Pyramiding novel EMS-generated mutant alleles to improve fiber quality components of elite upland cotton germplasm.

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Highlight

- Two population were developed by crossing different EMS-generated mutant lines with superior fiber qualities
- Population R was developed to improve four fiber attributes (micronaire, length, strength, and elongation) simultaneously
- Population S was developed by double crossing four mutants with improved fiber length
- Both populations showed significant improvement in different fiber attributes when compared to parental lines, while population S exceeded expectations

Abbreviations

HVI - High Volume Instrument; AFIS - Advanced Fiber Information System; EMS - Ethyl methanesulfonate; MIC - micronaire; LEN - fiber length; STR - fiber strength; ELON - fiber elongation, UI - Uniformity index; SFC - Short Fiber Content.

Abstract

Improvement of cotton fiber quality, hampered by historical genetic bottlenecks, may benefit from the use of EMS-induced mutants that are largely free of linked unfavorable alleles often associated with the use of secondary and tertiary *Gossypium* gene pools. Here we intercrossed seven EMS-generated improved fiber quality mutant lines to produce two populations, one (pop. R) focused on improving four fiber attributes (micronaire, length, strength and elongation) and the other (pop. S) to pyramid superior alleles for fiber length. The overall average of both populations was significantly improved for micronaire, fiber length, fiber strength, uniformity and short fiber content compared to parental lines, with 39 lines in pop. R and 71 in pop. S showing significant improvement for four or more traits. Multiple lines in these populations showed improvement for all six fiber traits tested. Fiber length of populations S and R was significantly higher than the original (non-EMS mutated) parents (ACALA1517-99, TAM94L25), local elite germplasm (GA230) and other commercial checks (DeltaPine 393 and Fiber Max 832). As expected, average fiber length of pop. S was significantly higher by 4.2% than pop. R. Surprisingly, pop. S was also significantly better than pop. R in micronaire, fiber strength, uniformity and short fiber content, adding further support to hypotheses about the complex nature of cotton fiber QTLs and the corollary that selection for one fiber quality trait may also increase values of other traits. New allele combinations from these mutant lines show promise for improving fiber qualities beyond the levels of current elite varieties.

Keywords: crop improvement, functional genomics, EMS mutagenesis, allele stacking

1. Introduction

Despite its global importance in contributing about one-third of the raw material used by textile industries and its cultivation in about 100 countries (CONSTABLE *et al.* 2015), Upland cotton (*Gossypium hirsutum L.*) improvement has been constrained by several historical genetic bottlenecks (ULLOA AND MEREDITH JR 2000; PATERSON *et al.* 2004; TYAGI *et al.* 2014; BOOPATHI AND HOFFMANN 2016). These bottlenecks are results of polyploid formation, domestication, human movement of small germplasm samples and intensive breeding for yield components (LUBBERS AND CHEE 2009).

One can generally generate non-lethal variation in genomes using mutagenesis techniques such as EMS (Ethyl methanesulfonate), offering potentially useful alleles for crop improvement with little of the 'linkage drag' that is common to alleles introgressed from exotic germplasm. Researchers have identified EMS mutations conferring discrete morphological traits such as trichome variations (PATEL *et al.* 2016), naked seed (PATEL *et al.* 2014; KONG DEPEI 2017), short fiber mutants (KONG DEPEI 2017; NAOUMKINA *et al.* 2017), albino cotyledons and leaves, red-violet leaves and stems, and multilayered bracts (KONG DEPEI 2017). Multiple years of field trials showed that mutant lines with improved fiber properties can be developed through mutation breeding (AULD *et al.* 2000; PATEL *et al.* 2014) but only a handful of attempts have been made to transfer EMS alleles into elite backgrounds and none to our knowledge have investigated combining such alleles in elite or mutant backgrounds (BECHERE *et al.* 2007; BECHERE *et al.* 2011).

