Proceedings of the Sixth Academic Sessions, University of Ruhuna 2009
Vol. 6 pgs 32 - 38

Preliminary study of the segregating pattern of anthocyanin pigmentation as a marker to facilitate tea breeding

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Abstract

Anthocyanin is one of the plant pigments that have been widely used as a morpho-chemical marker in characterization of several tea cultivars since it is an easily observable phenotypic trait. Hence, it has a potential to be used as a selection criterion in tea breeding program. This research was aimed at studying the segregating pattern of anthocyanin pigment in the shoot using the population of tea which shows a great variation in the expression of the trait. A progeny consisted of 248 individuals generated from a cross between two parental clones TRI 2043 and TRI 2023, which showed extreme phenotypes for the anthocyanin pigment (pigmented/non-pigmented) was used in the study. All individuals of the progeny were characterized morphologically for presence or absence of purple pigments as well as intensity of the pigments using Royal Horticultural Society (RHS) colour chart for anthocyanin pigmentation. For the confirmation of this morphological characterization, anthocyanin content was determined by spectrophotometry. According to the results, colour variation of tea shoots throughout the population confirms the highest segregation for anthocyanin pigments, a transgressive segregation. A continuous variation of the frequency distribution of anthocyanin concentration of individuals in the population revealed that there is no normal Mendelian segregation for the anthocyanin pigmentation. Therefore, there is no major gene inheritance of the trait but a polygenic inheritance can be observed and this trait can be considered as governed by quantitative trait loci (QTL).

Keywords: tea breeding, morpho-chemical markers, segregating pattern, Mendelian segregation, Anthocyanin pigmentation

Introduction

Tea is one of the most important non-alcoholic beverages worldwide that rank second only to water. In recent times it is gaining further popularity as an important 'health drink' in view of its purported medicinal value. Sri Lanka is one of the largest tea exporter in the world. More than 90% of the production is aimed for export market, earning Sri Lankan Rs. 112 billion (Anonymous, 2008).

Continuous attempts are being made to improve quantity and quality of the end product to maximize profits in order to sustain the industry. Among the several factors that contribute for productivity in tea plantations, use of improved cultivars is ascribed as the most important component. Conventional tea breeding is well established and contributed much for tea improvement than non conventional tea breeding over the past several decades in Sri Lanka. Tea cultivar development was made possible due to character segregation and transmission of parental characters to their offspring, mainly due to its allogamous nature.

Most plant breeders refer Mendelian's principles as laws in their breeding experiments to have a basic understanding of how traits are inherited from one generation to the next. All phenotypic traits do not segregate according to the Mendelian laws.

Several morpho-chemical markers are available for superior cultivar identification. These markers are greatly influenced by environmental factors and show a continuous variation with high degree of plasticity. Tea

breeders use morphological markers such as leaf size, pubescence, pigmentation and biochemical markers such as total catechin/ polyphenol content, caffeine content (Mondal *et al.*, 2004). Anthocyanin pigmention which is characterized by its unique colour in plant tissues has been widely used as a prominent descriptor for characterization of tea germpalsm and identified as an easily observable phenotypic trait (Gunasekare and Pieris, 2006; Piyasundara *et al.*, 2008).

Anthocyanin pigment is a water soluble flavonoid (Janna *et al.*, 2006). Next to chlorophyll, anthocyanin is the most important group of plant pigments visible to the human eye. They are non toxic pigments, responsible for some colour of most part of the plant such as leaves, fruit, stem, flower petal. It provides wide range of colours such as orange, red, pink, scarlet, mauve, violet and blue (Obute and Adubor, 2007). They have long been the subject of investigation by botanists and plant physiologists because of their roles as pollination attractants and phytoprotective agents (Gould, 2004) and their importance in taxonomic studies. Today, interest in anthocyanin pigments beside colour attractions has intensified because of their possible health benefits with antioxidant properties (Wang *et al.*, 1997) as measured by the oxygen radical absorbingcapacity (ORAC) assay (Wang *et al.*, 1996).

Naturally occurring anthocyanins are in equilibrium between the color flavylium cation and the colorless hydrated form. The equilibrium is driven to the left as pH decreases and to the right as it increases.

