Differential variability of maize (*Zea mays* L.) inbred lines to moisture-stress at reproductive stages and DNA methylation studies of identified contrasting inbred lines under moisture-stress conditions

ABSTRACT

An investigation was undertaken to assess the effect of moisture stress during the reproductive initiation stage on the quality and quantity of pollen grains produced in eight inbred lines under greenhouse conditions in three blocks viz., well-watered, stress I (for 21 days from 28 days after sowing) and stress II (for 22 days from 32 days after sowing). Moisture stress significantly affected the number of pollen grains per anther and other plant growth-related traits. The moisture stress effect was not uniform across inbred lines. The inbred lines UASBM22, UASBM13, UASBM09 and UASBM11 recorded a significant reduction in the total number of pollen grains per anther and an increase in pollen sterility while in inbred lines, UASBM06, UASBM14, UASBM02 and UASBM10, the moisture stress effect was not significant. The changes in the DNA methylation pattern in leaves and immature anthers under moisture stress of the contrasting inbred lines (UASBM06 and UASBM13) were studied through methylation-sensitive random amplification polymorphism. An increase in total DNA methylation level in both leaves and anthers was observed in drought tolerant inbred line, UASBM06 under stress while the increase was only in the leaves of the susceptible inbred line UASBM13. Leaves and anthers of UASBM06 showed hypermethylation compared to UASBM13 in moisture-stress conditions. In maize, increased DNA methylation seems to be an important mechanism associated with drought responses which probably regulates the methylation-sensitive gene expression and acclimation responses in maize.

Keywords: *Moisture-stress, pollen grains, DNA methylation, methylation sensitive-random amplification polymorphism*

INTRODUCTION

Maize (*Zea mays* L.) is the third most important cereal crop in India after rice and wheat and plays pivotal role in agricultural economy as food for larger section of the population, raw materials for industries and feed for animals. Maize is cultivated globally in about 160 countries and contributes approximately 50 *per cent* (1031.86 MT) to global grain production (FAOSTAT, 2019). Globally, it is second most valuable crop in terms of acreage with a cultivated area of 191.90 million hectares, having a production of 1077.98 million metric tonnes and a productivity of 5.62 metric tonnes per hectare. In India, it covers an area of 9.02 million hectares securing fourth position in the world area-wise with a production of 27.71 million metric tonnes and a productivity of 3.07 metric tonnes per hectare (FAOSTAT, 2019; India Agri stat, 2020).

Maize production and productivity are mainly reduced by a number of biotic and abiotic stresses. Among all the abiotic factors, drought is one of the major environmental constrains, that limits the productivity of crop (Hassan *et al*., 2016) by affecting the growth, physiology and metabolism of plants. Being drought sensitive crop, maize is affected at each and every stage of growth and development i.e., affects the plant from seedling to maturity especially during its reproductive stage because it leads to increased anthesis silking interval, sterile pollen and no seed set (Sah *et al*., 2020). According to Khodarahmpour and Hamidi (2012) drought stress at the vegetative, pollination and grain filling periods can cause losses in maize yield by 15, 40, and 60 *per cent* respectively. As much as 90 *per cent* of maize yield can be reduced if the crop is exposed to drought stress from a few days before tassel start to emerge till the commencement of grain filling stage (Awosanmi *et al*., 2016; Daryanto *et al*., 2016). Response of the maize crop to climate depends entirely on the genetic and physiological make-up of the hybrid/variety being grown, and interactions with prevailing climatic conditions. Therefore, all genotypes of maize are not equally affected by drought due to high level of variability in genetic background of this crop. Differential variation in maize landrace genetics is natural and would be helpful in coming up with breeding advancement (Reif et al., 2004).

Plants are continually confronted with biotic and abiotic challenges, and as a result, they have developed amazing ability to control their physiological and developmental machinery in response to these pressures through gene expression variations (Zhao et al., 2007). The response to abiotic stressors is complicated, involving numerous processes such as genetic and epigenetic pathways to adapt to the changing environment. Detection, quantification, and use of natural and/or induced genetic diversity coming from DNA sequence variation are required for breeding any crop, including maize. However, there has recently been a surge in interest in exploiting variation caused by factors other than DNA sequence differences. Variation caused by epigenetics is one of the most prominent. DNA methylation, histone modifications, RNA interference, and other processes are thought to play a role in epigenetics (Springer and Schmitz, 2017).

DNA methylation, one of the most important epigenetic mechanisms in plants, is known to impact gene expression when plants are exposed to abiotic stress, such as drought. Methyl groups are added to the 5th carbon atom of the cytosine nitrogenous base of DNA sequence to produce 5-methylcytosine. It alters gene expression without altering DNA sequence. In plants, cytosine DNA methylation can occur in any context (CG, CHG, and asymmetric CHH, where H is A, C, or T), with CG being the most often methylated dinucleotide (Springer and Schmitz, 2017). Cytosine methylation regulates gene expression by influencing protein binding to DNA and chromatin structure (Osabe et al., 2014). The majority of methylation in plants is evident in the transposon-rich heterochromatic area, repeated sequences, and regions producing small interfering RNAs (Zhang *et al*., 2006). Methylation of a gene's promoter region can make it inactive, whereas demethylation can make it active again. Promoter methylated gene expression is tissue-specific (Zhang et al., 2006, Feng et al., 2010, Zemach et al., 2010). The expression pattern of drought stress response genes is influenced by DNA methylation status, suggesting that DNA methylation may play a role in drought response and tolerance.

For any exact selection of crop varieties, breeders must recognise causes of phenotypic variability. If epigenetic alteration may yield desired phenotypes, there is no need for selection pressure on specific gene(s). This lessens selection pressure on genetic variety, resulting in less genetic erosion (Gallusci et al., 2017). Plants collect both DNA sequence-dependent (genetic) and DNA sequence-independent (epigenetic) variation during evolution and adaptation to maximise heritable phenotypic differences to deal with environmental disruption (Tirnaz and Batley, 2019). As a result, DNA sequence variation alone has been found to be insufficient to explain heritable phenotypic variation in various instances. A better knowledge of the effect of epigenetic variation such as DNA methylation on plant phenotypic, in addition to genetic variations, has created a chance to speed up the crop development process (Merce et al., 2020). Thus, DNA methylation can broaden the sources of phenotypic variation for use by breeders (Gallusci et al., 2017; Tirnaz and Batley, 2019) and would be valuable for a better understanding of the expression profile of genes involved in drought adaptation. In order to take advantage of DNA methylation-induced phenotypic variation in crop breeding, scientists must first determine (1) the extent of DNA methylation variation, (2) the extent to which DNA methylation variation is associated with economically important quantitative traits, and (3) the extent to which superior genotypes linked to methylation marks are stably inherited (Kumar et al., 2017).

Considering all the points mentioned above, the present work was carried out to study the effect of moisture stress during the reproductive stage on the quality and quantity of pollen grains produced in different maize inbred lines. Further an attempt was made to understand the epigenetic changes in leaves and immature anthers of the contrasting maize inbred lines under moisture stress through methylation sensitive random amplification polymorphism.

MATERIAL AND METHODS

A. Experimental details

Eight homozygous contrasting maize inbred lines for drought tolerance viz., UASBM22, UASBM13, UASBM06, UASBM09, UASBM02, UASBM14, UASBM10, UASBM11 which were developed in the Department of Plant Biotechnology, UAS Bangalore were selected for the present study. The inbred lines were sown in pots of size 30 × 30 cm, filled with 13 kg of potting mixture (soil + FYM in 1:1 ratio) under greenhouse conditions. Totally nine plants (one plant per pot) were grown for each inbred line and were divided into three blocks with three pots per inbred line per block. All the seventy-two plants from the three blocks were watered daily with 1litre of water per pot to maintain the field capacity. One block of three plants per inbred line were watered daily and the pots were maintained at field capacity till maturity. The second and third block plants were subjected to moisture stress by providing limited water at early reproductive initiation stage. The second block with three plants per inbred line was subjected to water stress from 28 days after sowing (DAS) for 21 days and the third block of three pots per inbred line was

subjected to water stress from 32 DAS for 22 days. During stress period, limited water was applied to the pots to induce moisture stress.

Analysis of soil moisture content

Soil water status in the stressed pots was daily monitored using soil moisture indicator developed by Sugarcane Breeding Institute, Indian Council of Agricultural Research (ICAR-SBI), Coimbatore and marketed by Tech Source Solution, Bengaluru. The sensor rods of the instrument were inserted to a depth of 22 cm (Gao *et al*., 2010) the switch of the indicator was pressed and held till the LED stopped at a particular colour thus, indicating soil moisture content as described by ICAR-SBI Coimbatore as given in Table 1.

