

Stomatal Density as a Selection Criterion for Developing Tea Varieties with High Physiological Efficiency

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Abstract

Stomata, the small opening in leaf connecting plant with atmosphere, play pivotal roles in global water and carbon cycles. Stomata regulate the two key important physiological functions *viz.* photosynthesis and transpiration and thus are crucial for performance of crop species in changing climatic conditions. Although environmental factors influence the density and size of stomata, the genetic control of this character is evident. High variation for this character is reported from different crop species and thus improvement of this character is possible through appropriately designed breeding strategy. In tea, stomatal density varied from 44.67 mm⁻² to 108 mm⁻² and this character exhibited a significant and positive correlation with photosynthetic rate (Pn) and water use efficiency. A high value for heritability and genetic advance was detected for this character in tea, indicating its tight genetic control. Thus stomatal density may be an important selection criterion and development of physiologically efficient tea clones and seed varieties may be possible by improving stomatal density through a suitably designed breeding strategy.

Keywords: Heritability; Photosynthesis; Stomata; Transpiration; Water use efficiency

Introduction

Tea [*Camellia sinensis* L (O) Kuntze; family Theaceae] is grown in a wide range of soil and climatic conditions in both the hemisphere from 43 °N in Georgia to 34 °S in South America, and from 150 °E in New Guinea to 60 °W in Argentina (Bezbaruah 1975). Tea is a perennial woody plant and conventionally, two leaves and a bud is plucked from regularly pruned tea bushes to make this non-alcoholic but widely consumed beverage. Tea is propagated through vegetative clones and seed varieties and selection and hybridization program are the most common breeding methods adopted to develop new clones and seed varieties.

The drastic changes in climatic conditions are likely to impact the tea cultivation in north east India adversely (Dutta 2014) and as such, development of physiologically efficient tea clones and seed varieties is necessary to keep the industry on its feet.

Stomata, the small aperture, connecting plant with the atmosphere, present in both the surfaces (amphistomatous leaves) or in the lower surface (hypostomatous leaves) and regulate important physiological functions such as photosynthesis and transpiration (Parkhurst 1978). In trees, stomatal density (SD) often ranges from 30 to 1190 mm⁻² (Willmer and Fricker 1996), and often a negative

relationship between SD and stomatal size has been observed (Hetherington and Woodward 2003). Although significant progress has been made in understanding the regulatory network of stomatal movement (Hetherington and Woodward 2003), less attention has been paid to the effects of stomatal density and size on photosynthesis and transpiration (Wang *et al.* 2007).

The number and distribution of the stomata plays an important role in determining the rate of gas exchange and water loss from a leaf. For example, we might hypothesize that a tea clone with high density of stomata will have a greater rate of transpiration and as such, will be more prone to drought damage. Even in conditions when soil moisture is not a limiting factor for plant growth, the control of water loss is important to avoid leaf dehydration due to excessive transpiration, particularly in tall trees that may exhibit reduced hydraulic conductance (Hubbard *et al.* 1999). Adams *et al.* (2012) from their investigation reported that plants with low stomatal density (SD) and high stomatal size (SS) may be well suited to growth under predicted future atmospheric CO₂ environments and/or water-scarce environments.

Stomatal density and distribution, and epidermal features may affect gas exchange quite remarkably and exhibited relationships with key environmental factors such as elevated CO₂ concentration, heat stress, salt stress, drought, precipitation change and plant density (Schluter *et al.* 2003; Nilson and Assmann 2007; Xu *et al.* 2008)

Information on how stomata are distributed on the leaf surface (one or both sides) is of major interest, not only because stomatal location can

affect CO₂ diffusion into the carboxylation sites, but also because latent heat transfer (amount of energy used in transpiration) is related to the placement of stomata on the leaf surface. Thus, information on variation of stomatal density and distribution is of significant importance for plant breeder as this character can affect CO₂ diffusion into the carboxylation sites and also the amount of energy used in transpiration (Jarvis and McNaughton 1986).

Although stomatal density is affected by environmental factors, SD is a quantitative character and its genetic control is evident (Nadeau and Sack 2002; Gailing *et al.* 2008). However, Zhang *et al.* (2012) suggested that the extent to which genetics and the environment determine stomatal initiation and development is species-specific.