Sax (1923) first reported association of a simply inherited genetic marker with a quantitative trait in plants when he observed segregation of seed size associated with segregation for a seed coat colour marker in beans (Phaseolus vulgaris L). Haskell (1954) demonstrated linkage of flowering time (a quantitative trait) in Raspberry with a simply inherited gene for flower colour. Anthocyanin pigmentation has been widely used as genetic marker in many other crops such as maize (Robinett *et al.*, 1995), soybean (Groose and Palmer, 2003), teosinte (Lauter *et al.*, 2004), apple (Change *et al.*, 2007),). However, there has been no study reported on anthocyanin pigments as a genetic marker in tea.

The objective of this research was to study the segregating pattern of anthocyanin pigmentation in a population of tea with a great variation in the expression of anthocyanin pigment thereby exploring the possibility of using it as a marker in the tea breeding program.

Materials and methods

Plant material

The study is carried out at the plant breeding division in Tea Research Institute, Thalawakele, Sri Lanka. Seed population established in Field No. 10, St Coombs estate was used for the study.

Leaf material comprising of two leaves and a terminal bud was sampled from 248 individuals of the F1 (segregating) progeny in the population and their parents, TRI 2043 and TRI 2023 clones. Individuals in the progeny were tagged assigning the labels during the sampling.

Evaluation of pigment distribution in shoots

Two parental plants and individual plants in the progeny were evaluated for the distribution of anthocyanin pigmentation.

Each individual in the progeny were morphologically categorized as pigmented and non-pigmented individuals. The pigment distribution was recorded by the presence of pigments in first leaf, second leaf, third leaf, petiole and/or stem (Figure 1).

Assessment of colour intensity

Second leaf was used as standard to evaluate colour variation phenotypically to express intensity of the leaf colouration using the colour chart developed by the Royal Horticultural Society (RHS, 2007) color chart.

Determination of anthocyanin concentration

Then anthocyanin pigments of those individuals was quantified by using spectrophotometry after extraction of anthocyanin from freshly harvested second leaf using 1% (v/v) HCl according to the procedures described by Harbone (1967) with some modifications.

(A) (B) (C)

Figure 1. Variation of anthocyanin distribution in various plant parts among individuals. Presence of anthocyanin in (A) leaf, stem and petiole; (B) leaf and petiole; (C) petiole only

Anthocyanin content in extracts of second leaf was measured quantitatively by their absorption at 530 nm using UV Visible spectrophotometer (Jenway) against a blank (480 µl methanol 1% HCl and 320 µl distilled water for a total of 800 µl). Absorbance values obtained for anthocyanin pigments were expressed in mole per liter ($molL^{-1}$) by using the law of Beer – Lambert ($A= \epsilon CL$). The molar absorption coefficient was used 30,000 mol/l cm (Mark *et al.*, 2002). One hundred and fifty individuals from the progeny were chosen for anthocyanin extraction depending on their colour codes that given according to the RHS color chart.

Statistical data analysis

Data of anthocyanin distribution were subjected to Chi squared (χ^2) test to check whether the segregating pattern follow the Mendelian law. Mean anthocyanin quantity, of both individuals and parental clones were calculated and cluster analysis was carried out for morphological characterization based on presence or absence of pigments using SAS (SAS, 1999) software package.

Frequency distribution of anthocyanin pigments of individuals was plotted for the intensity of leaf colour and for total anthocyanin content. Finally the correlation analyse between the morphological characterization based on RHS colour chart and anthocyanin content were analyzed using SAS (SAS, 1999). For correlation analysis, colour codes used for morphological characterization of leaf colour were converted into a numerical scale according to the order given in the RHS color chart.

One way ANOVA was done to analyze variance of anthocyanin concentration among individuals in the progeny. Mean separation was done using Dunnet's t-test to study the variation of anthocyanin concentration of individuals in the progeny comparing with parental clones.

Results and discussion

TRI 2043 has characteristic purple pigmentations in immature leaves, petiols and stem whereas it is absent in TRI 2023. Tea is a highly cross pollinating and heterozygous species. The high degree of variation and heterozygosity found in tea suggests that traits segregation in the F1 progeny itself. Therefore, to study the trait segregation in tea, F1 progeny could be used. Two parents used to generate the segregating progeny have shown extreme phenotypes for various traits such as purple pigmentation, pubescence in young leaves and sensitivity to blister blight disease. Hence, the resultant F1 progeny among the cross between those two parents has significance in studying the segregation of anthocyanin pigments. This progeny was a result of the open pollination between those two parental clones and they were planted with parental clones in the same field to minimize environmental effects within the population.