Table 1: Soil moisture content reading using soil moisture indicator in maize

Colour of the LED (10)	Soil moisture status	Inference
Blue (3 levels)	Ample moisture	No need for irrigation at all
Green (3 levels)	Sufficient moisture	Immediate irrigation may not be necessary
Orange (1 level)	Low moisture	Irrigation advisable
Red (3 levels)	Very low moisture	Immediate irrigation

Depending on the colour of the LED glow, moisture content was decided and plants were watered based on the requirement for survival under moisture stress. For moisture stressed plants, the colour of the LED glow was maintained at orange throughout the day. When the LED glow reached first red, 200 ml of water was given to the plants to ensure their survival, yet maintaining low soil moisture content as indicated by orange LED glow. After 21 days of moisture stress (at 48 DAS) for second block and 22 days of moisture stress (at 54 DAS) for third block, the stress was relieved and 1 litre of water was given till maturity every day maintaining blue LED glow. It has been observed from our previous experiments that the moisture stress from 36 to 44 days depending on the duration of the inbred line affects the process of microsporogenesis in maize. The control plants (Block 1) were watered with 1litre of water every day till maturity to maintain ample soil moisture content as indicated by blue colour LED glow.

Morphological characterization

Maize inbred lines exhibited a wide variation with respect to morphological features. Plant height, days to tasselling, days to silking, days to anthesis, anthesis silking interval, tassel length, spike length, spikelet length, anther length, total pollen grains per anther and *per cent* pollen grain sterility were recorded using standard protocol.

Number of pollen grains per anther

Number of pollen grains produced per anther was counted in both moisture stressed and well-watered plants. First and fourth primary branches from the bottom of the tassel of each individual plant in which the anthers were about to dehisce the following day were selected. These primary branches were collected in a petri dish, brought to the laboratory and incubated at 70 °C in an oven for 24 hours. After incubation, one spikelet each was collected from 5^{th},10^{th},15^{th} and 20^{th} position of the first primary tassel branch and fourth primary tassel branch. For each plant 8 anthers were used. One anther from each of the spikelet was carefully removed and transferred to 1.5 ml Eppendorf tube containing 1 ml of 5 *per cent* tween20 solution. The tubes were sonicated at 70 amplitudes to completely release the pollen grains in to the solution. The sample was mixed thoroughly to ensure uniform distribution of pollen grains in the solution. From each tube, three replications of 1µl sample were drawn and dispensed on each side of Neubeur counting chamber German-hemacytometer and the total number of pollen grains in 1ml sample was counted using projection microscope Euromex-Holland, model- CMEX DC.300x at a magnification of 10x. For each anther, three samples were drawn and the average number of pollen grains per anther was determined for each position. The average number of pollen grains per anther was calculated for each first and fourth primary tassel branch separately for the inbred line.

Per cent pollen sterility

The fully circular (turgid), non-transparent pollen grains were considered as fertile pollen grains while the irregular shaped (flaccid), transparent pollen grains were considered as sterile pollen grains (Mohapatra *et al*., 2020). The total number of sterile pollen grains per anther was recorded and the p*er cent* pollen sterility was calculated as follows:

$$Per\ cent\ \text{pollen sterility} = \frac{\text{Number of sterile pollen grains}}{\text{Total pollen grain per anther}}\ X\ 100$$

Data analysis

Completely randomized factorial (factorial CRD) analysis was carried out using the recorded observations. The first factor was the three levels of treatment (control, stress I and stress II) and the second factor was the eight maize inbred lines. The analysis of variance was performed for the traits recorded to ensure the existence of significant differences between treatments and their interaction.

B. Methylation-sensitive random amplification polymorphism in the stressed leaves and immature anthers of contrasting maize inbred lines

Experimental material

Based on *per cent* pollen sterility and number of pollen grains per anther produced under drought in the previous above experiment, UASBM06 was selected as drought tolerant and UASBM13 was selected

as drought susceptible for the present study. These two contrasting inbred lines were sown in pots of size 30 × 30 cm, filled with 13 kg of potting mixture (soil + FYM in 1:1 ratio) under greenhouse conditions. Six plants were grown for each inbred line. Out of six, three plants for each inbred line were grown till anthesis without any water stress. The pots were watered daily with 1 litre of water per pot to maintain the field capacity. Another set of three plants per inbred line was subjected to water stress during microsporogenesis. The time and duration of stress for both resistant and susceptible inbred lines were decided based on the previous experiment (A). The inbred lines differed for days to anthesis. The inbred line UASBM13 was early while UASBM06 was late in the earlier experiment. Thus, moisture stress was given to both inbred lines on different dates and for different duration such that it matches the microsporogenesis stage. UASBM06 was subjected to moisture stress from 36 DAS for 22 days and UASBM13 was subjected to moisture stress from 26 DAS for 22 days as mentioned in experiment A.

Selection of immature anther and leaf for DNA isolation

The inbred line UASBM13 was early whereas UASBM06 was late in days to tasselling. The water stressed plants delayed tassel initiation and anthesis in both the inbred lines. For UASBM13 the control plants were dissected on 44 DAS for harvesting immature anthers at microsporogenesis stage and for water stressed plants, the stage was achieved on 54 DAS. Similarly, for UASBM06 the control plants were carefully dissected on 59 DAS and for water stressed plants, the stage was achieved on 62 DAS. Immature tassels were harvested and immediately wrapped in aluminium foil and brought to laboratory for isolation of anthers. Immature anthers of size 2 mm were carefully dissected from spikelet of immature tassels in a laminar air flow and used for DNA isolation. The anthers of size 2 mm were removed from the entire immature tassel of both control and water stressed plants of UASBM13 and UASBM06 and was used for DNA isolation by using modified Cetyl Trimethyl Ammonium Bromide (CTAB) method. Similarly, uppermost leaf was cut from both control and water stressed plant of both inbred lines from which tassel was dissected. The leaf sample was collected on the same day of tassel dissection and immediately wrapped in aluminium foil and brought to laboratory to isolate DNA. The genomic DNA was extracted from the leaves and immature anthers of contrasting inbred lines of maize by following CTAB method of DNA extraction.

Method of genomic DNA isolation

The DNA from immature anther and leaf samples of both UASBM13 and UASBM06 was isolated. Two grams of fresh leaves and anthers dissected from entire tassel of maize plants of both the inbred lines were collected as mentioned earlier and grounded to a fine powder using liquid nitrogen in a pestle and mortar separately. To this, 1 ml of CTAB extraction buffer pre-warmed at 65 °C in a water bath was added and the contents were transferred to 2 ml Eppendorf tube. 5 µl of 10 mM RNase A was added to each tube and inverted thoroughly to remove RNA contamination. The sample containing tubes were incubated at 65 °C for 30 min in the water bath with intermittent mixing at every 10 min. The samples were then removed from water bath and kept outside for 5-10 min for thawing. The samples were centrifuged at 12000 rpm for 15 min at 4 °C. The supernatant was transferred to fresh 2 ml Eppendorf tube and an equal volume of

chloroform: isoamyl alcohol (24:1) was added and mixed by gently inverting the tubes. The tubes were centrifuged at 12000 rpm for 10 min at 4 °C. This step was repeated till a clear supernatant was obtained. The supernatant was carefully transferred to a fresh 1.5 ml sterile Eppendorf tube and an equal volume of pre-chilled isopropanol was added. The tubes were gently inverted and incubated at -20 °C overnight. The samples were centrifuged at 12000 rpm for 10 min to pellet down the DNA. The supernatant was discarded. The pellet was washed with 70 *per cent* ethanol and air dried for 2 hours such that no alcohol trace is present. The pellet was dissolved in 50 µl of Tris EDTA (10 mM Tris Cl and 1 mM EDTA) buffer and stored at -20°C for future use.

Assessment of DNA quality and quantity

The quality and quantity of DNA was assessed on 0.8 *per cent* agarose gel. 100 ml of 1x TBE (0.89 M Tris, 0.89 M boric acid and 0.02 M EDTA, pH 8) buffer was added to 0.8 g agarose in conical flask and heated till the agarose completely melted. The solution was cooled partially and 5 µl of ethidium bromide (10 mg/ml) was added and mixed well. The agarose solution was poured into gel tray with combs and allowed to solidify. 2.5 µl genomic DNA was mixed with 0.5µl of loading dye and loaded into the wells of 0.8 *per cent* agarose gel. The gels were then electrophoresed at 80 V for 2 hours. The DNA bands on the gels were visualized and documented using Alpha digidoc 1000 gel documentation system (Alpha Innotech Corporation, USA). The quality/purity of extracted genomic DNA was assessed by checking the shearing of DNA and contamination with RNA. The quantity and purity of the extracted genomic DNA was also assessed using Nano drop spectrophotometer. Based on the absorbance at 260 nm, the quantity and the purity were determined. With a pure sample of DNA, the ratio of absorbance at 260 nm and 280 nm (OD (260) /OD (280)) is 1.8. Ratio less than 1.8 indicates that the preparation is contaminated either with phenol or proteins. Value higher than 1.8 indicates the presence of RNA in the preparation.

Detection and quantification of DNA methylation variations using Methylation Sensitive Amplification Polymorphism (MSAP) assay

MSAP (Methylation Sensitive Amplification Polymorphism) is one of the most widely used methods for determining DNA methylation changes in plants. It involves visualising PCR fragments on gel after cleaving genomic DNA with methylation sensitive restriction enzymes and amplification with random primers.

