Research Article *Journal of Plantation Crops, 2013, 41(3): 389-397*

Assessment of morphological, physiological and molecular characteristics of cocoa accessions from Central and South America in relation to drought tolerance

S. Elain Apshara*, M.K. Rajesh[1] and D. Balasimha

Central Plantation Crops Research Institute, Regional Station, Vittal - 574 243, Karnataka
[1]Central Plantation Crops Research Institute, Kasaragod - 671 124, Kerala

(Manuscript Received: 08-07-13, Revised: 01-08-13, Accepted: 02-09-13)

Abstract

Eleven cocoa accessions, representing collections from five central and south American countries, were assessed for their morphological, molecular and physiological parameters. Growth characters were observed in three year old plants and initial pod yields were recorded. Photosynthesis, related parameters and chlorophyll indices, measured during two seasons, showed significant differences between non-stress and stress periods as well as among the genotypes. The transpirational water loss was reduced with increased stomatal closure, which is a favourable drought trait in crops. The results indicated that the genotypes showing higher water potential and Fv/Fm ratio can be considered as drought tolerant. The rank sums of these parameters showed that genotypes JA-1/19, POU-16/A and SC-4 were the most drought-tolerant. Microsatellite markers were used to assess the extent of genetic diversity between clones. The amplification of DNA from the 11 accessions using the 15 microsatellite loci revealed a total of 80 consistent and scorable alleles with an average of 5.33 alleles per locus and all the loci were 100 per cent polymorphic, the most polymorphic locus being mTcCIR33 with 8 alleles. The observed heterozygosity ranged from 0.36 to 0.63 with an average of 0.52. The inbreeding co-efficient (*f*) ranged from -0.22 (mTcCIR8) to 0.58 (mTcCIR40) with an average of 0.32. The microsatellite marker analysis revealed that the genotypes possess a wide genetic diversity. The drought tolerant types identified in this study *viz.*, JA-1/19, POU-16/A and SC-4 could be used for cultivation in areas with moisture deficient stress and in selective cocoa breeding programs for drought tolerance.

Keywords: Cocoa, drought tolerance, physiology, diversity

Introduction

Cocoa (*Theobroma cacao* L.), is an evergreen tree native to the deep tropical regions of South America and the seeds are extensively used in cocoa powder and chocolate industry. The tree is adapted to shade conditions and is widely grown as under-storey crop of perennial trees in its natural habitat. Its cultivation is spread over Central and South America, West Africa and South-East Asia. In southern India, cocoa is grown in the states of Karnataka and Kerala traditionally as mixed cropping in arecanut and coconut plantations. It is gaining importance and area expansion in other non-traditional regions of Tamil Nadu and Andhra Pradesh. Arecanut, to a considerable extent, and coconut, on a limited scale, are irrigated crops. The microclimatic conditions in these areas are congenial for cocoa cultivation and so there has been a continuous effort in importing cocoa germplasm from different centres and assessing their adaptability and suitability under Indian conditions.

Cocoa plants are susceptible to environmental stresses, especially altering temperatures and drought (Balasimha *et al*., 1991; Baligar *et al*., 2008; Daymond and Hadley, 2004; Daymond *et al*., 2011; Joly and Hahn, 1989; Raja Harun and Hardwick,

**Corresponding Author: elain_apshara@yahoo.co.in*

1988a, 1988b). Efforts made earlier to identify drought tolerance characters among cocoa accessions found five tolerant varieties (Balasimha *et al*., 1985; Balasimha and Rajagopal, 1988). Some of the parents were selected along with high yielding lines for selective breeding (Balasimha *et al*., 1999). Balasimha and Rajagopal (1988) found that stomatal conductance was reduced by high photosynthetically active radiation, low relative humidity and moisture stress in cocoa. The photosynthetic rate was influenced by light, temperature and vapour pressure deficit (Balasimha *et al*., 1991). Chlorophyll fluorescence measurements are useful techniques for assessing plant stress responses (Daymond and Hadley, 2004). This technique has been used to study light and stress responses in cocoa (Balasimha, 1992). The chlorophyll fluorescence and photosynthesis data indicate the high adaptation of cocoa leaves to understorey conditions.

The development and validation of microsatellites or simple sequence repeat (SSR) markers in cocoa (Lanaud *et al*., 1999) has tremendously increased the efficiency of molecular characterization of cocoa germplasm accessions. Since its discovery, SSR-based molecular analysis has been routine in characterization of cocoa germplasm accessions (Efombagn *et al*., 2006; Zhang *et al*., 2009) and these studies have revealed the extent of genetic diversity in cocoa and also, information regarding its possible origin (Zhang *et al*., 2009a).

The present study examines changes in morphological, photosynthetic characteristics and the microsatellite diversity in cocoa genotypes of five geographic origins in relation to their drought tolerance/susceptibility.