Bio-water saving, coined by Lun Shan in 1991, emphasizes the physiological and ecological bases of water saving by crops in agriculture (Sun *et al.* 2005). The core of bio-water saving is to increase “water use efficiency” (WUE) of plants, which is defined as a ratio of photosynthesis rate to transpiration rate (Smith 1980). The balance between carbon gain through photosynthesis and water loss through transpiration may also affect instantaneous WUE. In the context of climate smart agriculture, information on these aspects may play a crucial role in developing climate efficient clones.

Information regarding the variability and genetic control of the characters under investigation are the prerequisite for improvement of the characters through selection and/or hybridization. Establishment of a correlation of the

stomatal density with the important physiological traits will help the plant breeder in their selection program to identify tea bushes with better physiological efficiency and in identification of parents in hybridisation program to develop physiologically efficient clones. Keeping this in mind, in this investigation we studied the variability and genetic control of stomatal density and of key physiological traits in 50 elite germplasm of tea maintained in the field gene bank at the National Active Germplasm Site (NAGS) of Tocklai Tea Research Institute, Tea Research Association, Jorhat, Assam, India.

Materials and Methods

Fifty garden series tea clones (Table 1) were selected for this investigation. All these clones were propagated through single leaf cutting and were maintained as block of two rows with 10 bushes in each row. The plantation was done in 1985. For all the clones, similar cultural and crop management practices were followed to minimize the experimental error in the field. Data on stomatal density and physiological traits were recorded in three randomly chosen bushes of each germplasm and from each of the chosen bushes, three matured leaves from the top layer of the bush table were taken randomly. These permanent leaves of the tea bush are known as the maintenance leaves and these leaves contribute the maximum amount of photosynthetase to the growing shoot. In N.E. India, photosynthesis in tea was observed to

continue in the month of December when the mean minimum temperature was lower than 13°C and the tea bushes were completely dormant (Manivel 1980).

Measurement of area and stomatal density

For measuring area and stomatal density, three fully expanded leaves from three randomly selected plants of each clone were plucked in the morning hrs. in a bubble wrapped sample collection bag. The leaf area was measured by a portable Leaf Area Meter (Model LI 3000C, LICOR) following the instruction given in manual with the instrument.

For measuring stomatal density, the abaxial epidermis of the leaf was cleaned first using degreased cotton. An area measuring ~2-3 cm² between the central vein and the leaf edge was carefully smeared with nail varnish at top, middle and bottom portion of leaf for approximately 20 min. The thin film (approximately 5 mm²) was peeled off from the leaf surface, mounted on a glass slide, immediately covered with a cover slip, and then lightly pressed with a fine point tweezers. Numbers of stomata (s) for each film strip were counted under light microscope (40 × objective) of CARL ZEISS (model 2138878), Germany. An ocular scale (Netz mikrometer, Back Kassel, Germany) of 1 mm² was fixed with the eyepiece of the microscope and stomatal counts were made on three microscopic fields per impression. Stomatal density was expressed as density per mm².

Table 1. Tea germplasm included in the present investigation

SL. No.	Germplasm	Origin	SL. No.	Germplasm	Origin
1	KP1/43	Ch x As	26	124/35/18	As x Ind
2	KP 6/37	Ch x Ind	27	124/48/8	Ind
3	KP 1/41	Ch x As	28	180/3	Ind x As
4	KP 4/10	Ch x Ind	29	107/17	Ind x Ch
5	KP 1/7	Ch x As	30	16/10/22	As x Ch
6	KP 1/1	Ind x Ch	31	170/39	As x Ch
7	Go H 33	Ch x As	32	16/11/12	Ch x As
8	BAG 10	Ch x As	33	128/27/16	Ch
9	KOL 26	Ch x As	34	8/5.	As x Ind
10	MR 76	Ch x Ind	35	16/2/15	Ch x Ind
11	TJ37	As x Ch	36	427/5	Ch x Ind
12	DP 36	Ch x As	37	15/6/2	Ch x As
13	KG 5	Ch x As	38	299/9	As hy
14	GT 30	Ch x Ind	39	124/24/1	Ind
15	DP 10	Ch x As	40	14/6/28	Ch
16	TJ 34	Ch x Ind	41	23/16	Ch x As
17	BAG 35	Ch x Ind	42	427/7	Ch
18	Hunuwal	Ch x As	43	177/3	Ind x Ch
19	CP 13	Ch x As	44	170/12	As x Ch
20	CP 23	Ch x As	45	3/ 28.	As x Ind
21	DHUL 41	Ch x Ind	46	297/9	Ind
22	BAG 20	As x Ind	47	299/8	As x Ch
23	TRA/TR/2/11	Ch X As	48	179/4	extreme Cam
24	3/218	As x Ch	49	128/26/2	extreme Ch
25	317/6	Ch	50	177/5	As x Ind