Principle of MSAP

Methylation Sensitive Amplification Polymorphism (MSAP) involves the utilisation of isoschizomers, which are a pair of restriction enzymes like *MspI* and *HpaII* that detect and cleave the same tetranucleotide sequence, 5'-CCGG-3',3'-GGCC-5', but differ in their sensitivity to the methylation status of cytosine residues. *HpaII* (methylation-sensitive restriction enzyme) identifies only hemi-methylated external cytosine (HMeCCG) recognition sequences, whereas *MspI* (methylation insensitive restriction enzyme) detects only hemi or fully methylated internal cytosine sequences (HMeCG or MeCG). Both enzymes do

not digest sequences that are fully methylated at the external cytosine (MeCCG) or hemi or fully methylated at both the internal and external cytosines (hyper-methylated) (HMeCHMeCG or MeCMeCG). However, CCGG sequences free of any methylation are digested by both enzymes (Schulz *et al*., 2013).

Digestion of DNA using restriction enzymes (*Msp*I & *Hpa*II)

Restriction enzymes *MspI* and *HpaII* which were procured from *New England Biolabs* (*NEB*) were used to digest DNA to analyse the methylation status of the genome. One microgram of DNA from both leaf and immature anthers of both control and water-stressed UASBM13 and UASBM06 inbred lines were digested separately with 1 µl restriction enzymes *MspI* and *HpaII* in different tubes. The protocol followed for digestion of DNA samples was as per the procedure provided by the *New England Biolabs* (*NEB*) and the composition of restriction digestion, incubation time and temperature are given in Table 2.

Table 2: Protocol for digestion of maize DNA samples using restriction enzymes

Sl.no.	Component	*MspI*	*HpaII*
1	Restriction enzyme	1 µl	1 µl
2	DNA	1 µg	1 µg
3	10X NE Buffer	5 µl (1x)	5 µl (1x)
4	Total Rxn volume	50 µl	50 µl
5	Incubation temperature	37°C	37°C
6	Incubation time	Overnight	Overnight
7	Enzyme inactivation	Not inactivated	Inactivated at 80°C for 20 min

Polymerase chain reaction for amplification of DNA samples

Genomic DNA which was digested by two enzymes viz., *MspI* and *HpaII* were separately used for amplification using RAPD primers (Labra *et al*., 2002; Cai *et al*., 1996; Karaca *et al*., 2019 and Sapna *et al*., 2020). Name and sequence of twenty random primers which were used for amplification is given in Supplementary Table 1. The polymerase chain reaction (PCR) was carried out in a Master Cycler Gradient, Eppendorf, Hamburg, Germany. The PCR components and amplification conditions used are given below in Tables 3 and 4 respectively.

Table 3: Components of the PCR reaction mixture

Sl.no.	Component	Concentration	Quantity of each component in µl (For 10 µl)
1	Nuclease free water	-	6.92
2	Taq polymerase buffer with MgCl2	10x	1
3	dNTPs	2mM	0.4
4	Primer	10pmol	0.8
5	Taq polymerase enzyme	1U/µl	0.2
6	Template DNA/genomic DNA	-	0.8

Table 4: PCR Amplification conditions

Sl.no.	Steps	Temperature (°C)	Duration	Cycles
1	Initial denaturation	**95**	**3 min**	**1**
2	Final denaturation	**95**	**1 min**	
3	Annealing	35	**1 min**	45
4	Extension	**72**	**90 sec**	
5	Final extension	**72**	**10 min**	1
6	Final hold	4		

Agarose gel Electrophoresis and separation of PCR-amplified genomic fragments

The PCR products were resolved on 1.5 *per cent* agarose gel along with 100 bp DNA ladder and visualized and documented using Alpha digidoc 1000 gel documentation system (Alpha Innotech Corporation, USA) and based on the presence of the bands, scoring was done.

Scoring inbred lines for DNA methylation types

Scoring was done firstly on the basis of presence or absence of bands as 1 or 0 respectively in all the treatments and then they were classified as given below. For each inbred line and treatment (stress/control), the bands were classified as given below. (i) Type-1 (Non-methylation): When inbred lines whose control (uncut) sample and *MspI & HpaII* digested samples produced amplicons. The amplicons were scored as (1,1), representing non-methylation at 'CCGG' sequences (ii) Type-2 (Internal methylation): When inbred lines whose control sample and the sample digested only by *MspI* produced amplicons. The

amplicons were scored as (1,0), representing internal cytosine full methylation at 'CCGG' sequences (iii) Type-3 (External methylation): When inbred lines whose control sample and the sample digested only by *HpaII* produced amplicons. The amplicons were scored as (0,1), representing external cytosine hemi-methylation at 'CCGG' sequences (iv) Type-4 (Full methylation): When inbred lines whose control sample produced amplicons but the samples digested by any of the two-restriction enzyme failed to produce the amplicons, corresponding to those produced by control sample. The genotypes were scored as (0,0), representing full/hyper methylation in both cytosines of 'CCGG' sequences (Schulz *et al*., 2013). Based on the scoring patterns of methylation, the loci generated by each of the twenty RAPD primers were detected. The Type 1, Type 2, Type 3, Type 4 were compared to find *per cent* no methylation, internal methylation, external methylation and fully methylation as per the given formulae, and further counted for polymorphism for methylation under stress and control treatments in both the inbred lines.

RESULTS

Analysis of variance

Analysis of variance was performed for quantitative traits viz., plant height, days to tasselling, days to silking, days to anthesis, anthesis silking interval, tassel length, spike length, spikelet length, anther length, total pollen grains per anther and *per cent* pollen grain sterility. The results from the analysis of variance showed a significant effect of moisture stress on all the growth parameters. The inbred lines showed highly significant variation for all thirteen characters. The analysis of variance also showed that the interaction effect between inbred lines and stress treatments was significant for all the investigated traits (Table 5).

Table 5. MSS of growth parameters among 8 inbred lines under control (C) and stress (S1, S2) conditions

Source of variation	df	PH	DT	DS	DA	ASI
Inbred (A)	7	12103.49**	367.20**	542.79**	940.09**	24.71**
Stress (B)	2	25074.15**	483.18**	386.54**	358.93**	57.93**
Interaction (AxB)	14	698.40**	10.51**	23.73**	720.15**	35.53**
Error	48	233.36	3.85	5.04	5.92	4.20

****significant P<0.01; *significant at P<0.05**

Table 5 continued…

Source of variation	df	TL	SL	SpL	AL	TPC	PST
Inbred (A)	7	87.95**	56.64**	2.67**	5.75**	343006.87**	1196.40**
Stress(B)	2	974.88**	436.63**	12.98**	1.42**	1250687.60**	2503.54**
Interaction (AxB)	14	42.42**	27.05**	1.35**	0.65**	407980.97**	676.95**
Error	48	12.35	7.67	0.21	0.10	69512.69	87.82

****significant P<0.01; *significant at P<0.05**

PH: Plant height at maturity, DT: Days to Tasselling, DS: Days to Silking, DA: Day to Anthesis, ASI: Anthesis Silking Interval, TL: Tassel Length, SL: Spike Length, SpL: Spikelet Length, AL: Anther Length, TPC: Total pollen grains per anther, PST: *per cent* Pollen grain sterility

Effect of moisture stress on quantitative traits

It has been observed that moisture stress had significant effect on all the quantitative traits. The plant height, tassel length, spike length, spikelet length, anther length and total pollen grains per anther recorded significantly lower values in both stressed treatments as compared to well-watered treatment (control) (Table 6). An increase in the mean value of days to tasselling, days to silking, days to anthesis, anthesis silking interval and pollen sterility was observed under stress treatments compared to well-irrigated treatment. Thus, there was a negative impact of drought on flowering and growth-related traits (Figure 1).

Table 6: Mean performance of 8 different maize inbred lines across 3 treatments for quantitative traits

Inbred line	PH (cm)	DT (days)	DS (days)	DA (days)	ASI (days)	TL (cm)	SL (cm)	SpL (mm)	AL (mm)	TPC	PST (%)
UASBM22	147.33	56.44	58.45	59.707	1.56	21.99	18.06	5.16	3.21	1625.00	43.57
UASBM06	218.94	68.45	69.89	70.67	1.89	20.59	11.94	6.27	4.47	1178.24	31.50
UASBM13	98.67	54.33	57.22	67.17	10.17	12.61	10.45	4.88	3.18	985.27	57.40
UASBM14	151.94	64.00	71.11	68.78	2.33	22.39	15.03	5.52	3.59	1136.57	28.74
UASBM02	152.56	70.89	77.67	76.00	3.67	18.46	10.72	5.78	3.75	1197.03	34.50
UASBM10	200.27	68.33	74.78	72.44	2.55	21.13	12.78	6.41	5.06	1180.55	28.40
UASBM09	160.08	71.33	75.56	73.11	2.44	18.76	11.83	5.84	4.54	1242.83	29.97

UASBM11	143.25	65.11	65.00	71.55	3.55	18.37	12.60	5.16	2.75	1424.63	53.09
CD@ 5%	14.48	1.86	2.13	2.31	1.94	3.33	2.63	0.44	0.30	249.90	8.88
CD@1%	19.32	2.48	2.84	3.08	2.59	4.44	3.50	0.58	0.40	333.36	11.85
Control	193.59	59.83	64.21	64.29	0.00	26.63	17.78	6.48	4.10	1509.86	26.63
Stress I	154.31	66.29	70.00	71.54	1.54	15.24	9.78	5.18	3.63	1113.66	43.55
Stress II	129.49	68.46	71.92	75.00	3.08	15.99	11.21	5.22	3.73	1115.28	45.07
CD@ 5%	8.87	1.14	1.30	1.41	1.19	2.04	1.60	0.27	0.19	153.03	5.44
CD@1%	11.8	1.52	1.74	1.88	1.59	2.72	2.14	0.36	0.25	204.14	7.26