Materials and methods

Eleven genotypes of cocoa (*Theobroma cacao* L.) from five countries *viz*., Columbia (Santa Cruz; SC-1, SC-9, SC-4), Brazil (Rio Branco; RB-33/3), Peru (Pound; POU- 16/A, POU-7/B), Costa Rica (Rosario Izupa Marico; RIM-41, RIM-189) and Ecuador (Bolivar; B-12/2, Javilla; JA-1/19, Amazon; AMAZ-15) were imported through International cocoa quarantine centre, University of Reading, U.K. These were planted in the field at a spacing of 2.7 m x 5.4 m during 2007, under 2.7 m x 2.7 m spaced areca palms at Central Plantation Crops Research Institute (CPCRI), Regional Station, Vittal, Karnataka. The following growth characters were observed in these genotypes. The plant height was taken from ground level upto the tip of the canopy and expressed in meter and trunk girth was measured at 15 cm height from base. The first branching or the jorquette height was measured on the main stem from ground level. Spread of the canopy was measured in both East-West and North-South directions. The canopy area was calculated from the mean growth parameters considering the canopy as cone shaped using the formula πrl, whereas $r = (EW+NS)/4$ and $l = \sqrt{r^2 + h^2}$, h = canopy height. Pod yields were also observed per plant basis and the count of healthy pods per tree per year is given along with average number of beans and fermented, dried single bean weight.

Field measurements were done using a portable photosynthetic system LCA-4 (ADC Bioscientific Ltd, UK) for photosynthesis, plant efficiency analyzer (Hansatech Instruments Ltd., UK) for chlorophyll fluorescence, pressure chamber (Soil Moisture Corp., USA) for water potential (WP) (Balasimha, 1992; Balasimha, *et al*., 1999; Scholander *et al*., 1965). Three replicated plants from each genotype were sampled and six leaves were measured per plant with at least 4-6 values in each leaf. Fully expanded healthy third to fourth leaf from distal portion was used for measurements in non-stress (December) and stress (May) seasons during 2009-2010. The stress was imposed by withdrawing irrigations during March-April. The level of soil moisture was reduced to about 60 per cent of field capacity (20%) to induce stress.

A total of 15 highly polymorphic SSR primer pairs specific to cocoa from the list of international microsatellite markers for global cocoa fingerprinting project (Cryer *et al*., 2006) were used in the present study. Initially, the use of the reported microsatellite markers required optimization of the assay. The annealing temperatures were determined for each primer pair using gradient PCR. Once optimized, the PCR reaction was conducted in volumes of 20 µl containing 35 ng genomic DNA, 0.2 µM each of forward and reverse primers, 50 µM

of each dNTPs (M/s Bangalore Genei Pvt. Ltd., Bangalore), 1X buffer (10 mM Tris-Hcl (pH8.3), 50 mM KCl, 1.5 mM $MgCl_2$) and 0.3 Unit of *Taq* DNA polymerase (M/s Bangalore Genei Pvt. Ltd., Bangalore). PCR amplifications were performed on a BIORAD gradient thermal cycler with a PCR profile of 94 °C for 5 min followed by 30 cycles of 1 min at 94 °C, 2 min at the different annealing temperatures standardized for the individual SSR locus, and 2 min at 72 °C with a final extension for 5 min at 72 ºC. After amplification, 4 µl of 6X loading dye was added to each of the amplified products and these were separated on 4 per cent agarose gel running in TBE buffer (45 mM Tris-Borate, 1mM EDTA, pH 8.3), stained with ethidium bromide and visualized in a gel documentation system. The alleles were scored individually based on comparison with the molecular weight.

The expected and observed heterozygosity, the mean number of alleles per polymorphic locus, the fixation index across the cocoa accessions were worked out using the software GDA (Genetic Data Analysis) (Lewis and Zaykin, 2002). A cluster analysis was performed on the similarity matrix using the unweighted pair group method with arithmetic averages (UPGMA) and the resultant phenogram was constructed. The per cent polymorphic loci, expected and observed heterozygosity, F-statistics and PCA were worked out for the cocoa populations using the software GENALEX (Peakall and Smouse, 2006).

Results and discussion

Growth parameters

The mean values of growth parameters recorded in three-year old trees of 11 cocoa clones are given in Table 1. The plant height ranged from 1.7 m to a maximum of 2.9 m and girth from 16.0 cm to 27.3 cm. The first branching height ranged from a lowest of 0.55 m to the highest of 1.28 m and the trees had 3.33 to 9.57 branches among the clones. The canopy area compiled from the East-West and North-South spread showed a small canopy of 4.66 m^2 to a big canopy of 13.2 m^2. All clones started yielding and showed increasing trend from third to fifth year with low pod yield of 3 pods $tree^{-1}$ $year^{-1}$ to high pod yield of 31 pods $tree^{-1}$ $year^{-1}$. Number of beans per pod ranged from 30 to 45 and single dry bean weight from a minimum of 0.8 g to a maximum of 1.2 g recorded among genotypes. These growth and yield observations are very preliminary and to be continued throughout the crop period to assess their adaptability, stability and productivity in the introduced environment. Selection of clones based on high yield along with drought tolerance and utilizing them in hybridization program is suggested.