Experiments on gene or QTL pyramiding have been conducted in different plant species with a major focus on developing lines resistant to biotic or abiotic stresses (GREGORIO *et al.* 2002;

ATKINSON AND URWIN 2012). In cotton, QTL pyramiding has been reported to improve fiber qualities such as fiber length and strength (WANGZHEN *et al.* 2005; DONG *et al.* 2009; YUAN *et al.* 2014).

Here, we developed two double-cross populations using previously identified mutant lines (PATEL *et al.* 2014), one combining four lines that showed improvement for multiple fiber traits (fiber length, strength, fineness and elongation) and another combining four lines that all had strikingly improved fiber length. Such populations allow us to investigate interactions between different fiber traits, effects of allele pyramiding for the same or different fiber traits, and the possibility to break negative associations between yield and fiber quality components by crossing novel alleles generated by EMS mutagenesis.

2. Materials and Methods

2.1. Plant sources and population development

A total of seven mutant lines (Table 1) were used to develop two populations. Four lines, namely Acala 1517-99-M1903 (fiber length, herein abbreviated LEN), Acala 1517-99-M1793 (fiber strength, STR), TAM94L25-M2925 (fiber elongation, ELON), and TAM94L25-M2877(micronaire, MIC) were used to develop 'pop. R'; and four lines, namely, Acala 1517-99 -M1903, Acala 1517-99 -3028, TAM94L25-M926, and TAM94L25-M2888 showing improved LEN were used to develop pop. S. These lines were selected from a set of 157 mutant lines in two different genetic backgrounds [*G. hirsutum* viz. TAM94L25 (SMITH 2003) and Acala 1517-99 (CANTRELL *et al.* 2000)] that showed striking improvement over wild type progenitor ('parent') or control lines. The pilot results were supported by replicated trials in multiple environments (PATEL *et al.* 2014). In a greenhouse at Athens, GA (Summer, 2012), four crosses of two mutant lines each (one Acala, one TAM) were made to develop F1's and in an off-season nursery in Tecoman, Mexico (Winter, 2012), the F1 hybrids were further crossed with each other, thus developing double-cross populations that combine four mutant lines (Table 2).

2.2. Field trial and data collection

A total of 100 F2 progenies from each population were grown in Watkinsville, GA (soil type-fine, kaolinitic, thermic typic kanhapludults) in May 2013. In 2014, a total of 95 individuals from pop. R and 94 from pop. S were evaluated at two locations with two replications (i.e., Watkinsville and Tifton), in a randomized complete block design (RCB). The soil type at Watkinsville, GA was Appling Coarse Sandy Loam (fine, kaolinitic, thermic typic kanhapludults) and at Gibbs farm, Tifton, GA was Tifton loamy sand (fine, loamy, siliceous, thermic Plinthic kandiudult). For all three environments both parents TAM94L25 and ACALA1517-99, TXA (TAM94L25 x ACALA1517-99), plus three checks GA230 (PVP 201500309), Fiber Max 832 (PVP 9800258), and Delta Pine 393 (PVP 200400266) were replicated 10 times for each replication in the field. A total of 35 seeds were planted in a single-row plot of 3m, with plots spaced 1 m apart. Agronomic practices like weeding, irrigation, fertilizer application and pest management were conducted as per standard cotton growing practices. To obtain fiber samples, bolls were hand-picked in Athens (November 25, 2013) and Tifton (October 26, 2014) while seed cotton samples were collected from machine harvested cotton in Athens (November 19, 2014) and ginned using a 20-saw gin (DENNIS MFG. CO., INC.). Lint weight and seed weight (seed plus fuzz) were measured, and lint percent (lint weight X 100/seed cotton weight) was calculated. Samples of 10 grams of cotton fiber were sent to Cotton Inc. to measure HVI fiber properties, namely upper half mean fiber fineness or micronaire (MIC), fiber length (LEN), fiber strength (STR), fiber elongation (ELON), Uniformity index (UI) and Short Fiber Content (SFC).