PH: Plant height at maturity, DT: Days to Tasselling, DS: Days to Silking, DA: Day to Anthesis, ASI: Anthesis Silking Interval, TL: Tassel Length, SL: Spike Length, SpL: Spikelet Length, AL: Anther Length, TPC: Total pollen grains per anther, PST: *per cent* Pollen grain sterility

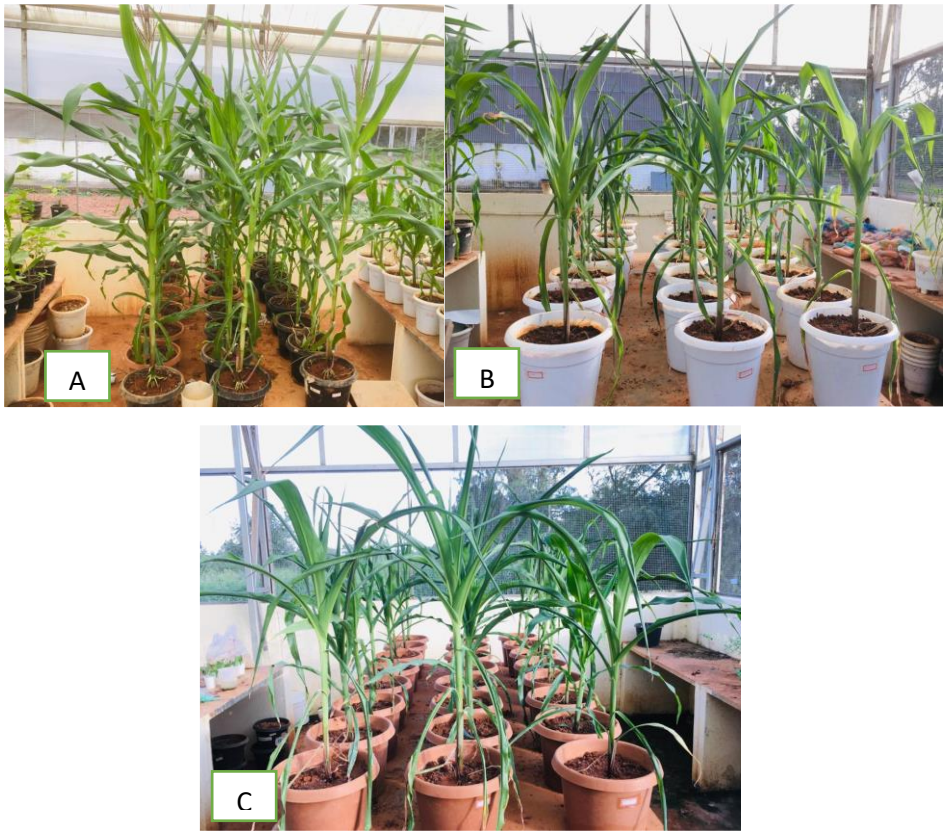


Fig 1: Severe leaf wilting and leaf rolling in different maize inbred lines under moisture stress when compared to control
A: Control B: Stress I treatment B: Stress II treatment

The primary objective of the study was to determine the effect of early reproductive stage stress on quality and quantity of pollen grains produced and pollen sterility under moisture stress during early reproductive stage. Pollen quality can be estimated on the basis of vigour and fertility.

The number of pollen grains produced per anther was measured from the anthers collected from I and IV primary tassel branch positions on the tassel. Reduction in total number of pollen grains produced per anther was observed in moisture stressed plants as compared to well-watered plants. The mean total number of pollen grains produced per anther was 1113.66, 1115.28 and 1509.86 respectively in stress I, stress II, and well-watered treatments. Similarly, increased pollen grain sterility was observed under water-stressed treatments as compared to well-watered treatment (Figure 2). The mean *per cent* pollen grain sterility was 43.55, 45.07, and 26.63 *per cent* in stress I, stress II and well-watered treatments respectively. The mean number of pollen grains produced per anther decreased significantly in both stress treatments and it was observed from the results that both stress treatments had the same effect on the mean number of pollen grains produced per anther. Similarly, the mean pollen sterility significantly increased in both stress treatments and it was observed that stress treatment II had considerably higher pollen sterility as compared to stress treatment I. The result is in accordance with Meghana and Ravikumar (2018) who observed a significant (P<0.001%) reduction in a number of pollen grains per anther under moisture stress in the maize F_2 population. Water-deficit stress causes developmental defects in the tapetum and a lack of starch accumulation in pollen grains leading to pollen sterility (Saini *et al*., 1984; Nguyen *et al*., 2009; Ji *et al*., 2010). Pollen sterility could be due to decreased accumulation of starch in pollen grains which is attributed to the decreased activity of vacuolar and cell wall invertases and other enzymes involved in carbohydrate metabolism.

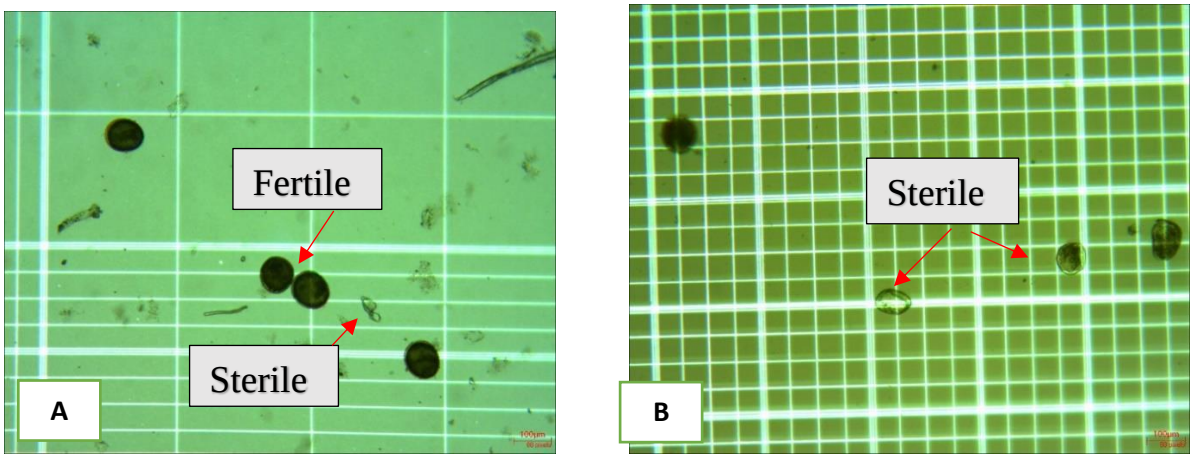


Figure 2: Higher frequency of sterile and irregular shaped pollen grains in the maize inbred line under moisture stress as compared to those in well-watered condition

A: Higher frequency of fertile pollen grains in well-watered treatment

B: Higher frequency of sterile pollen grains in moisture stress treatment

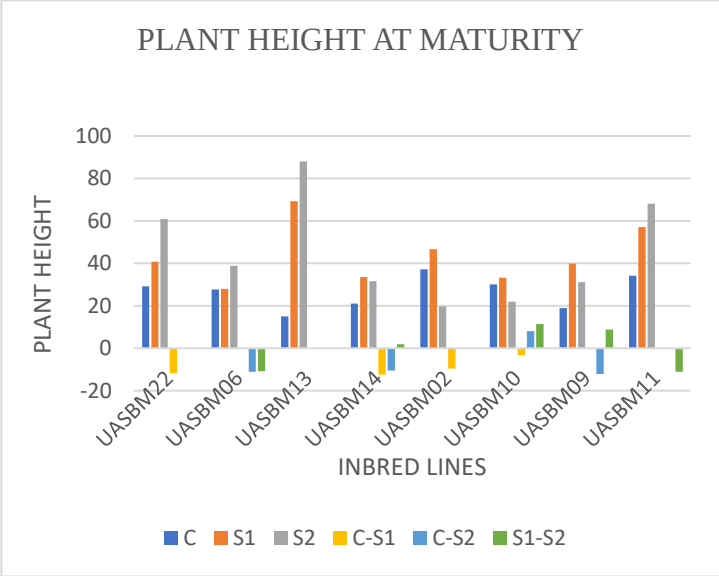
Differential response of inbred lines to moisture stress

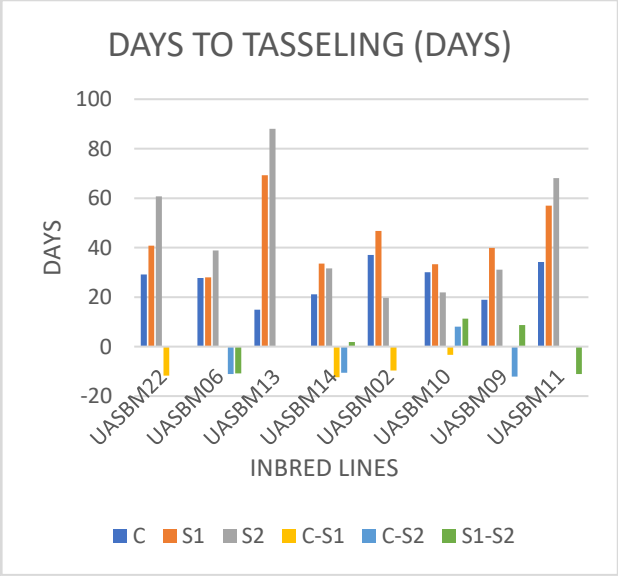
The mean performance of different inbred lines across 3 main treatments for quantitative traits showed significant variation for plant height, days to tasselling, days to silking, days to anthesis, anthesis silking interval, tassel length, spike length, spikelet length, anther length, total pollen grains per anther and *per cent* pollen grain sterility (Table 2). The significant differences among the genotypes for different traits studied show that the maize genotypes have diverse genetic backgrounds and variation among inbred lines for all the traits can be exploited for specific purposes in breeding programs.