Photosynthetic parameters

The photosynthesis and related parameters in eleven genotypes were measured at two seasons (Table 2). There were significant differences in these parameters between non-stress and stressed seasons

Table 1. Growth and yield parameters of selected clones of different geographical origin

Clones	Height (m)	Girth (cm)	HAFB* (m)	Branches No.	Canopy area (m^2)	No. of pods $tree^{-1}$			No. of beans pod^{-1}	1 dry bean wt. (g)
						2010	2011	2012		
SC1	2.80	26.1	0.83	9.57	13.2	3.57	9.08	14.0	35	1.0
SC9	2.28	22.0	0.91	4.25	7.41	5.58	7.50	12.0	30	1.0
SC4	2.25	21.5	0.91	4.60	6.70	2.00	4.50	15.3	35	1.1
RB33/3	2.54	27.3	1.28	4.89	8.12	1.11	12.1	31.0	35	1.0
POU 16/A	2.00	19.0	0.81	4.50	5.32	1.00	10.5	18.0	36	1.0
POU 7/B	2.65	26.3	1.05	5.00	6.83	1.25	8.00	12.7	39	1.2
RIM 41	2.90	22.0	0.88	3.50	6.99	1.75	9.50	10.3	37	0.8
RIM 189	2.13	20.5	0.85	4.50	6.02	2.25	6.75	9.00	30	1.0
B 12/2	1.70	16.0	0.55	3.67	5.03	4.33	3.00	7.67	34	0.8
JA 1/19	1.85	20.0	0.62	3.50	4.66	2.50	10.5	18.3	45	1.1
AMAZ 15	2.20	19.7	1.04	3.33	5.65	1.00	7.50	13.0	33	0.8
CV%	16.4	16.0	22.9	40.6	34.6	60.7	36.6	41.2	13.1	14.3
CD	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

*HAFB: Height at first branching

also between the genotypes. Similarly, the leaf water potential was reduced and showed genotypic variations (Table 2). Stomatal conductance also showed similar decreases. This fact confirms the earlier results that transpirational water loss is reduced with increased stomatal closure, which is a favourable drought trait in cocoa (Balasimha *et al*., 1991, 1999). Similar results on stomatal resistance and drought resistance association (Nunes, 1967; Joly and Hahn, 1989) and reduced transpiration rate (Segbor *et al*., 1981) have been reported earlier. It is possible that the ability to tolerate drought results from stomatal regulation, thus reducing transpirational water loss.

photochemical reaction was affected due to stress. Chlorophyll fluorescence has been used as a tool to screen cocoa for drought tolerance (Balasimha and Namboothiri, 1996). Similarly, study of genotypic differences in chlorophyll fluorescence in cocoa genotypes in response to high temperature has been done (Daymond and Hadley, 2004). The ratio of variable to maximal fluorescence (Fv/Fm) is an important indicator of drought tolerance. The results indicated that the genotypes showing higher water potential and Fv/Fm ratio can be considered as drought tolerant traits. The rank sums of these parameters were calculated based on the relationships to drought tolerance, which showed

Table 2. Photosynthetic characteristics in cocoa genotypes

Genotype	Pn (µmol m^{-2} s^{-1})		Tran (mmol m^{-2} s^{-1})		Gs (mol m^{-2} s^{-1})		CO_2 internal (ppm)	
	Non-stress	Stress	Non-stress	Stress	Non-stress	Stress	Non-stress	Stress
SC1	6.12	4.24	3.02	1.99	0.18	0.11	266	281
SC9	4.57	2.30	2.48	1.56	0.15	0.10	273	297
SC4	4.82	3.74	2.49	1.74	0.12	0.09	235	271
RB33/3	5.39	2.75	2.27	1.34	0.12	0.06	241	249
POU 16/A	4.75	3.65	1.96	1.55	0.11	0.06	220	224
POU 7/B	5.87	2.38	2.63	1.05	0.15	0.05	240	234
RIM 41	6.45	3.12	2.32	1.84	0.15	0.13	222	339
RIM 189	6.06	2.99	2.46	1.76	0.15	0.13	257	331
B 12/2	5.87	3.15	1.88	1.12	0.09	0.07	235	275
JA 1/19	5.50	3.68	2.00	0.95	0.09	0.05	230	195
AMAZ 15	3.55	3.44	1.31	1.27	0.05	0.04	206	255
Mean	5.28	3.27	2.22	1.42	0.12	0.08	268	238
Sig Genotype	**		**		**		**	
Season	**		**		**		**	

The Pn/gs ratio increased with stress and showed variations among the genotypes (Table 3). This increase in the Pn/gs ratio leads to a decrease in Ci suggesting that mesophyll factors are not affected much in cocoa. Despite stomatal control limiting Pn, there was small change in WUE during stress as compared to non-stress conditions. This type of relationship has been attributed to adaptation for water deficit stress conditions in cocoa (Balasimha, 1993; Balasimha and Rajagopal, 1988). There was high intrinsic WUE (Pn/E) in some genotypes, which was reduced under stress situations (Table 3).