2.3. Data analysis

Data was analyzed using SAS 9.4 (**SAS** Institute Inc., Cary, NC, USA). The program statement, “Proc CORR” was used to determine correlations between fiber traits. Heritabilities of fiber traits were calculated using parent-offspring regression by the SAS “Proc REG” statement. The contribution and significance of genotype, environment and interaction between genotype and environment for fiber traits was calculated using the SAS statement “Proc GLM”. The means of each fiber trait of pop. R and pop. S were compared with means of wild type progenitors, checks and each other by Fisher’s LSD test at alpha level of 0.01 to identify significant differences. The average of the top 10 lines for the focal fiber trait(s) of the respective populations was compared with TXA (TAM94L25 X ACALA1517-99) to estimate percentage gains in these lines and assess their potential merit in breeding programs to improve fiber traits. For the analysis, genotype, environment, replication and selection (top 10 lines) were considered fixed variables.

3. Results

3.1. Heritability of fiber traits and association between the fiber traits

Parent-offspring regression was done to calculate heritability between F2 and F3 for each fiber trait. Overall, Lint_% showed the lowest heritability (0.25) while LEN showed the highest (0.44) (Table 3). Trends of heritability for different fiber traits were consistent with previous reports (HERRING *et al.* 2004).

Correlation coefficients were used to study association between fiber traits. In both populations, positive correlation was seen between LEN and UNIF, LEN and STR, and STR and UNIF, indicating that simultaneous improvement of LEN, STR and UNIF is possible. For MIC and SFC, a negative correlation of these traits with other fiber traits is favorable as low values of each of these traits are preferred. In both populations, SFC showed negative correlation with LEN, UNIF, STR and ELON, which means improving one or more of these fiber traits may also improve SFC. There was a negative correlation between MIC and LEN in both populations. In pop. S, there was favorable correlation between MIC and STR, and MIC and SFC; and unfavorable correlation between MIC and lint%. In pop. R, ELON and UNIF had no correlation but a positive correlation in pop. S. Lint% was positively correlated with ELON in both populations. LEN and STR had minor negative correlation with lint% in pop. S (Table 4).

3.2. Analysis of Variance

Analysis of variance in both populations showed significant difference between genotypes and between environments but no significant genotype X environment interaction. In pop. R, the contribution of genotype to overall variance was lowest for ELON (19.3%) and highest for LEN (50.5%) and in pop. S, contribution of genotype to overall variance was lowest for ELON (21.5%) and highest for MIC (48.8%) (Table 5).

3.3. Fiber traits

3.3.1. MIC (fiber fineness)

Both populations showed significant improvement for MIC when compared to TAM (TAM94L25), ACA (ACALA1517-99), TXA and DP (Delta Pine 393), but no significant difference was found between these populations and the commercial line FM (Fiber Max 832). The overall mean of Pop. R showed 4.5 % improvement compared to TXA, and pop. S showed 7.3% improvement with significantly better MIC than pop. R. Totals of 23 and 49 lines of pop. R and pop. S respectively showed significant improvement over both parents and TXA with maximum improvements of 17.2% and 19.4% in pop. R and pop. S, respectively (Table 6).

3.2.2. LEN, UNIF, STR AND SFC

Both populations showed significant improvement for LEN, UNIF, STR and SFC with respect to all parents and checks, with the average of pop. S significantly better than pop. R for LEN, UNIF, STR and SFC. Compared to TXA, pop. R showed an average 3.4%, 1.3%, 6.4%, and 8.8% improvement for LEN, UNIF, STR and SFC, respectively; while pop. S showed 7.8%, 2.1%, 12.8% and 13.7%. The number of lines exceeding parental values for LEN, UNIF, STR and SFC, respectively was 55, 43, 38, and 59 for pop. R; and 78, 74, 87, and 87 for pop. S. The maximum improvements in LEN, UNIF, STR and SFC, respectively, were 10.5% (over TXA), 4%, 16.6%, and 19.5% in pop. R; and 14.9% (over TXA), 4.3%, 21.8%, and 24.8% in pop. S (Table 6).