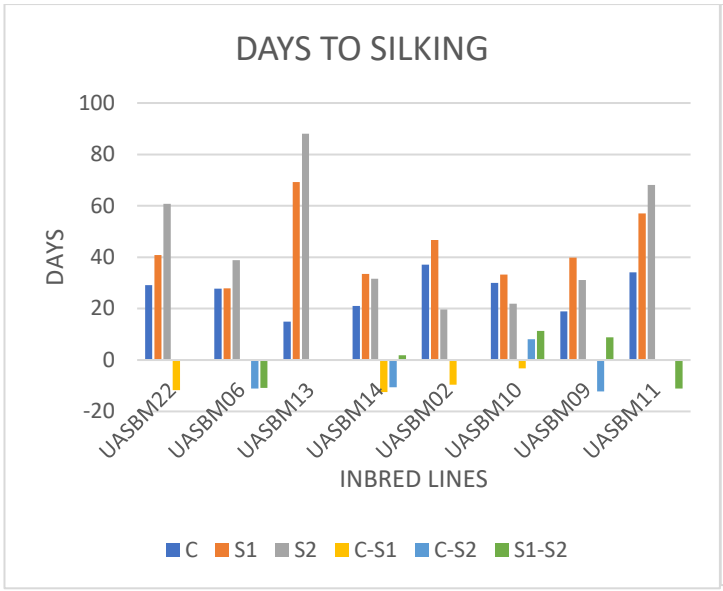
Differential effect of moisture stress treatments on maize inbred lines

The effect of moisture stress was not uniform across all the eight inbred lines under study (graph 1).

Graph 1: Mean performance of different maize inbred lines for quantitative traits in control and water-stressed treatments



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UASBM22
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UASBM13
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INBRED LINES
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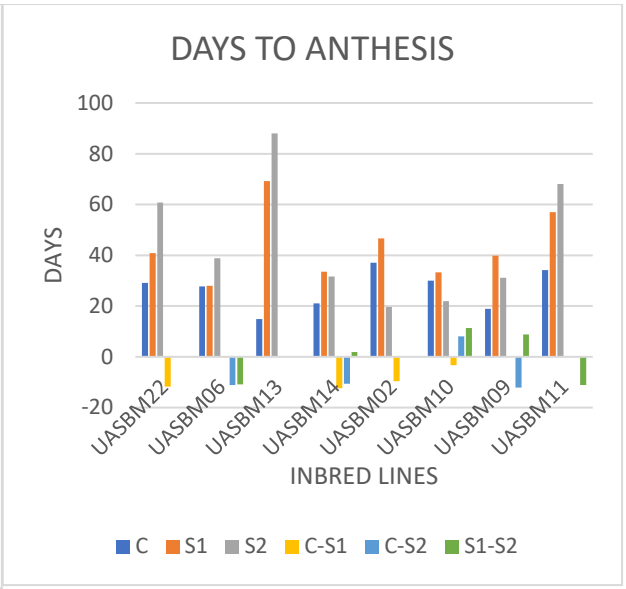
S1-S2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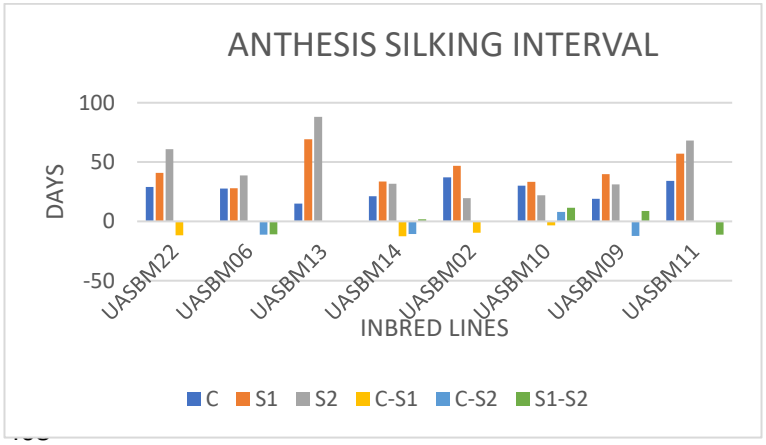
C-S1
C-S2
S1-S2

407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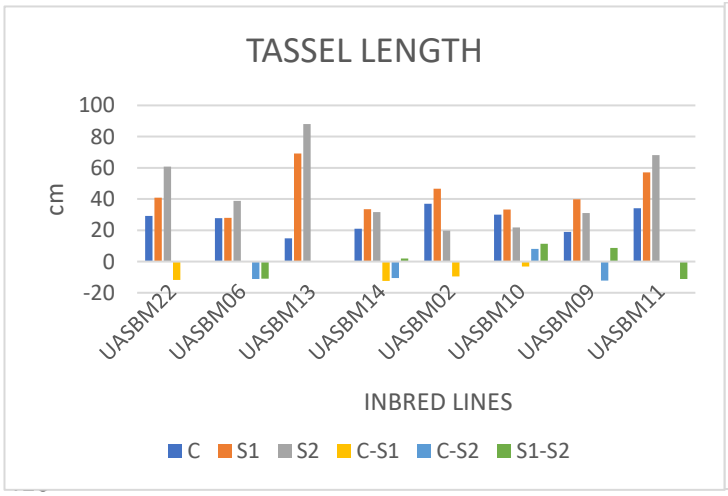
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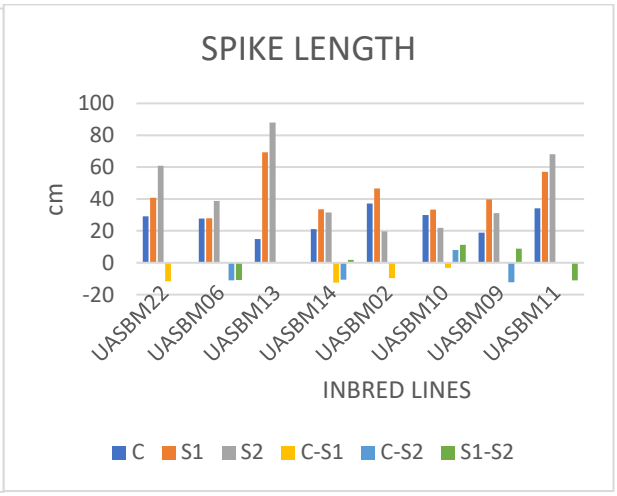
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UASBM02
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INBRED LINES
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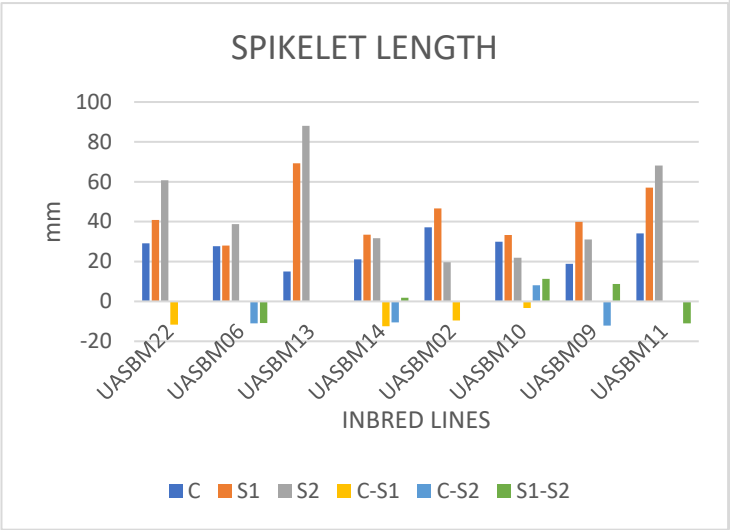
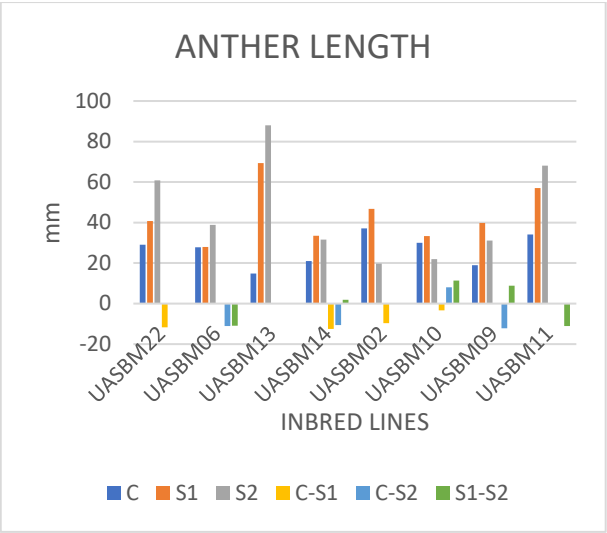
C-S2
S1-S2