Morphological characterization based on standard plant descriptors (IPGRI, 1997) in relation to purple pigmentation clearly showed a wide variation in terms of presence or absence of purple pigments depending on its presence in different plant tissues. The dendrogram constructed using average linkage cluster analysis method separated 150 phenotypes into 13 clusters (Figure 2).

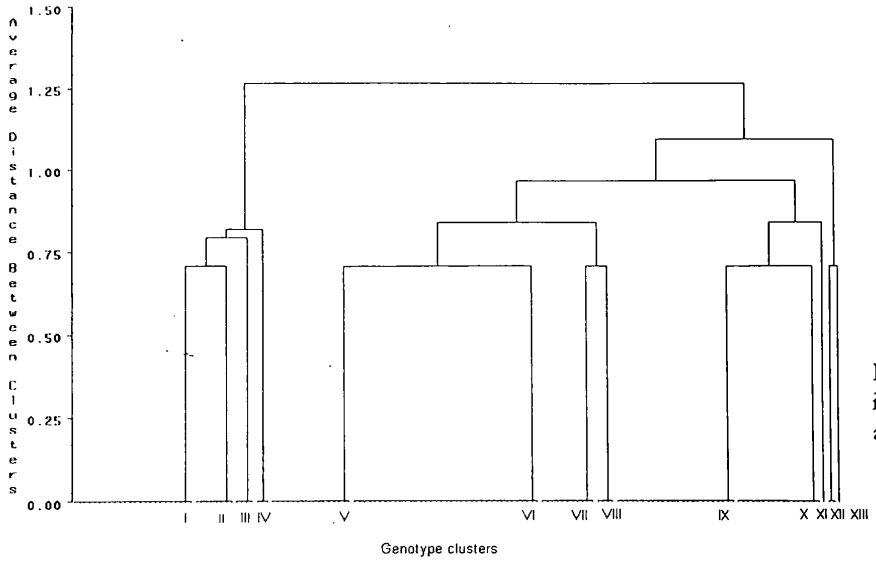

Figure 2. Phenotype clusters of individuals in the progeny for anthocyanin pigment distribution.

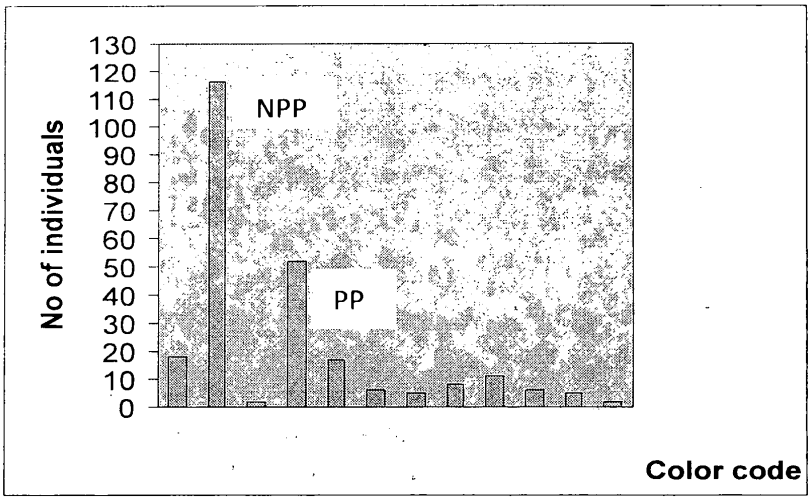

Figure 3 Variation in anthocyanin pigments in the population according to the coluor chart, after arranging colour codes into a linear scale of the colour chart. (NPP- Non pigmented parent, PP- Pigmented parent), representative colour codes for given numbers in X axis are shown as follows (**1**-N 37A, N137B, **2**- 144A, **3**- N144A, **4**-146A, 146B, 148A, **5**-152A, 152B, **6**-165A, 166A, 166B, 177A, 183A, **7**- 199A, **8**- N199A, **9**- N199B, **10**- N199C, N199D, **11**- 200B, 200C, 200D, **12**- N200A)

According to the data generated from morphological characterization of the progeny for the presence of purple pigments in various plant parts (first leaf, second leaf, third leaf, petiole, stem) revealed a great variation in the intensity of the pigments that was observed visually. Based on the categorization of individuals, using the RHS colour chart have showed that individuals in the progeny belong to 12 different color groups (Figure 3)

However, the grouping made by distribution of anthocyanin in various parts of the plant (Figure 2) and the grouping made by colour intensity (Figure 3) cannot be correlated.