3.3.3. ELON

For ELON, pop. R showed significant improvement compared to TAM and TXA, but no significant difference from ACA and the two elite checks. Pop. S showed significant improvement over TAM but no significant improvement over TXA and FM, and was significantly inferior to ACA, DP and pop. R. Compared to TXA, the mean of pop. R showed 15.3% improvement whereas pop. S showed 5.2% improvement. No line in either population had significantly higher ELON than ACA, but 39 and 9 genotypes in pop. R and pop. S, respectively, showed significant improvement over TXA.

3.3.4. Lint %

Both populations had significantly lower lint% compared to all the parental and checks. Average of pop. R was 6.5% lower and pop. S was 7.7% lower than TXA. No line in either population was significantly better than the parental lines. However, 60 and 41 genotypes in pop. R and pop. S, respectively, had improved fiber quality and were not significantly different for lint% than the parental lines.

4. Discussion

Building on recent evidence that EMS-induced mutants may contribute substantially to mitigating a lack of genetic diversity owing to genetic bottlenecks during cotton evolution, domestication, selection and crop breeding practices (BECHERE *et al.* 2007; BROWN *et al.* 2012; PATEL *et al.* 2014), the present research validates additional EMS-mutants for roles in cotton fiber quality (beyond what were validated in a companion study, Patel et al. unpublished) and explores the effects of pyramiding multiple mutants.

Surprisingly, pop. S, combining multiple mutations that alone each improve LEN, also had better MIC, STR, UNIF and SFC than pop. R, combining mutants that alone improve LEN, MIC, STR and ELON, which suggests that it is possible to improve multiple fiber attributes by targeting single fiber quality traits such as LEN. This might be due to the presence of fiber QTL hotspots comprised of dozens of genes with coordinated expression during different stages of fiber development (PATERSON *et al.* 2012). Thus, by editing a single gene through EMS-mutagenesis or other mechanisms, we might affect the function of other genes that might produce additional improvements.

SFC and UNIF were not directly targeted in this research (although SFC is clearly related to LEN) but we still found striking improvement in both mutant populations compared to all parents and checks. SFC is a major factor contributing to irregularity in yarn and reducing its strength (THIBODEAUX *et al.* 2008; CAI *et al.* 2011). The number of neps (small knots of entangled fibers in fabric) that reduce the overall quality of yarn is also positively associated with SFC (VAN DER SLUIJS AND HUNTER 1999; ULLOA 2006). Here we found lines showing 19.5% (relative to TXA) and 24.8% reduced SFC content in pop. R and pop. S, respectively. Compared to TXA, pop. R showed an average 8.8% improvement for SFC, while pop. S showed

13.7%; indeed, in each population, the majority of lines (59 and 87 respectively) exceeded the best parental value for SFC.

Lint % is an important component of cotton yield. Pop. R had better lint% than pop. S, but both populations had lower lint% than parents and checks. This was expected as yield components are strongly negatively associated with fiber quality (MEREDITH 1984; CLEMENT *et al.* 2012; CONSTABLE *et al.* 2015). Still, multiple lines in both populations had improved fiber qualities with no adverse effect on lint%, suggesting that negative association between fiber traits and yield components could be overcome, as also suggested in previous research (CLEMENT *et al.* 2015). Intermating among such lines coupled with recurrent selection may weaken negative associations and produce lines with superior fiber qualities and adequate Lint%. Similar strategies have been suggested by CLEMENT *et al.* (2012) to break negative correlations between yield components and fiber quality.