411





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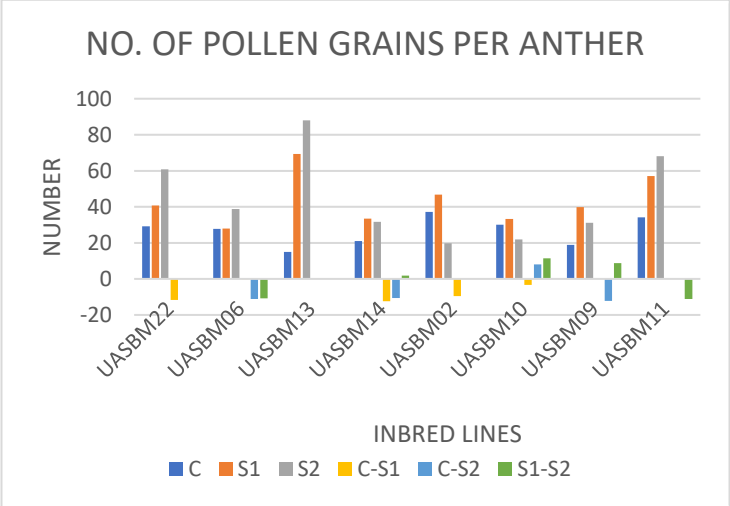

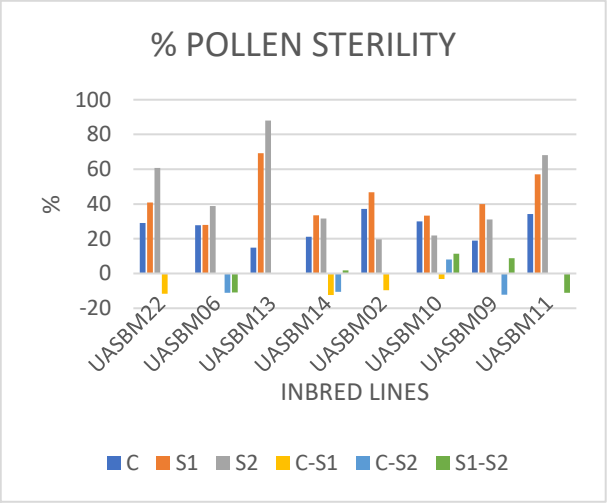

415

416 **Evaluation of methylation-sensitive random amplification polymorphism (MS-RAPD) in**
417 **the stressed leaves and immature anthers of contrasting maize inbred lines**

418

419 MS-RAPD technique was applied to investigate the variation in DNA methylation pattern
420 in the leaves and immature anthers of contrasting maize inbred lines in response to drought stress
421 during the early reproductive stage. The contrasting inbred lines viz., UASBM06 (tolerant) and
422 UASBM13 (susceptible) for pollen quantity and sterility under moisture stress were selected for
423 the study. The *per cent* pollen sterility of UASBM06 and UASBM13 under stress treatment I was
424 0.20 and 54.36 *per cent* respectively while in stress treatment II it was 11.07 and 73.08 *per cent*
425 respectively. The total number of pollen grains per anther in UASBM06 and UASBM13 under
426 stress I. treatment was 1055.56 and 753.65 respectively while in stress II treatment it was 1291.67

and 896.61 respectively. Hence, UASBM06 was considered as drought tolerant and UASBM13 was considered as drought susceptible inbred line for the present study.

Detection and quantification of DNA methylation variation in leaf and immature anthers

Cytosine methylation patterns in the immature anthers and leaf of moisture-stressed and control plants of UASBM06 and UASBM13 maize inbred lines were assessed by MS-RAPD. Using 20 MS-RAPD primers, a total of 96 and 114 bands were revealed in anthers of UASBM06 and UASBM13 respectively and a total of 91 and 115 bands were revealed in leaves of UASBM06 and UASBM13 respectively. The banding patterns of anther and leaf of both the genotypes under control and moisture stressed conditions were compared for identifying changes in cytosine methylation patterns under moisture stress (Plate 1 and 2) (Supplementary Table 2, Table 3). According to the presence or absence of the bands from specific isoschizomer digestions (Schulz *et al*., 2013) the amplified DNA fragments could be divided into four types: type I represents the band presence for both enzyme combinations; type II is the band presence only for *MspI*; type III is the band presence for *HpaII*; and type IV represents the band absence for both enzyme combinations. In the present study, type II and type III represent cases of hemi-methylated bands while type IV represents fully methylation bands.

Comparative DNA methylation analysis of maize leaves and immature anthers under drought conditions revealed that leaves and immature anthers may differ greatly in the level and pattern of DNA methylation, with more changes occurring in the leaves than in the immature anthers. When measured by the total number of polymorphic bands and percentage of total methylated bands (type II+ type III+ type IV), DNA methylation level of UASBM06 ranged from 56.58 *per cent* (48 bands) to 72.24 *per cent* (68 bands) in leaves and from 57.07 *per cent* (54 bands) to 66.33 *per cent* (64 bands) in anthers under control and water deficit treatments respectively. On the other hand, DNA methylation level of UASBM13 ranged from 51.87 *per cent* (58 bands) to 61.35 *per cent* (76 bands) in leaves and from 48.64 *per cent* (58 bands) to 43.70 *per cent* (52 bands) in anthers under the control and water deficit treatments respectively (Table 7). The results obtained in the present study are found consistent with previous reports showing that drought could induce changes in DNA methylation/demethylation across the plant genome in species such as rice (Wang *et al*., 2011; Sapna *et al*., 2020). Among these methylated loci, fully methylated loci were more common than hemi methylated loci in stress conditions in both anthers and leaves except UASBM06 anther where external methylation was more common. It was observed from the methylation pattern that fully methylated loci were more in anther of UASBM06 (24.49 *per cent*) than in UASBM13 (22.68 *per cent*) in stress treatment. Moreover, fully methylated loci increased in case of stress condition in both genotypes. Hemi-methylated

loci (including internal methylation and external methylation) also increased in stress treatment in anther of UASBM06 as compared to UASBM13. The hemi-methylation pattern was calculated during water stress treatment and it was observed that internal methylation decreased in case of UASBM06 anther in stress treatment (9.08 *per cent*) whereas external methylation increased in case of stress treatment (32.75 *per cent*). Similarly, external methylation decreased during stress (14.09 *per cent*) as compared to control treatment (16.83 *per cent*) whereas internal methylation decreased in UASBM13 anther (6.92 *per cent*). It was observed that the demethylation percentage (non-methylation) was more in drought susceptible inbred line, UASBM13 (54.30 *per cent*) compared to drought tolerant inbred line UASBM06 (33.67 *per cent*). Moreover, demethylation percentage was found to increase in stress treatment in drought susceptible inbred line (UASBM13) whereas it decreased in case of drought tolerant inbred line (UASBM06) (Table 8).

Table 7: Total Methylation *per cent* in control and stress treatments in anther and leaf of maize inbred line UASBM06 and UASBM13

Sample	**UASBM 06**			**UASBM13**		
	Control	**Stress**	**% Change**	**Control**	**Stress**	**% Change**
Anther	57.07	66.33	+16.22	48.64	43.70	-10.15
Leaf	56.58	72.24	+27.67	51.87	61.35	+18.27

Table 8: Average methylation pattern in the anther of maize inbred line, UASBM06 and UASBM13 under control and stress treatments

Sl.no.	**Inbred line**	**Type 1: *per cent* nonmethylation**		**Type 2: *per cent* Internal methylation**		**Type 3: *per cent* External methylation**		**Type 4: *per cent* Full methylation**	
		(1,1)		**(1,0)**		**(0,1)**		**(0,0)**	
		C	**S**	**C**	**S**	**C**	**S**	**C**	**S**
1	**UASBM06**	43.83	33.67	18.16	9.08	22.92	32.75	16.00	24.50
2	**UASBM13**	51.36	54.30	12.11	6.92	16.83	14.09	19.69	22.68

Similarly, it was observed in the leaves that fully methylated loci were more in the leaves of UASBM06 (39.41 *per cent*) compared to UASBM13 (23.06 *per cent*) under stress condition (Table 13). The external methylation percentage was found to be 16.51 *per cent* in the leaf of UASBM06 while it was 22.70 *per cent* in UASBM13. The internal methylation percentage was observed to be 16.33 *per cent* in leaf of UASBM06 while it was 15.59 *per cent* in UASBM13. However, the external methylation percentage decreased in case of UASBM06 in stress conditions (16.51 *per cent*) whereas it increases in case of UASBM13 (22.70 *per cent*). The internal methylation in case of UASBM06 (16.33 *per cent*) and of UASBM13 (16.51 *per cent*) was almost at par under stress. It was observed that the demethylation percentage (non-methylation) was higher in drought susceptible inbred line, UASBM13 (35.43 *per cent*) compared to drought-tolerant inbred line UASBM06 (26.75 *per cent*) (Table 9). The demethylation percentage was found to decrease in case of stress conditions in both inbred lines.

