeo (1966b) the major part of activity of this enzyme is not in the

chloroplast but it is bound on the precipitable particles. Further, the position of polyphenol oxidase coincides with those of the markers of peroxisomes, catalase and malate dehydrogenase, but not with those of mitochondria and chloroplasts, cytochrome oxidase and chlorophyll (Kato *et al.* 1976). There is also evidence (Wickremasinghe *et al.* 1967) that a major portion of the enzyme is localised in the leaf epidermis with the young leaves having the enzyme both in the upper and lower epidermis, but in older leaves the enzyme is restricted to the lower epidermis only.

Polyphenol oxidase (PPO) oxidises polyphenol and this enzyme activity steers the commercial processing of tea leaves. Polyphenol oxidase is involved in the manufacture of black tea from fresh green tea leaves and it is responsible for the formation of aromatic compounds by oxidising the substrate (Srivastava 1986). PPO oxidised thearubigins (TR) which gives the positive quality of tea (Ullah *et al.* 1984). The specific activity of PPO with peroxidase, responsible for formation of brown components known as teaflavins during commercial tea processing, particularly highest activity was noted in rolling process than fermentation (Mahanta *et al.* 1993).

The advances in the understanding of reaction mechanism relating to polyphenol oxidase was critically reviewed by Mayer (1987) with special emphasis on methodology, occurrence, properties and physiological functions. It has also been reported that the activity of the

enzyme polyphenol oxidase was observed higher in the leaves of the tea clone than the seed cultivar. As a result of this PPO activity, clonal tea has a better quality than seedling tea (Van Lelyveld and Oe Rooster 1986).

This result of polyphenol oxidase activity obtained from five selected clonal tea leaves of Darjeeling hills partially agree with the result presented by Wickremasinghe (1974), excepting root and seed PPO activity, while estimating the distribution of various chemical compounds in the entire tea bush including PPO activity.

This quantitative estimation of polyphenol oxidase activity may have a scope for utilisation in commercial objective of tea manufacture in Darjeeling hills, because of the fact that the outstanding feature of tea manufacture (in fermentation stage) is the formation of theaflavins, an essential product derived from polyphenol oxidase activity (Coggon *et al.* 1973; Kato *et al.* 1976, Raymond-Miller *et al.* 1990), which is one of the determining factors of tea quality (Ullah *et al.* 1984). Another interesting feature is that with natural ageing of tea leaves, polyphenol activity is found to increase and this fact is true for all the cases (Tolbert 1973). Thus, with the natural ageing of tea leaves, phenol content is found to be decreased, which can be correlated with the rise in PPO activity in such tea leaves. Fig. –39 shows that phenol content in young leaves of T-78 is 3.14 times higher than that of old leaves and in the same clone, PPO activity in the young leaves is about 1.55 times

higher in comparison to mature leaves. Thus, it may be concluded that increased PPO activity is actually responsible for the fall of phenol content during natural ageing of tea leaves as observed in five selected clones of Darjeeling hills.