Chlorophyll fluorescence indices also showed differences between genotypes and seasons (Table 4). Due to stress, the values decreased suggesting the

Table 3. Photosynthesis ratios and leaf water potential

Genotype	WUE (Pn/E)		Pn/gs		WP (bars)	
	Non-stress	Stress	Non-stress	Stress	Non-stress	Stress
SC1	2.07	2.16	38.92	40.34	-5.73	-6.57
SC9	1.92	1.58	35.77	29.05	-6.17	-7.33
SC4	2.13	2.14	57.62	40.49	-6.80	-6.67
RB33/3	2.39	2.12	46.90	49.50	-5.90	-6.52
POU 16/A	2.42	2.99	62.46	73.01	-6.67	-7.52
POU 7/B	2.25	2.27	43.98	47.48	-5.77	-8.40
RIM 41	2.86	1.70	58.24	21.59	-6.00	-6.77
RIM 189	2.50	1.73	42.04	24.56	-5.87	-6.60
B 12/2	3.20	3.40	68.66	68.81	-6.27	-7.53
JA 1/19	2.97	4.21	72.36	86.58	-5.83	-6.98
AMAZ 15	2.74	2.79	85.09	70.19	-5.17	-7.38
Mean	2.50	2.46	55.64	50.15	-6.02	-7.12
Sig Genotype	**		**		ns	
Season	ns		ns		**	

Table 4. Chlorophyll fluorescence indices in cocoa genotypes

Genotype	Fo (units)		Fm (units)		Fv (units)		Fv/Fm	
	Non stress	Stress	Non stress	Stress	Non stress	Stress	Non stress	Stress
SC1	649	799	3,734	3,051	2,997	2,252	0.820	0.708
SC9	715	854	3,441	3,104	2,748	2,250	0.791	0.720
SC4	750	722	3,248	2,999	2,496	2,276	0.767	0.756
RB33/3	736	999	3,453	3,571	2,816	2,673	0.783	0.718
POU 16/A	726	804	3,556	3,026	2,834	2,131	0.794	0.725
POU 7/B	803	915	3,907	3,487	3,104	2,517	0.794	0.733
RIM 41	671	861	3,483	3,466	2,876	2,593	0.809	0.751
RIM 189	702	862	3,111	3,472	2,354	2,610	0.771	0.752
B 12/2	699	870	3,384	2,964	2,690	2,096	0.792	0.706
JA 1/19	744	830	3,202	3,761	2,347	2,931	0.767	0.779
AMAZ 15	767	730	3,748	3,312	2,966	2,582	0.792	0.775
Mean	724	841	3479	3292	2748	2447	0.789	0.738
Sig	Genotype	**		**		**		**
	Season	**		**		**		**

that genotypes JA-1/19, POU-16/A and SC-4 were to the most drought tolerant (Table 5).

A more detailed assessment of genotypic responses to water stress conditions was done using a grouping analysis based on the variables shown in Table 5. The ratios between values obtained from stress and non-stress conditions among the 11 genotypes were used to build a similarity matrix and dendrogram (Fig.1). The results showed genotypic variations and least difference obtained among the traits in genotypes indicate relatively higher tolerance. The results indicated existence of three groups, based on similarity value cut-off Rescaled Value Distance of 5. However, the groups did not show any direct relation to drought tolerant character as per the rank sum analysis.

The results on genetic variations in cocoa are consistent with earlier studies on photosynthetic

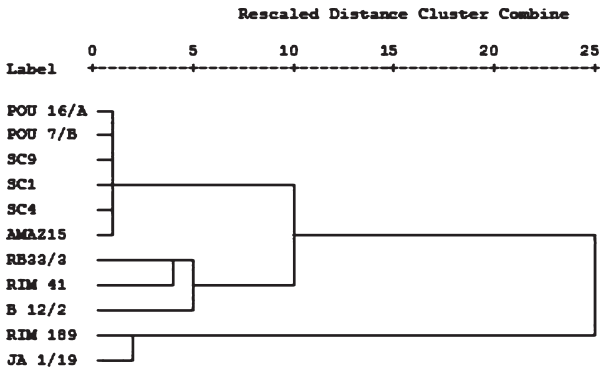

Fig. 1. Euclidian distance-based grouping analysis of cocoa genotypes subjected to stress, using the differences between non stress and stress values for physiological variables shown in Table 5

characteristics (Balasimha, 1991 & 1999; Baligar *et al*., 2008; Daymond and Hadley, 2004; Daymond *et al*., 2011). These genetic differences can be further utilized in breeding and selection programmes with photosynthesis characters and chlorophyll

Table 5. Differences in values from non stress to stress seasons

Genotype	Pn	Tran	Gs	CO2 int	Pn/E	Pn/gs	WP	Fo	Fm	Fv	Fv/Fm	Rank sum
SC1	1.880	1.030	0.070	-16.0	-0.090	-1.421	0.834	-150	684	745	0.112	10
SC9	2.270	0.910	0.060	-23.0	0.330	6.720	1.163	-139	338	498	0.071	9
SC4	1.080	0.720	0.020	-37.0	-0.003	17.126	-0.133	28	249	226	0.011	3
RB33/3	2.640	0.920	0.060	-8.0	0.260	-2.600	0.617	-263	-118	143	0.065	5
POU 16/A	1.110	0.410	0.050	-5.0	-0.570	-10.546	0.850	-78	531	712	0.069	2
POU 7/B	3.490	1.580	0.100	5.0	-0.026	-3.497	2.633	-111	421	588	0.061	11
RIM 41	3.330	0.480	0.020	-117.0	1.158	36.650	0.767	-190	20	284	0.058	7
RIM 189	3.070	0.700	0.020	-82.0	0.526	17.489	0.733	-159	-361	-252	0.019	4
B 12/2	2.730	0.760	0.030	-39.0	-0.194	-0.147	1.266	-171	-580	593	0.086	8
JA 1/19	1.820	1.882	0.040	35.0	-1.237	-14.225	1.150	-86	-558	-584	-0.012	1
AMAZ 15	0.110	0.040	0.010	-49.0	-0.520	14.820	2.216	37	435	346	0.017	6