Multiples lines in each population showed improvement for more than one fiber trait, making them well suited for direct use to improve fiber quality in mainstream breeding programs. In pop. S, a total of 61 lines showed improvements for four or more fiber traits

29 lines showed improvements for four fiber traits, 39 for five fiber traits, and three for six fiber traits (LEN, STR, UNIF, MIC, ELON and SFC), with multiple lines showing no significant difference for ELON and lint% when compared to parental lines and TXA. This further supports our hypothesis that crossing mutant lines for improved for one fiber trait can simultaneously improve other fiber traits. For pop. R, a total of 39 lines showed improvements for four or more fiber traits 22 lines showed improvement for four fiber traits, 16 for five and one for six (LEN, STR, UNIF, MIC, ELON and SFC) when compared to parental lines and TXA.

In summary, the present research shows the opportunity for simultaneously improving multiple traits and the merit of pyramiding independent EMS-induced mutants for a trait. Further work is needed to determine if this is a general trend or peculiar to these particular sets of mutants, and to investigate consequences for yield components and other traits. It would of course be interesting to identify such mutants that presumably have pleiotropic effects on multiple fiber traits. The ability to manipulate germplasm containing discrete mutations affecting fiber traits provides new insight into cotton breeding strategies, that may inform fiber improvement programs using natural or induced alleles.

CRediT authorship author statement

Jinesh Patel: Methodology, Data curation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Sameer Khanal:** Investigation. **Rahul Chandnani:** Investigation. **Jeevan Adhikari:** Investigation. **Ismail Brown:** Investigation**. Peng W. Chee:** Investigation. **Don C. Jones:** Investigation. **Andrew H. Paterson:** Conceptualization, Supervision, Funding acquisition, Methodology, Data curation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Table 1- Superior fiber quality mutant lines selected from Patel *et al.* (2014) for population development

Mutant id	**Mean**	**Control mean**	**Significance**	**Background**	**Improvement in**	**% improv.**
926	1.26	1.15	0.0001	TAM 94L25	LEN	9.00%
1903	1.3	1.18	0.0001	Acala 1517-99	LEN	9.60%
2888	1.25	1.15	0.0001	TAM 94L25	LEN	8.60%
3028	1.27	1.18	0.001	Acala 1517-99	LEN	7.20%
2925	8.68	5.78	0.0001	TAM 94L25	ELONG	50.00%
2877	3.94	4.83	0.0001	TAM 94L25	MIC	18.40%
1793	37.11	33.84	0.005	Acala 1517-99	STR	9.70%

Table 2- Crossing scheme of F1 hybrids to study effect of combination of different novel alleles on fiber traits

Pop id	**Crosses between F1 hybrid**	**Fiber trait targeted**	**Mutant parental lines**	**Population size**
Pop. R	1903-1 X 2925-1	(LEN +ELON) X (MIC + STR)	Acala 1517-99 + TAM 94L25	95
	2877-2 X 1793-1			
Pop. S	926-4 X 3028-2	LEN	Acala 1517-99 + TAM 94L25	94
	2888-1 X 1903-3			

Table 3- Parent-offspring regression estimates of heritability for seven cotton fiber traits across two mutant-containing populations

	Athens 14	**Tifton 14**	**Total_14**
MIC_A13	0.21	0.37	0.29
LEN_A13	0.46	0.42	0.44
UNIF_A13	0.28	0.3	0.29
STR_A13	0.36	0.39	0.37
ELON_A13	0.34	0.31	0.33
SFC_A13	0.37	0.46	0.41
Lint %_A13	0.21	0.3	0.25

Table 4-Correlations between seven cotton fiber traits in two mutant-containing populations

	MIC	**UHM**	**UI**	**STR**	**ELO**	**SFC %**
Pop. R						
UHM	-0.24*					
UI	0	0.50*				
STR	0	0.55*	0.54*			
ELO	-0.10	-0.15	0.16	-0.08		
SFC	-0.03	-0.53*	-0.83*	-0.56*	-0.24*	
Lint %	0.14	-0.08	0.12	0.11	0.39*	-0.11
Pop. S						
UHM	-0.50*					
UI	-0.10	0.47*				
STR	-0.21*	0.40*	0.44*			
ELO	0.12	-0.13	0.35*	0.10		
SFC	0.26*	-0.70*	-0.80*	-0.47*	-0.28*	
Lint %	0.39*	-0.22*	0.06	-0.19*	0.45*	0.03