Measurement of leaf photosynthesis, transpiration and water use efficiency

Data for photosynthetic rate (Pn), transpiration rate (E) and WUE were measured in three attached matured fully expanded leaves from the same three bushes of each clones used for measuring the stomatal density. Pn and E were measured using portable photosynthetic system (CIRAS II model) under field conditions. The physiological data were

recorded during November 2014 to February 2015. Readings were taken only after 30 s when the values did not fluctuate.

Statistical analysis

Genotypic and phenotypic variances, genotypic and phenotypic coefficient of variability and broad sense heritability were calculated according to Singh & Chaudhary (1985). Narrow sense

heritability and expected genetic advance at 5% selection intensity were calculated as Johnson *et al.* (1955).

Results

The maximum and minimum value and mean of the characters under investigation for the tea germplasm considered in this investigation are presented in the Table 2. Wide range of variation was observed for all the characters studied in this investigation. China clone 128/27/16 showed the minimum leaf area whereas maximum leaf area was found in Assam-China hybrid 3/218.

Maximum value for stomatal density was recorded in the extreme China type clone (128/26/2) whereas minimum value was recorded in China X Ind hybrid clone KP 4/10. Germplasm of China X Assam origin *viz.* Kol 26 and CP 13 exhibited highest value for E whereas germplasm of China origin 427/7 exhibited the lowest value for E. Germplasm of China X Assam origin KP 1/41 recorded lowest values for both WUE & Pn. Germplasm 15/6/2, also a germplasm of China X Assam origin, exhibited the highest value for WUE. Highest value for Pn was recorded in China X Assam clone 16/11/12.

Table 2. Maximum, minimum and mean values of the characters under investigation

Character	Unit	Maximum value	Genotype	Minimum value	Genotype	Mean value
LA	cm ²	68.85	3/218	5.51	128/27/16	29.92
SD	No./mm ²	88.33	128/26/2	44.67	KP 1/10	67.95
E	m.mol/m ² /s	2.86	KOL 26 CP 13	1.50	427/7	2.14
WUE	μmol/m.mol	6.23	15/6/2	3.86	KP1/41	4.79
Pn	μmol/m ² /s	11.9	16/11/12	7.58	KP1/41	9.61

LA: Leaf Area, SD: Stomatal Density, E: Evapotranspiration, WUE: Water Use Efficiency, Pn: Photosynthetic rate

Genetic analysis of the morpho-physiological characters under investigation was carried out and the results are presented in Tables 3 and 4. A significant variation for all of the characters was detected in the studied germplasm. Thus further improvement of the characters under investigation is possible through breeding for desirable traits.

Selection for the traits having high heritability coupled with high genetic advance is likely to accumulate more additive genes leading to further

improvement of their performance. In the present study, heritability and genetic advance were calculated to identify the characters highly responsive to breeding exercises. High heritability and high genetic advance was recorded for the character stomatal density followed by leaf area. Moderate heritability as well as moderate value for genetic advance was found in transpiration rate and photosynthetic rate, whereas low value of heritability and genetic advance was recorded for

Table 3. Analysis of variance for morpho-physiological characters involving 50 germplasm of tea

Source of variation	df	Mean Squares				
		LA	SD	E	WUE	Pn
Replication	2	103.5	702.1	3.99	116.20	263.77
Treatment	49	450.07**	515.92**	0.46**	1.43**	4.14**
Error	98	35.76	12.48	0.21	1.19	2.20

** Statistically significant at $p \leq 0.01$

LA: Leaf Area, SD: Stomatal Density, E: Evapotranspiration, WUE: Water Use Efficiency, Pn: Photosynthetic rate

Table 4. Estimates of genetic parameters of morpho-physiological traits for 50 germplasm in tea