fluorescence as a selection tools (Balasimha and Namboothiri, 1996; Daymond and Hadley, 2004; Daymond *et al*., 2011). Thus, the 11 genotypes used in this study revealed a general decrease in physiological parameters under stress and showed superiority of three genotypes for drought tolerance.

Analysis of genetic diversity using microsatellite markers

Microsatellite markers (SSR) were studied to see the level of genetic diversity among the 11 cocoa genotypes. All the 15 microsatellite loci tested produced consistent and scorable alleles (Fig. 2). The amplification of DNA from the 11 accessions using the 15 microsatellite loci revealed a total of 80 alleles with an average of 5.33 alleles per locus. The number of alleles per locus ranged from 3 to 8 and all the loci were 100 per cent polymorphic (Table 6). The most polymorphic locus was mTcCIR33 with 8 alleles, while mTcCIR1 and mTcCIR8 were the least polymorphic with 3 alleles each. The mean alleles per polymorphic loci (A_p) also ranged from 3 to 8.

The observed heterozygosity ranged from 0.36 to 0.63 with an overall average of 0.52. For all loci, expect mTcCIR8, the expected heterozygosity was higher than the observed. The inbreeding co-efficient (*f*) ranged from -0.22 (mTcCIR8) to 0.58 (mTcCIR40) with an average of 0.32. Maximum genetic identity (0.64) was between RIM-189 and RIM-41, while the least (0.07) was between JA1/19 and RIM-189 (Table 7, Fig. 3).

Table 6. Description of the microsatellite loci with the primer-pair statistics, total number of alleles (A), mean alleles per polymorphic loci (A_p), expected heterozygosity (gene diversity; H_e), observed heterozygosity (H_o), inbreeding coefficient (*f*) and linkage group localization

No.	Locus	Primer sequence (5' - 3')	T_m(^{0}C)	A	A_p	H_e	H_o	*f*	Linkage group
1	mTcCIR1	FP:GCAGGGCAGGCTCAGTGAAGCA							
		RP:TGGGCAACCAGAAAACGAT	60	3	3	0.68	0.54	0.20	1
2	mTcCIR 8	FP:CTAGTTTCCCATTTACCA							
		RP:TCCTCAGCATTTTCTTTC	52	3	3	0.42	0.50	-0.22	9
3	mTcCIR 17	FP:AAGGATGAAGGATGTAAGAGAG							
		RP:CCCATACGAGCTGTGAGT	58	4	4	0.64	0.36	0.44	4
4	mTcCIR 7	FP:ATGCGAATGACAACTGGT							
		RP:GCTTTCAGTCCTTTGCTT	58	4	4	0.76	0.45	0.42	7
5	mTcCIR 18	FP:GATAGCTAAGGGGATTGAGGA							
		RP:GGTAATTCAATCATTTGAGGATA	58	4	4	0.71	0.64	0.11	4
6	mTcCIR 12	FP:TCTGACCCCAAACCTGTA							
		RP:ATTCCAGTTAAAGCACAT	55	7	7	0.82	0.64	0.23	4
7	mTcCIR 6	FP:TTCCCTCTAAACTACCCTAAAT							
		RP:TAAAGCAAAGCAATCTAACATA	56	7	7	0.77	0.64	0.18	6
8	mTcCIR 15	FP:CAGCCGCCTCTTGTTAG							
		RP:TATTTGGGATTCTTGATG	55	6	6	0.81	0.64	0.23	1
9	mTcCIR 24	FP:TTTGGGGTGATTTCTTCTGA							
		RP:TCTGTCTCGTCTTTTGGTGA	58	5	5	0.75	0.55	0.29	1
10	mTcCIR 22	FP:ATTCTCGCAAAAACTTAG							
		RP:GATGGAAGGAGTGTAAATAG	52	6	6	0.73	0.36	0.52	1
11	mTcCIR 40	FP:AATCCGACAGTCTTTAATC							
		RP:CTTAAATGTTATGTGTATGC	52	6	6	0.84	0.36	0.58	3
12	mTcCIR 33	FP:TGGGTTGAAGATTTGGT							
		RP:CAACAATGAAAATAGGCA	52	8	8	0.88	0.55	0.39	4
13	mTcCIR 26	FP:GCATTCATCAATACATTC							
		GCACTCAAAGTTCATACTAC	52	4	4	0.72	0.36	0.51	8
14	mTcCIR 25	FP:CTTCGTAGTGAATGTAGGAG							
		T RP:TAGGTAGGTAGGGTTATCT	52	6	6	0.80	0.64	0.22	6
15	mTcCIR 11	FP:TTTGGTGATTATTAGCAG							
		RP:GATTCGATTTGATGTGAG	52	7	7	0.84	0.55	0.37	2
			Average	**5.33**	**5.33**	**0.75**	**0.52**	**0.32**	