* shows significance at $p < 0.0001$

Table 5- Variance components for seven cotton fiber traits across two mutant-containing populations

Fiber traits	**Source**	**DF**	**SS**	**MS**	**F Value**	**% Contribution**	**DF**	**SS**	**MS**	**F Value**	**% Contribution**
		Pop. R					**Pop. S**				
MIC	**G**	94	31.3	0.33	3.20*	33.6	93	44.4	0.48	4.27*	48.8
	E	2	17.6	8.8	84.40*	18.9	2	4.95	2.47	22.12*	5.4
	G*E	185	24.6	0.13	1.28	26.4	186	20.69	0.11	0.99	22.7
	Error	190	19.8	0.1			188	21.03	0.11		
Len	**G**	94	0.65	0.01	4.23*	50.5	93	0.78	0.01	4.01*	45.3
	E	2	0.04	0.02	11.89*	3.0	2	0.08	0.04	19.64*	4.8
	G*E	185	0.29	0	0.95	22.4	186	0.46	0	1.19	27.0
	Error	190	0.31	0			188	0.39	0		
UI	**G**	94	269	2.86	2.11*	32.8	93	290.26	3.12	2.04*	29.4
	E	2	51	25.5	18.79*	6.2	2	155.76	77.88	50.84*	15.8
	G*E	185	243	1.31	0.97	29.6	186	252.16	1.36	0.88	25.6
	Error	190	258	1.36			188	288	1.53		
STR	**G**	94	832	8.85	2.85*	41.6	93	635.8	6.84	2*	32.5
	E	2	52.9	26.5	8.52*	2.7	2	75.97	37.99	11.1*	3.9
	G*E	185	523	2.83	0.91	26.2	186	598.83	3.22	0.94	30.6
	Error	190	590	3.11			188	643.59	3.42		
ELON	**G**	94	77.8	0.83	4.89*	19.3	93	55.04	0.59	3.37*	21.5
	E	2	257	129	760*	64.0	2	136.31	68.15	389*	53.3
	G*E	185	34.9	0.19	1.11	8.7	186	31.4	0.17	0.96	12.3
	Error	190	32.2	0.17			188	32.98	0.18		
SFC	**G**	94	61.6	0.65	2.05*	31.1	93	82.49	0.89	2.5*	33.4
	E	2	16.2	8.09	25.35*	8.2	2	36.74	18.37	51.7*	14.9
	G*E	185	59.5	0.32	1.01	30.1	186	61.02	0.33	0.92	24.7
	Error	190	60.7	0.32			188	66.8	0.36		
Lint %	**G**	94	1012	10.8	1.35*	20.9	93	1370.1	14.73	2.62*	26.5
	E	2	1264	632	79.51*	26.1	2	1574.6	787.28	140*	30.5
	G*E	185	1058	5.63	0.71	21.9	186	1163.8	6.26	1.11	22.5
	Error	190	1502	7.95			188	1057.7	5.63		

Table 6 – Comparing of each fiber trait between two population, parental and check lines