Table 9: Average methylation pattern in the leaf of maize inbred line, UASBM06 and UASBM13 under control and stress treatments

Sl.no.	Inbred line	Type 1: *per cent* nonmethylation		Type 2: *per cent* Internal methylation		Type 3: *per cent* External methylation		Type 4: *per cent* Full methylation	
		(1,1)		(1,0)		(0,1)		(0,0)	
		C	S	C	S	C	S	C	S
1	UASBM06	46.08	26.75	11.92	16.33	27.58	16.51	17.08	39.41
2	UASBM13	51.34	35.43	17.46	15.59	21.06	22.70	13.36	23.06

Interestingly, the results of differential DNA methylation pattern between the two contrasting maize genotypes revealed that drought stress enhanced the methylation rate in the leaves of tolerant genotype (UASBM06) by 27.67 *per cent* and by 18.27 *per cent* in the susceptible genotype (UASBM13). It was also observed that drought stress increased the methylation rate in the immature anthers of tolerant genotype (UASBM06) by 15.64 *per cent* while it was found that there was a decrease in the methylation rate in the immature anthers of UASBM13 by 10.15 *percent* (Table 7). Drought increased the total DNA methylation level in both leaves and anther in drought tolerant inbred line, UASBM06 whereas only in leaves of drought susceptible inbred line, UASBM13. Leaves and anthers of UASBM06 show more hypermethylation as compared to UASBM13 in moisture stress condition.

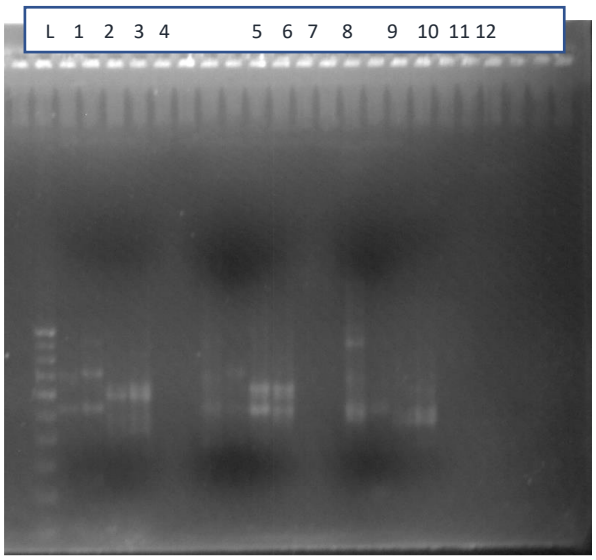

Plate 1: Banding pattern of MS-RAPD in the anther of maize inbred lines, UASBM13 and UASBM06

L:100 bp DNA ladder 1: uncut UASBM13-control 2: uncut UASBM13-stress
3: uncut UASBM06-control 4: uncut UASBM06 -stress 5: *HpaII* digested UASBM13-control
6: *HpaII* digested UASBM13-stress 7: *HpaII* digested UASBM06-control
8: *HpaII* digested UASBM06-stress 9: MspI digested UASBM13-control
10: *MspI* digested UASBM13-stress 11: *MspI* digested UASBM06-control
12: *MspI* digested UASBM06-stress

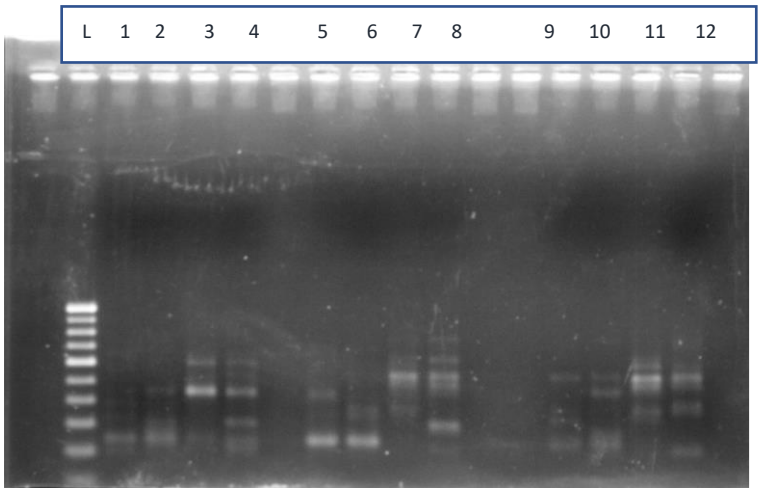

Plate 2: **Banding pattern of MS-RAPD in the leaf of maize inbred lines, UASBM13 and UASBM06**

L:100 bp DNA ladder 1: uncut UASBM13-control 2: uncut UASBM13-stress
3: uncut UASBM06-control 4: uncut UASBM06 -stress 5: *HpaII* digested UASBM13-control
6: *HpaII* digested UASBM13-stress 7: *HpaII* digested UASBM06-control
8: *HpaII* digested UASBM06-stress 9: MspI digested UASBM13-control
10: *MspI* digested UASBM13-stress 11: *MspI* digested UASBM06-control
12: *MspI* digested UASBM06-stress

Discussion

Drought, as a major abiotic stress, causes significant constraints on crop yield (Iqbal *et al*., 2020). Thus, it is the primary focus of breeders to elaborate and develop drought-tolerant varieties. The physiological period at which moisture stress prevails and its extent and extremity period determine the level of moisture stress induced yield reduction in maize. In the present study, 2 stress treatments were given in maize inbred lines, and out of the 2 stress treatments imposed; Besides prolonged days to tasselling, the stress II treatment affected days to silking and anthesis silking interval on tassel length, spike length, primary tassel branch length. In addition, stress II treatment also had higher effect on number of pollen grains per anther and pollen sterility as compared to stress I treatment. Therefore, stress II treatment had a more prominent effect on maize inbred lines. Stress exposure causes meiotic defects or premature microspore abortion in male reproductive organs, leading to male sterility. Thus, stress II treatment will be better than stress I treatment for inducing reproductive stress in maize and to select drought-tolerant genotypes in maize.

Considerable variability was observed in the eight inbred lines for response to drought stress. Based on the combined effect of drought on different traits viz., plant height at maturity, days to anthesis, anthesis silking interval, tassel length, spike length, primary tassel branch length, spikelet length, anther length, number of pollen grains per anther and *per cent* pollen sterility, the inbred lines viz., UASBM06, UASBM14, and UASBM10 were considered as drought tolerant inbred lines and the inbred lines UASBM13, UASBM11, UASBM22, UASBM02, and UASBM09 were considered as drought susceptible. The inbred line UASBM06 was considered as the most drought tolerant lines as it had the same number of total pollen grains per anther as that of control with minimum *per cent* pollen sterility followed by low reduction in other traits under moisture stress conditions. Among all the susceptible lines, UASBM13 was considered as most drought-susceptible line as anthesis did not occur, depicting that it was most severely affected by the drought stress during the early reproductive stage. Conclusively, in the present study UASBM06 was considered as drought tolerant and UASBM13 was considered as drought susceptible inbred line for further analysis. However, there is a need to confirm the tolerance of these inbred lines under field conditions over seasons and locations before using them as parental lines in the development of drought-tolerant hybrids.

It was also observed that drought stress increased the methylation rate in the immature anthers of tolerant genotype (UASBM06) by 15.64 *per cent* while it was found that there was a decrease in the methylation rate in the immature anthers of UASBM13 by 10.15 *per cent*. Drought increased the total DNA methylation level in both leaves and anther in drought tolerant inbred line, UASBM06 whereas only in leaves of drought susceptible inbred line, UASBM13. Leaves and anthers of UASBM06 shows more hypermethylation as compared to UASBM13 in moisture stress condition. Abid *et al*., 2017 noticed that drought stress reduces the methylation level in two faba bean genotypes, irrespective of their tolerance level. Similar results were also found by Liang *et al*., 2014 who reported that DNA methylation increased in *Populus trichocarpa* under drought stress. In some rice genotypes, it has been observed that drought stress increases DNA methylation and only 70 *per cent* of the total changes in DNA methylation reset to the

normal level even after recovery in non-drought conditions (Wang *et al*., 2011). Drought-induced hypermethylation has been found to play a primary and direct role in reducing the metabolic activity in pea root tips after a 72-hour water deficit (Bracale *et al*., 1997; Labra *et al*., 2002). Similarly, Suji and Joel, 2010 reported drought-induced hypermethylation and hypomethylation in drought-tolerant and drought susceptible varieties of rice, respectively which is by the results obtained in the present study where it was found that water stress induces hypermethylation in drought-tolerant inbred lines and hypomethylation in drought susceptible maize inbred lines. Thus, altered methylation in response to drought stress was probably involved in environmental stress acclimation.

Conclusion

We conclude from the above findings that moisture stress had a significant effect on all the quantitative traits viz., plant height, spad chlorophyll meter reading, days to tasselling, days to silking, days to anthesis, anthesis silking interval, tassel length, spike length, primary tassel branch length, spikelet length, anther length, total pollen grains per anther and *per cent* pollen grain sterility. An increase in the mean value of days to tasselling, days to silking, days to anthesis, anthesis silking interval and *per cent* pollen sterility was observed under stress treatments compared to well-irrigated treatment. Thus, there was a negative impact on flowering and growth-related traits.