The frequency of distribution of colour categories showed a large number of individuals in the progeny (116) skewed towards non-pigmented parent, TRI 2023 and revealed a segregating ratio of 3: 1, (non-pigmented : pigmented) (χ^2 = 1.3) closely associated with Mendelian law of segregation. If there is a normal Mendelian segregation of this trait, two discrete groups should be identifiable. However, within the group of pigmented individuals, a great variation in the colour and the intensity was noted. Due to greater variation observed morphologically in the intensity of the pigment in the population, it cannot be confirmed that this trait follows Mendelian inheritance as revealed from the morphological characterization.

The observed morphological variation of purple pigment distribution (Figure 1) was not sufficient to confirm the segregation pattern of the trait. According to Harborne (1967), phenotypic estimation of colour in plant tissues does not provide conclusive evidence because, colour expressed in plant tissues is a combined effect of anthocyanin and chlorophylls. Perhaps, phenotypic expression of anthocyanin is often masked by chlorophyll. This suggests that those qualitative measurements of colour intensities were

not strong enough to study the pigment inheritance. Therefore, more accurate quantification of purple pigments which could be due to anthocyanin content is required to confirm segregation ratio obtained from morphological characterization of the population as well as to see whether there is any correlation between qualitative and quantitative estimations (Figure 4).

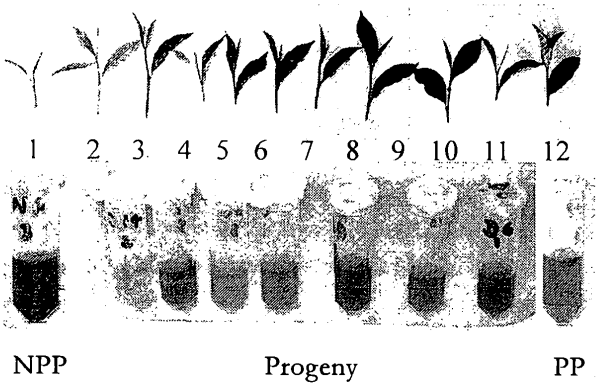

Figure 4. Illustration to show segregation of anthocyanin pigmentation between extreme phenotypes (NPP-Non pigmented parent, PP-pigmented parent)

In order to arrive at an accurate estimation of anthocyanin content, spectrophotometry was used. According to the Dunnet's t-test of anthocyanin content measured in the second leaf, the progeny showed a significant variation among the individuals as well as with their parents. Seven individuals (F10, F5, D1, D12, G4, D13, E6) recorded significantly higher anthocyanin content whereas majority of the individuals had significantly lesser content of anthocyanin than the pigmented parent, TRI 2043. As compared to the non-pigmented parent, TRI 2023, majority of the individuals of the progeny showed significantly higher anthocyanin concentration (Table 1 and Figure5). However, there were no any individual with significantly lesser content of anthocyanin than recorded in TRI 2023 as expected. These data showed extreme anthocyanin quantities than the both parental quantities and provide the conclusive evidence for the segregation of anthocyanin pigmentation as a Transgressive segregation according to the Vicente and Tanksley, (1993).

Between the morphological characterization based on RHS colour chart and anthocyanin content, a positive significant correlation (r^2=0.568) was observed. This indicates that qualitative estimates using RHS colour chart could be used as a measure of anthocyanin content with reasonable accuracy without using spectrophotometric method. The frequency distribution obtained for anthocyanin concentrations of individuals showed a continuous variation and hence it can be suggested that this trait is governed by polygenic inheritance. Unlike monogenic traits, polygenic traits do not follow a pattern of Mendelian inheritance. Their phenotypes typically vary along a continuous gradient depicted by a bell shaped curve. All genes have an effect on expression of the character (trait) in different quantities. This is evident from the frequency distribution histogram (Figure 5) obtained for anthocyanin concentration.

One of the practical implications of the outcome of this study could be related to identification of individuals having higher anthocyanin content in a highly segregating population for anthocyanin pigments.