Genetic parameter	LA	SD	E	WUE	Pn
Environmental variance	35.76	12.48	0.21	1.18	2.20
Genetic variance	138.10	167.81	0.06	0.06	0.49
Phenotypic variance	173.86	180.29	0.27	1.24	2.68
Heritability	0.79	0.93	0.23	0.05	0.18
Genetic advance	21.57	25.75	0.25	0.11	0.61
Genetic advance %	72.12	37.89	11.56	2.37	6.36
GCV%	39.28	19.06	11.66	5.17	7.25
PCV%	44.08	19.76	24.20	23.28	17.03
CV%	19.99	5.19	21.21	22.72	15.41
SE (d)	4.88	2.88	0.32	0.77	1.04

LA: Leaf area, SD: Stomatal Density, E Evapotranspiration, WUE: Water Use Efficiency, Pn; Photosynthetic rate

WUE. Thus stomatal density and leaf area can be improved easily through appropriately designed breeding strategy.

Estimates of phenotypic coefficients of variation (PCV) were slightly higher than the corresponding genotypic coefficients of variation (GCV) for stomatal density and leaf area, indicating that the characters were less influenced by the environment. Therefore, selection on the basis of phenotype alone can be effective for the improvement of these traits. However, for other characters *viz.* E, WUE and Pn, PCV values differed widely from the corresponding GCV value, indicating high environmental influence in the expression of these characters. As such,

phenotypic selection cannot be expected to bring a stable improvement of these characters. Thus an indirect selection for character/s that has/have high correlation with the physiological characters may be a useful strategy to further improve the characters.

Correlations among the characters under investigation are presented in Table 5. Stomatal density exhibited a strong positive correlation with WUE. Since WUE has a positive correlation with Pn, selection on the basis of stomatal density may lead to improvement in both WUE and Pn. Leaf area did not show correlation with any of the physiological characters. As such, leaf area cannot be an important character for developing

physiological efficient clones. Stomatal density was negatively correlated with transpiration rate, although at 10% level, and transpiration rate

showed a strong but negative correlation with the WUE.

Table 5. Pearson correlation coefficients (N = 50) for the characters under investigation

Variables	LA	SD	E	WUE	Pn
LA	1.000	-0.125	0.109	-0.056	0.138
SD		1.000	-0.238+	0.426**	0.113
E			1.000	-0.593**	0.394**
WUE				1.000	0.432**
Pn					1.000

⁺, ^{**} Significant at 10% and 1% probability level, respectively LA: Leaf Area, SD: Stomatal Density, E: Evapotranspiration, WUE: Water Use Efficiency, Pn: Photosynthetic rate

Discussion

In tea stomata was observed only on the abaxial surface and ranged from 44.67 mm² (clone KP 4/10) to 108 mm² (clone TRA/TR/2/11). Camarage *et al.* (2011) also reported that irrespective of species, stomata were located only on the abaxial surface (hypostomaty) in tree, with large variation in both stomata density (SD) and size (SS) among species. SD ranged from 110 mm² in *Neea altissima* to 846 mm² in *Qualea acuminata*.

In our investigation, leaf area did not show correlation with any of the physiological characters. Xu and Zhou (2008) also showed that stomatal density was not significantly associated with leaf area per plant.

The maximum or minimum value of any of the characters under investigation were not confined to the germplasm of specific origin. Germplasm of China X Assam origin seems to have highest as well as lowest values for WUE and Pn. Thus, from looking at the origin of the germplasm, it is not

wise to predict about the physiological performance of the germplasm. Further investigation on the genetic control of the physiological parameters and their correlation with the morphological characters is necessary to predict about the physiological efficiency of tea germplasm.

Stomatal behaviour or movement i.e. how stomata behave to the changes in environmental condition is a critical factor determining the response of the plant to abiotic stress condition. Actually, the physiological mechanisms of stomatal response are very complex and not yet fully understood to date (Sousa *et al.* 2006). For example, increased stomatal density in the *Arabidopsis* mutant *sdd1-1* exhibited no significant influence on the net photosynthetic rate (Schluter *et al.* 2003). However, Tanaka *et al.* (2013) indicated that increased stomatal density could increase the CO₂ gas exchange and the photosynthesis rate in *Arabidopsis thaliana*. In tea, SD did not show a strong correlation with Pn,

however, it exhibited a strong and positive correlation with WUE. This may be due to the significant but negative correlation of SD with E.