Pop id	Trait	Pop mean	TAM	ACA	TXA	% improvement to TXA	GA 230	Fiber Max 832	Delta Pine 393	# of improved lines	% improvement of best line to TXA	Difference in % to pop. R
R	MIC	4.3	4.53*	4.54*	4.5*	4.5	4.55*	4.26	4.57*	23	17.2	.
R	LEN	1.19	1.14*	1.12*	1.15*	3.4	1.17*	1.14*	1.13*	55	10.5	.
R	UNIF	83.72	82.33*	82.11*	82.66*	1.3	83.6*	82.96*	82.69*	43	4	.
R	STR	31.83	30.44*	29.15*	29.9*	6.4	30.48*	30.62*	30.33*	38	16.6	.
R	ELON	5.36	4.48*	5.37	4.65*	15.3	4.96	5.11	5.28	0	32	.
R	SFC %	7.7	8.57*	8.67*	8.44*	8.8	7.88*	8.35*	8.17*	59	19.5	.
R	Lint %	36.59	38.93	38.01	39.11	-6.5	41.04	40.17	39.49	0	5.5	.
S	MIC	4.17	4.53*	4.54*	4.5*	7.3	4.55*	4.26	4.57*	49	19.4	-3*
S	LEN	1.24	1.13*	1.12*	1.15*	7.8	1.17*	1.14*	1.13*	78	14.9	4.2*
S	UNIF	84.39	82.33*	82.11*	82.66*	2.1	83.6*	82.96*	82.69*	74	4.3	0.8*
S	STR	33.72	30.44*	29.15*	29.9*	12.8	30.48*	30.62*	30.33*	87	21.8	5.9*
S	ELON	4.89	4.48*	5.37	4.65	5.2	4.96	5.11	5.28	0	21.3	-8.8*
S	SFC %	7.28	8.57*	8.67*	8.44*	13.7	7.88*	8.35*	8.17*	87	24.8	-5.5*
S	Lint %	36.09	38.93	38.01	39.11	-7.7	41.04	40.17	39.49	0	2.2	-1.4*

Parental or checks cells with “*” are significantly inferior to population average by $p<0.01$

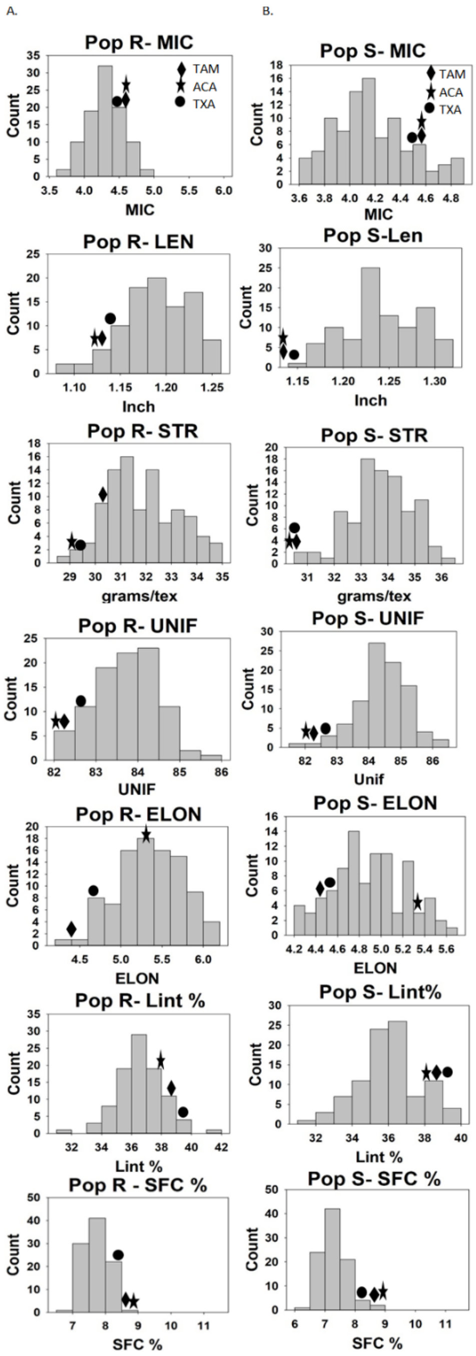

Figure 1- Distribution of genotypes in populations for different fiber traits. (A) pop. R and (B) pop. S