Table 1. Variations of anthocyanin quantities of selected individuals of progeny in comparison with the parents (NPP-Non pigmented parent, PP-pigmented parent) Quantity of anthocyanin of selected individuals of progeny representing the full spectrum of variation and of the two parents.

Mean Anthocyanin quantity (mol/l x 10^{-5})												
E19	**NPP**	D19	K1	F3	**PP**	K10	G17	D12	E6	D1	F5	F10
0.65	**1.52**	1.63	2.65	3.64	**4.03**	4.52	5.61	6.4	7.08	8.4	9.92	11.76

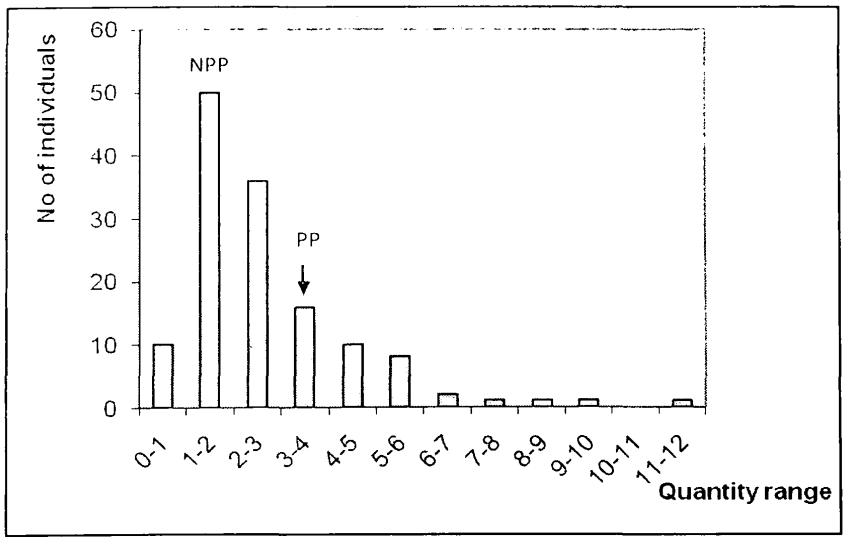

Figure 5. Frequency distribution of anthocyanin pigments based on anthocyanin content in each individual in the population

It was reported that high resistant levels found in TRI 2043 for blister blight disease (Islam *et al.*, 2005; Punyasiri *et al.*, 2005) is attributed to the high level of anthocyanins present and their ready conversion into proanthocyanidins *via* catechins. Results of the present study showed that there are seven individuals having more anthocyanin content (transgressive segregants) than the pigmented parent. Therefore, the higher levels of anthocyanin could exert the ability for higher level of blister blight disease resistance than the resistant parent, i.e. pigmented parent (TRI 2043). This source of resistance could be capitalized for developing new tea cultivars having higher resistance than found in the cultivated tea in tea breeding programmes. It has been reported that proanthocyanidins accumulates in tea plants upon infection by the pathogen causing Blister blight diseases (Punyasiri *et al.*, 2005).

Conclusion

Study reveals that this tea population is segregating for the trait of anthocyanin pigments. However, the trait does not follow normal Mendelian segregation ratios. Thus, it can be deduced that the trait is governed by polygenic inheritance and hence, inheritance of anthocyanin pigmentation is controlled by quantitative trait loci (QTLs) on the chromosome. Hybrids of crosses between individuals with favorable QTL genotypes can be easily screened using the morphological marker established in this study as there is a positive correlation between qualitative and quantitative estimates made for anthocyanin pigments. The RHS colour chart can be used to estimate the colour intensity of anthocyanin and could be used as a tool in measuring anthocyanin concentration as a morphological marker with reasonable accuracy in the selection of suitable cultivars for anthocyanin pigmentation without following a tedious process of chemical quantification of anthocyanin. Outcome of this study has important implications on the current tea breeding programs, to select appropriate clonal varieties, if a large number of plants have to be screened.

Acknowledgements

This research was supported by Department of Botany, University of Ruhuna, Sri Lanka and Tea Research Institute, Thalawakele, Sri Lanka. We thanks for Department of Zoology, University of Ruhuna for allowing us to do spectophotometric analysis and Prof. Saman Abeysinghe, Dept. of Botany, University of Ruhuna for his immense help in publishing this paper.

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