At the beginning of the investigation, we hypothesized that a clone with high density of stomata will have a greater rate of transpiration and as such, will be more prone to drought damage. But analysis of data showed that stomatal density is negatively correlated with transpiration rate, although at 10% level. Zhao (2015) reported significantly negative correlations of stomatal density with Pn and E demonstrating that higher stomatal density reduced leaf Pn and E. In contrary, Adams *et al.* (2014) reported from their investigation on two *Arabidopsis* mutants *epf1epf2* and *epf1epf2EPFL9OE*, both with increased stomatal density (D), that a strong correlation exists between D and transpiration rate as both these mutants transpire more than their control counterpart. This information in tea seems to have wide significance in developing climate smart clone, however it needs further investigation over the years to come into a conclusion.

Yang *et al.* (2007) and Xu and Zhou (2007) reported that the increase in stomatal density is positively correlated with WUE. In our investigation also, stomatal density exhibited a strong and positive correlation with WUE. An increase in WUE with high stomatal density might also indicate a high acclimation capacity to a gradually increasing water deficit, and suggest an evolutionary adaptation to environmental stresses (Xu and Zhou 2007). Low value for heritability and genetic advance was estimated for WUE, but WUE exhibited a strong and positive correlation with stomatal density. As such, WUE can be improved

through breeding for high stomatal density in tea. The present finding of correlation of stomatal density with the key physiological parameter has high significance in breeding tea clones for climate smart agriculture.

Investigation on opening-closing mechanism of stomata in response to drought experienced by tea may through some light on such findings. High photosynthetic rate is a desired character for a physiological efficient variety and in tea, it exhibited positive correlation with another desired character WUE. However, at the same time, Pn also showed a significant positive correlation with transpiration rate, a character that indicates loss of water and as such susceptibility to drought damage. A compromise between the two characters may lead to development of new clones with high Pn and WUE but low transpiration rate. Transpiration rate showed a strong but negative correlation with the WUE which seems to be obvious as high transpiration rate indicate significant loss of water through the stomata that results in low water use efficiency.

In severe drought condition, WUE showed a significant but negative correlation with stomatal density. Meng *et al.* (1999) attributed this negative correlation to the marked reduction of A due to the severe drought. Mishra *et al.* (2006) reported that ABA produced in the root in drought condition was translocated to leaf and induced stomatal closing and prevented stomatal opening. The closed stomata may be responsible for the low rate of photosynthesis in severe drought condition. In our investigation, although data were recorded in the drought period of the year, we did not find a significant correlation between stomatal density

and photosynthetic rate. This may be due to the fact that we measured photosynthetic rate in matured leaves (maintenance leaves) and stomatal opening/closing may not be much influenced by drought in matured tea leaves. Investigation in this aspect is required to come into a conclusion.

Some species have been reported as possessing generally high heritability (i.e., less sensitive to environmental change in their stomatal characteristics (Zhang *et al.* 2012), while others have been reported as being more sensitive to environmental factors (Schoch *et al.* 1980). In tea we found a high value for heritability, indicating its tight genetic control. Since stomatal density in tea exhibited a positive correlation with Pn and WUE, improvement of these two characters can be expected through breeding for high stomatal density.

Schutler *et al.* (2003) established that stomatal limitation of photosynthesis does not represent a major regulatory factor for CO₂ assimilation in *Arabidopsis* plants under constant environmental conditions and factors different from the CO₂ supply seem crucial for photosynthetic restriction under these conditions. Recent advances in stomatal development and the

identification of a number of genes eg., *ERECTA*, *SDD1*) regulating stomatal density make it possible to generate transgenic plants with different stomatal densities. Adams *et al.* (2012) opined that genetic manipulation of the EPF (Epidermal Patterning Factor) signaling pathway not only produced plants with dramatically altered stomatal densities but it also affected stomatal size, plant transpiration, rosette growth and tolerance to restricted water availability across a range of atmospheric CO₂ environments. In the near future, by using a unique approach to improving WUE such as decreasing stomatal density, it may be possible to reduce total water consumption and allow surplus water to be used in other capacities (Lawson *et al.* 2014). In tea, an appropriate breeding strategy to improve the stomatal density may lead to development of tea clones and seed variety with higher physiological efficiency.

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