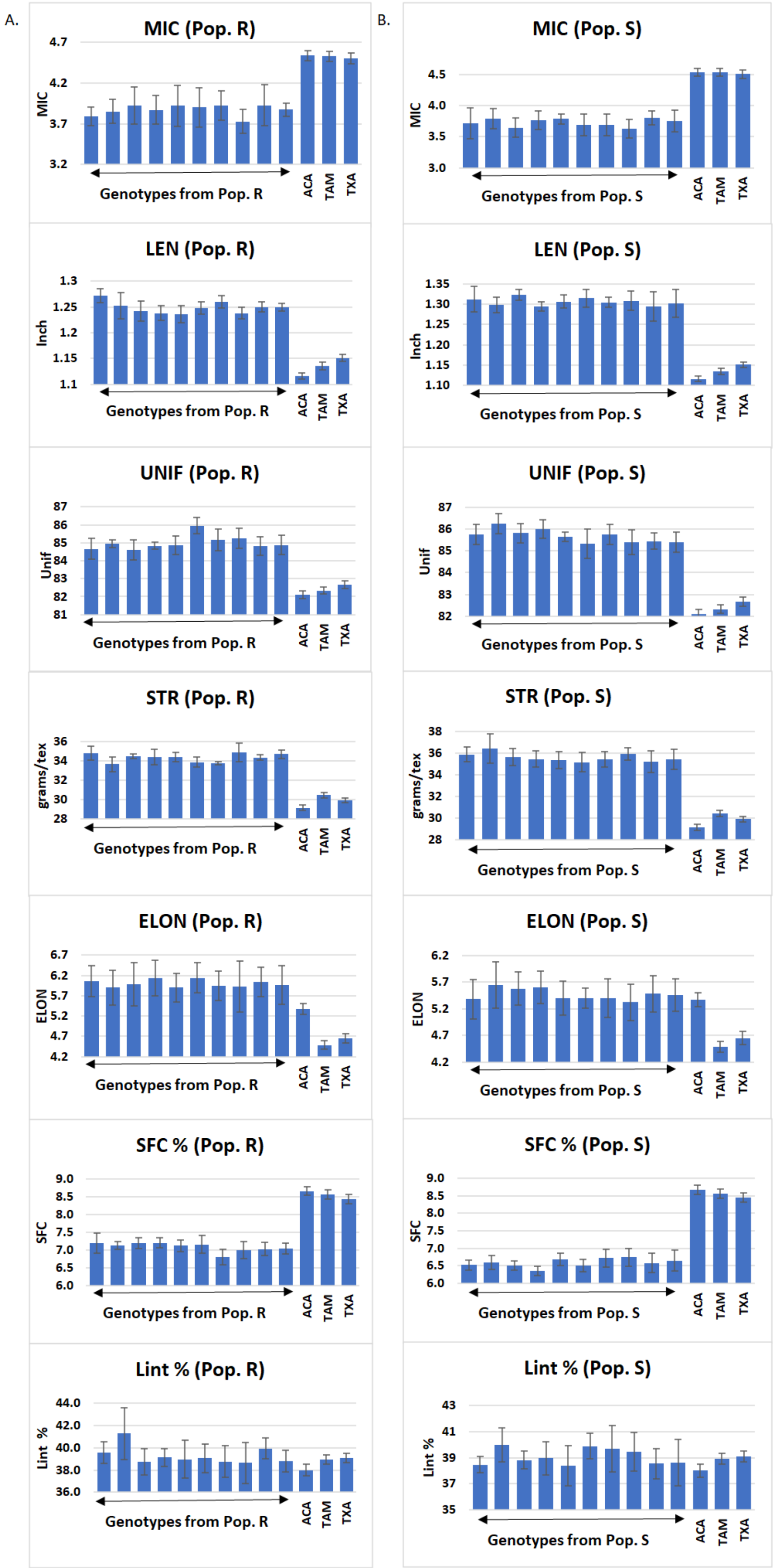

Figure 2- The 10 best lines for different fiber traits compared with parental lines. (A) pop. R and (B) pop. S