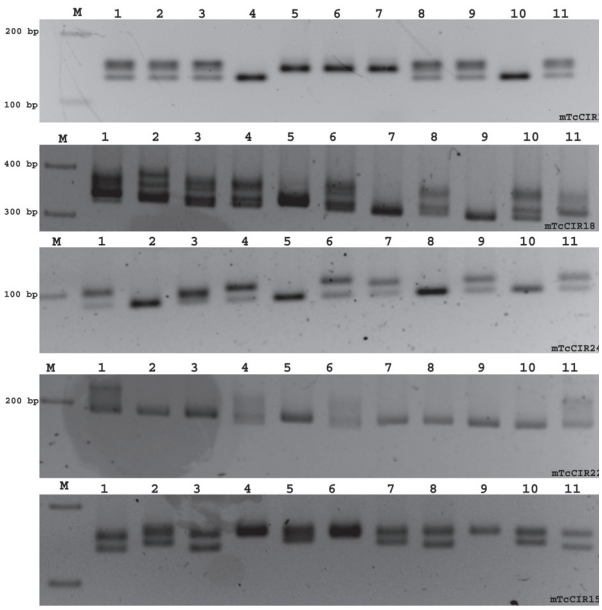

Fig. 2. Polymorphic banding patterns among the 11 cocoa genotypes
Lanes: M: 100 bp ladder; 1: AMAZ-15, 2: B-12/2, 3: JA-1/19, 4: POU-7/B, 5: POU-16/A, 6: RB-33/3, 7: RIM-41, 8: RIM-189, 9: SC-1, 10: SC-4, 11: SC-9

Organization of genetic diversity within groups

Gene diversity statistics were calculated based on the 15 polymorphic microsatellite loci for the accessions grouped into five regions or groups, according to the original location of collection. The Columbian and Ecuadorian genotypes had identical percentage of polymorphic loci (100%) while it was least in Peruvian genotypes (73.35%). The mean observed heterozygosity was 0.5. The Peru genotypes presented the lowest observed heterozygosity (0.3), while the genotypes from Costa Rica had the highest observed heterozygosity (0.67).

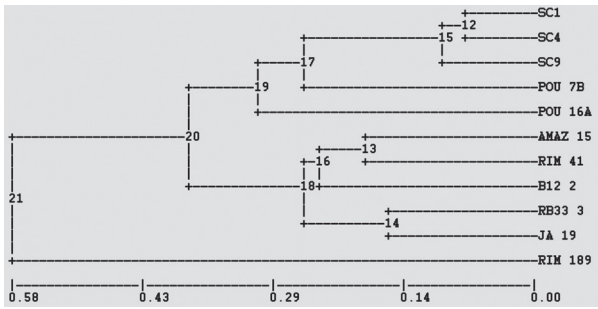

Fig. 3. Dendrogram of the 11 cocoa genotypes

The estimated F_{IS} values ranged from -0.48 (for the Costa Rican populations) to 0.1 (for the Peruvian genotypes) (Table 8). Based on average values for all loci, the values for F_{IS} and F_{IT} were -0.108 and 0.269 respectively, indicating an excess

Table 8. F-Statistics of Wright (1951) and estimates of N_m over all groups estimated for 15 microsatellite loci

Locus	FIS	FIT	FST	Nm
mTcCIR1	-0.33	0.22	0.42	0.35
mTcCIR 8	-0.64	-0.22	0.26	0.72
mTcCIR 17	-0.31	0.38	0.52	0.23
mTcCIR 7	0.01	0.41	0.40	0.38
mTcCIR 18	-0.05	0.11	0.16	1.34
mTcCIR 12	-0.01	0.23	0.24	0.79
mTcCIR 6	-0.12	0.15	0.26	0.69
mTcCIR 15	-0.10	0.22	0.29	0.62
mTcCIR 24	-0.14	0.19	0.29	0.63
mTcCIR 22	0.04	0.49	0.47	0.29
mTcCIR 40	0.04	0.54	0.52	0.23
mTcCIR 33	0.05	0.29	0.25	0.74
mTcCIR 26	0.05	0.50	0.48	0.27
mTcCIR 25	-0.16	0.18	0.29	0.62
mTcCIR R11	0.09	0.34	0.28	0.64
Mean	-0.11	0.27	0.34	0.57
SE	0.05	0.05	0.03	0.08