MS-RAPD technique was applied to investigate the variation in DNA methylation pattern in leaves and immature anthers of contrasting maize inbred lines, UASBM06 (tolerant) and UASBM13 (susceptible) in response to drought stress during the early reproductive stage. Drought increased the total DNA methylation level in both leaves and anthers in drought tolerant inbred line, UASBM06 whereas only in leaves of drought susceptible inbred line, UASBM13. Leaves and anthers of UASBM06 showed hypermethylation as compared to UASBM13 in moisture-stress conditions. An increase in global DNA methylation will tend to reduce global transcription and therefore, slow the energy consumption of the cell which is required during stress related to environmental challenges. The DNA methylated region-associated genes in drought tolerant line are mainly involved in stress response, programmed cell death, and nutrient reservoir activity, which may contribute to the constitutive drought tolerance (Wang *et al*., 2016). Receptor kinases, secondary messengers, regulatory proteins transcription factors, and transporters function together to sense the stress and take all necessary actions depending upon the plant's sensitivity to the stress. Thus, DNA methylation may cause activation or inactivation of the transcriptional processes for specific genes related to drought tolerance, and hence to improve maize adaptation to drought. However, the correlation between the methylation status of the CpG islands and gene expression needs to be established using contrasting maize genotypes under varied soil moisture regimes.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1: Primer name and sequence of primers used in the present study

Sl. No.	Primer	Sequence (5'-3')
1	FS-5	GGGATCCGGC
2	FS-28	TGGCCCCGGT
3	FR-27	ACGCGCGGGA
4	T7	GGCAGGCTGT
5	X11	GGAGCCTCAG
6	R2	CACAGCTGCC
7	U5	TTGGCGGCCT
8	U10	ACCTCGGCAC
9	U15	ACGGGCCAGT
10	U20	ACAGCCCCCA

11	AT03	GACTGGGAGG
12	AT04	TTGCCTCGCC
13	E1	CCCAAGGTCC
14	W15	ACACCGGAAC
15	OPE01	CCCAAGGTCC
16	OPG02	GGCACTGAGG
17	FS-15	ATCGGCTGGG
18	Y17	GACGTGGTGA
19	OPG14	GGATGAGACC
20	R15	GGACAACCAG

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Supplementary Table 2a: Methylation Pattern in UASBM 13 anther under control and stress condition

Sl no.	Primer	Type1: % Non-methylation		Type3: % Internal methylation		Type3: % External methylation		Type3: % Full methylation	
		(1,1)		(1,0)		(0,1)		(0,0)	
		C	S	C	S	C	S	C	S
1	FS-5	37.5	62.5	12.5	12.5	0	12.5	50	12.5
2	FS-28	57.14	57.14	0	0	28.57	0	14.29	42.86
3	FR-27	71.42	42.86	0	0	0	57.14	28.58	0
4	T7	66.67	83.33	0	0	33.33	16.67	0	0
5	X11	75	50	25	50	0	0	0	0
6	R2	57.14	42.85	14.28	0	14.28	42.85	14.28	14.28
7	U5	60	60	0	0	0	0	40	40
8	U10	80	80	0	0	0	0	20	20
9	U15	57.14	85.72	42.86	14.28	0	0	0	0
10	U20	25	50	50	25	25	0	0	25
11	AT03	28.57	85.71	14.28	0	42.85	14.28	14.28	0
12	AT04	75	75	0	0	25	25	0	0
13	E1	0	50	83.33	16.67	0	0	16.67	33.33
14	W15	0	14.28	0	0	14.28	0	85.71	85.71
15	OPE01	50	66.67	0	0	0	33.33	50	0
16	OPG02	60	20	0	20	20	0	20	60
17	FS-15	20	0	0	0	60	60	20	40
18	Y17	60	0	0	0	40	0	0	60
19	OPG14	66.67	100	0	0	33.33	0	0	0
20	R15	80	60	0	0	0	20	20	20
Average		51.36	54.30	12.11	6.92	16.83	14.09	19.69	22.68

Supplementary Table 2b: Methylation Pattern in UASBM 06 anther under control and stress condition

Sl no.	Primer	Type1: % Non-methylation (1,1)		Type2: % Internal methylation (1,0)		Type3: % External methylation (0,1)		Type3: % Full methylation (0,0)	
		C	S	C	S	C	S	C	S
1	FS-5	50.00	50.00	16.67	16.67	33.33	16.67	0	16.67
2	FS-28	20.00	20.00	40.00	0	0	40.00	40.00	40.00
3	FR-27	16.67	16.67	50.00	16.67	33.33	33.33	0	33.33
4	T7	25.00	25.00	50.00	25.00	25.00	25.00	0	25.00
5	X11	33.33	0	33.33	0	33.33	66.67	0	33.33
6	R2	33.33	50.00	33.33	16.67	0	0	33.33	33.33
7	U5	50.00	50.00	25.00	0	0	25.00	25.00	25.00
8	U10	66.67	66.67	0	16.67	16.67	16.67	16.67	0
9	U15	50.00	50.00	16.67	16.67	33.33	33.33	0	0
10	U20	40.00	40.00	0	0	20.00	40.00	40.00	20.00
11	AT03	40.00	20.00	0	20.00	40.00	40.00	20.00	20.00
12	AT04	50.00	25.00	0	0	25.00	25.00	25.00	50.00
13	E1	50.00	25.00	0	0	0	25.00	50.00	50.00
14	W15	66.67	33.33	0	0	0	33.33	33.33	33.33
15	OPE01	75.00	25.00	0	0	25.00	25.00	0	50.00
16	OPG02	40.00	60.00		20.00	60.00	0	0	20.00
17	FS-15	60.00	20.00	0	0	40.00	60.00	0	20.00
18	Y17	50.00	16.67	0	33.33	33.33	50.00	16.67	0
19	OPG14	20.00	20.00	20.00	0	40.00	60.00	20.00	20.00
20	R15	40.00	60.00	60.00	0	0	40.00	0	0
Average		43.83	33.67	18.16	9.08	22.92	32.75	16.00	24.50

Supplementary Table 3a: Methylation Pattern in UASBM 13 leaf under control and stress condition

Sl no.	Primer	Type1: % Non-methylation (1,1)		Type2: % Internal methylation (1,0)		Type3: % External methylation (0,1)		Type3: % Full methylation (0,0)	
		C	S	C	S	C	S	C	S
1	FS-5	75	50	0	0	25	25	0	25
2	FS-28	58	29	14	14	14	43	14	14
3	FR-27	57	43	0	43	43	0	0	14
4	T7	0	80	80	20	0	0	20	0
5	X11	75	50	25	0	50	0	0	0
6	R2	100	75	0	0	0	25	0	0
7	U5	67	67	0	0	33	33	0	0
8	U10	75	75	25	25	0	0	0	0
9	U15	66	50	17	33	0	0	17	17
10	U20	33	33	67	67	0	0	0	0
11	AT03	57	14	14	0	29	57	0	29
12	AT04	100	25	0	0	0	75	0	0
13	E1	23	11	33	11	33	0	11	78
14	W15	33	0	0	0	11	56	56	44
15	OPE01	12.5	12.5	0	25	25	12.5	62.5	50
16	OPG02	42.86	0	0	57.14	71.43	0	0	28.57
17	FS-15	37.5	37.5	12.5	0	25	37.5	25	25
18	Y17	50	16.67	16.67	16.67	16.67	50	16.67	16.67
19	OPG14	40	40	20	0	20	40	20	20
20	R15	25	0	25	0	25	0	25	100
Average		51.34	35.43	17.46	15.59	21.06	22.70	13.36	23.06

Supplementary Table 3b: Methylation Pattern in UASBM 06 leaf under control and stress condition

Sl no.	Primer	Type1: % Non-methylation (1,1)		Type2: % Internal methylation (1,0)		Type3: % External methylation (0,1)		Type3: % Full methylation (0,0)	
		C	S	C	S	C	S	C	S
1	FS-5	100	100	0	0	0	0	0	0
2	FS-28	16.67	0	50	33.33	33.33	0	0	66.67
3	FR-27	50	75	25	0	25	25	0	0
4	T7	80	80	0	20	20	0	0	0
5	X11	50	25	0	0	25	50	25	25
6	R2	66.67	0	16.67	0	0	33.33	16.67	66.67
7	U5	33.33	33.33	33.33	33.33	0	33.33	33.33	0
8	U10	66.67	66.67	33.33	16.67	0	0	0	16.67
9	U15	75	0	0	25	25	0	0	75
10	U20	20	0	60	0	20	60	20	20
11	AT03	40	0	0	60	60	0	0	40
12	AT04	40	20	20	20	40	0	0	60
13	E1	75	25	0	25	0	25	25	25
14	W15	40	20	0	40	20	0	40	40
15	OPE01	33.33	0	0	33.33	33.33	0	66.67	66.67
16	OPG02	50	50	0	0	0	0	50	50
17	FS-15	60	40	0	20	0	0	40	40
18	Y17	25	0	0	0	50	75	25	25
19	OPG14	0	0	0	0	100	0	0	100
20	R15	0	0	0	0	100	28.57	0	71.42
Average		46.08	26.75	11.92	16.33	27.58	16.51	17.08	39.41