Table 7. Genetic identity among the 11 cocoa accessions

	SC1	SC4	SC9	AMAZ-15	RB33/3	POU-7/B	POU-16/A	JA-1/19	B-12/2	RIM-189	RIM-41
SC1	1.00										
SC4	0.46	1.00									
SC9	0.52	0.60	1.00								
AMAZ-15	0.30	0.32	0.19	1.00							
RB33/3	0.38	0.26	0.19	0.34	1.00						
POU-7/B	0.55	0.47	0.29	0.40	0.37	1.00					
POU-16/A	0.22	0.42	0.36	0.47	0.31	0.51	1.00				
JA-1/19	0.24	0.19	0.10	0.39	0.60	0.31	0.29	1.00			
B-12/2	0.19	0.22	0.25	0.58	0.28	0.11	0.41	0.24	1.00		
RIM-189	0.20	0.19	0.17	0.29	0.14	0.20	0.22	0.07	0.38	1.00	
RIM-41	0.36	0.16	0.27	0.48	0.25	0.24	0.22	0.25	0.49	0.64	1.00

of heterozygotes. The co-efficient of differentiation (F_{ST}) among the five groups was estimated to be 0.342, indicating a high level of differentiation. The average gene flow (N_m) was 0.568. Nei's (1978) genetic identity was maximum (0.73) between Brazilian and Ecuadorian groups, while it was the least between Peruvian and Costa Rican groups (0.28).

The coefficient of differentiation, F_{ST}, was also estimated for all pairs of groups. The Brazilian and Costa Rican groups displayed the highest F_{ST} values, as the most distinct groups analyzed. The Brazilian and Ecuadorian groups presented the lowest F_{ST}, indicating the limited differentiation. The first axis explanied 45.4 per cent of the variation, the second axis 32.5 per cent of variation and the third axis, 15.1 per cent (Fig. 4).

In conclusion, the 11 genotypes studied from different geographic regions revealed that there was reduction in photosynthetic parameters under drought stress. There were high clonal diversity and those which had positive responses could be considered as drought tolerant. High yielding clones together with drought tolerance may be selected in the due course. No specific association of physiological responses to molecular results could be found. The interclonal genetic variability in physiological responses was not directly associated with microsatellites as they are genomic based, not necessarily associated with any particular phenotypic character (Hosbino *et al*., 2002). Similar results on physiological parametres with SSRs have been reported under waterlogging conditions (Bertolde *et al*., 2010). The identification of superior cocoa genotypes will help in improved strategies for cultivating in adverse conditions like drought and also can be used for selective breeding.

Table 9. Percentage polymorphic loci, observed and expected heterozygosity, unbiased heterozygosity and fixation index region-wise

Population	% P	H_o	H_e	UH_e	F
Columbia	100	0.62	0.53	0.66	-0.20
Brazil	80	0.47	0.46	0.61	-0.04
Peru	73.3	0.30	0.36	0.48	0.10
Ecuador	100	0.48	0.53	0.70	0.08
Costa Rica	93.3	0.67	0.46	0.61	-0.48
Mean	89.3	0.50	0.47	0.61	-0.12
SE	5.42	0.03	0.02	0.03	0.06

H_o = Observed Heterozygosity = No. of Hets / N
H_e = Expected Heterozygosity = 1 - Sum pi^2
UH_e = Unbiased Expected Heterozygosity = (2N / (2N-1)) * H_e
F = Fixation Index = (He - Ho) / He = 1 - (Ho / He)
Where pi is the frequency of the ith allele for the population & Sum pi^2 is the sum of the squared population allele frequencies.

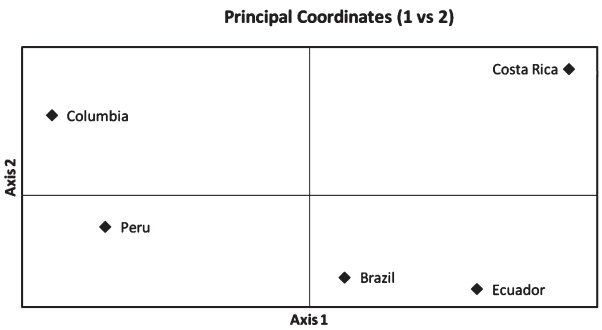

Fig. 4. PCO plot of the five cocoa groups

Acknowledgement

We are grateful to International Cacao Quarantine Centre, University of Reading, UK for supplying the cocoa germplasm materials. We also thank Mr. K.S. Muralikrishna and Mr. John Sunoj for help in photosynthesis measurements.

References

Balasimha, D. 1992. Net CO_2 assimilation and chlorophyll fluorescence in cocoa trees. *Plant Physiology and Biochemistry* **19**: 23-26.

Balasimha, D. 1993. Stomatal conductance and photosynthesis in cocoa trees. *Plant Physiology and Biochemistry* **20**: 16-18.

Balasimha, D. and Rajagopal, V. 1988. Stomatal responses of cocoa to changes in climatic factors. *Journal of Agricultural Science* **58**: 213-216.

Balasimha, D. and Namboothiri, C.G.N. 1996. Chlorophyll fluorescence of cocoas in relation to drought tolerance. *Plant Physiology and Biochemistry* **23**: 64-66.

Balasimha, D., Subramonian, N. and Subbaiah, C.C. 1985. Leaf characteristics in cocoa accessions. *Café Cacao The* **29**: 95-98.

Balasimha, D., Daniel, E.V. and Bhat, P.G. 1991. Influence of environmental factors on photosynthesis in cocoa trees. *Agriculture and Forest Meteorology* **55**: 15-21.

Balasimha, D., Anil Kumar, V., Viraktamath, B.C. and Ananada, K.S. 1999. Leaf water potential and stomatal resistance in cocoa hybrids and parents. *Plantations Recherche Developpement* **6**: 116-120.

Baligar, V.C., Bunce, J.A., Machado, R.C.R and Elson, M.K. 2008. Photosynthetic photon flux density, carbon

dioxide concentration and vapor pressure deficit effects on photosynthesis in cacao seedlings. *Photosynthetica* **46**: 216-221.

Bertolde, F.B., Almeida, A.A.F., Correa, R.X., Gomes, F.P., Gaiotto, F.A., Baligar, V.C. and Loguercio, L.L. 2010. Molecular, physiological and morphological analysis of water logging in clonal genotypes of cocoa. *Tree Physiology* **30**: 56-67.

Cryer, N.C., Fenn, M.G.E., Turnbull, C.J., Wilkinson, M.J. 2006. Allelic size standards and reference genotypes to unify international cocoa microsatellite data. *Genetic Resources and Crop Evolution* **53**: 1643-1652.

Daymond, A. and Hadley, P. 2004. Physiological characterization of cocoa germplasm. In: *Proc. International Workshop Cocoa Breeding for Improved Production systems.* (Eds). F. Bekele, F., End, M.J. and Eskes, A.B., 19-21 October 2003, Accra, Ghana: pp. 103-109.

Daymond, A., Tricker, P.J. and Hadley, P. 2011. Genotypic variation in photosynthesis in cacao is correlated with stomatal conductance and leaf nitrogen. *Biologia Plantarum* **55**: 99-104.

Efombagn, M.I.B., Sounigo, O., Nyassé, S., Manzanares-Dauleux, M., Cilas, C., Eskes, A.B. and Kolesnikova-Allen, M. 2006. Genetic diversity in cocoa germplasm of southern Cameroon revealed by simple sequences repeat (SSRS) markers. *African Journal of Biotechnology* **5**: 1441-1449.

Hosbino, A.A., Palmier, D.A., Bravo, J.P., Pereira, T.E.B., Lopez, C.R. and Gimenes, M.A. 2002. Marcador microssatelie na conservacao de germplasma vegetal. *Biotec. Ciencia Desenvol.* **29**: 146-150.

Joly, R.J. and Hahn, D.T. 1989. Net CO_2 assimilation of cocoa seedlings during periods of plant water deficit. *Photosynthesis Research* **21**: 151-159.

Lanaud, C., Risterucci, A.M., Pieretti, I., Falque, M., Bouet, A. and Lagoda, P.J. 1999. Isolation and characterization of microsatellites in cocoa. *Molecular Ecology* **8**: 2141-2152.

Lewis, P.O. and Zaykin, D. 2002. Genetic Data Analysis: Computer program for the analysis of allelic data. Version 1.1. Free program distributed by the authors over the internet from http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php.

Nunes, M.A. 1967. A comparative study of drought resistance in cacao plants. *Annals of Botany* **31**: 189.

Peakall Rod and Smouse, P.E. 2006. GENALEX 6: genetic analysis in Excel-Population genetic software for teaching and research. *Molecular Ecology Notes* **6**: 288-295.

Raja Harun, R.M. and Hardwick, K. 1988a. The effects of prolonged exposure to different light intensities on the photosynthesis of cocoa leaves. In: *Proc. 10th International Cocoa Research Conference*, 17-23 May, 1987, Santo Domingo, Dominican Republic, COPAL, Lagos, Nigeria. pp. 205-209.

Raja Harun, R.M. and Hardwick, K. 1988b. The effect of different temperature and water vapour pressure deficit on photosynthesis and transpiration of cocoa. In: *Proc. 10th International Cocoa Research Conference*, 17-23 May, 1987, Santo Domingo, Dominican Republic, COPAL, Lagos, Nigeria. pp. 211-214.

Scholander, P.F., Hammel, H.T., Bradstreet, E.D. and Hemingen, E.A. 1965. Sap pressure in vascular plants. *Science* **148**: 339-346.

Segbor, A., Djiekpor, E.K., Amefia, Y.K. and Partiot, M. 1981. Amelioration du cacaoyer; mise au point d'un test d'evaluation precoce de la resistance a la secheresse. In: *Proc. 7th International Cocoa Research Conference*, Lagos, Nigeria. pp. 131-133.

Zhang, D., Mischke, B.S., Johnson, E.S., Mora, A., Phillips-Mora, W. and Meinhardt, L.W. 2009. Molecular characterization of an international cocoa collection using microsatellite markers. *Tree Genetic Genomics* **5**: 1-10.

Zhang, D., Boccara, M., Motilal, L., Mischke, B.S., Johnson, E.S., Butler, D., Bailey, B.A., Meinhardt, L.W. 2009a. Molecular characterization of an earliest cacao (*Theobroma cacao* L.) collection from Peruvian Amazon using microsatellite DNA markers. *Tree Genetic Genomes* **5**: 595-607.