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EPIGENETIC ROLE OF DNA METHYLATION IN HYBRID VIGOR OF CYTOPLASMIC MALE STERILITY IN SUNFLOWER

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Abstract

The molecular epigenetic relationship between DNA methylation and hybrid vigor using different sources of cytoplasmic male sterility of sunflower (six A-lines, three R-lines, and their 18 F1's) has been investigated. Molecular analysis of DNA methylation using methylation-sensitive amplification polymorphism (MSAP), revealed that the used four primers succeeded in characterizing a total of 36 specific loci, 35 out of these loci found to be methylation-susceptible (MSL). Meanwhile, only 27 loci were polymorphic achieving were 77% polymorphism percentage of 77%. Differently methylated regions (DMRs) indicated a large convergence between A-lines and single hybrid populations, whereby the percentage of unmethylated state reached 23% and 22% in both populations, respectively. The highest percentage of non-methylated state (31%) was detected in R-lines, whereas the hybrids gave the highest percentage of hemimethylated state (13%) against their sterile maternal lines (A-lines) and fertility restorer lines (R-lines) which were 10% and 8%, respectively. A-lines showed the minimum percentage of the internal cytosine methylation (10%), where as, the parental testers and the resulted hybrids gained a slightly higher different percentage (14% and 13%, respectively). The epigenetic distinctness of sterile A-lines was clear as they showed the highest value for full methylation state (57%) against testers and diallel hybrids (46% and 50% respectively). Hybrids like A3XR2 showed a unique methylation pattern to occupy a single cluster. Hybrids with higher hybrid vigor (A2XR1, A4XR2, and A6XR1) gave the highest positive heterosis values for plant yield.

Key words: MSAP, CMS, Hybrid vigor, Epigenetic, DNA methylation.

Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important members of the Asteraceae family. The prestigious role of this crop is mainly based on its high oil content which reached 45%. There is always an urgent need for new genetic resources in any of the cultivated species such as sunflower that will minimize the genetic vulnerability (Yamgar, 2017).

Nevertheless, cropping systems in most parts of the world are highly dependent on a single source of Cytoplasmic Male Sterility, CMS still represents an effective tool in developing new genetic combinations that may widen the genetic base in different plant species and sunflower is on the top list (Gaurishankar, 2007).

Keeping this in view, developing heterotic groups is largely dependent on broadening the genetic gap between the crossed genotypes. In this context, the epigenetic modification can result in serious phenotypic alterations without changing the type or sequence of DNA nitrogen bases (Aydin *et al*., 2016). As the most prominent aspect of such modification, methylation of cytosine residue can control gene expression and chromatin remodelling which in turn regulate plant evolution, genome stability, responds to external factors, adaptation and development (Fulneček & Kovařík, 2014).

Therefore, DNA methylation could be exploited in developing the overall performance of many genotypes (Osabe *et al*., 2014). The MSAP (Methylation Sensitive Amplification Polymorphism) technique is actually a modified protocol of Amplified Fragment Length Polymorphism (AFLP), mainly using HpaII and MspI methylation-sensitive restriction enzymes. Both enzymes targeting the same sequence (3-CCGG-5), but in different sensitivity level to the methylated cytosine (Kanchanaketu *et al*., 2012 and Lauria *et al*., 2017).

The current study aimed to support prevailing theories of hybrid vigor and investigate the possible role of DNA methylation in drawing hybrid vigor of yield and its components traits by using MSAP technique in sunflower CMS inbred lines.

Materials and Methods

Genomic DNA extraction: A field study was conducted for two seasons (spring and autumn, 2016) in the Experimental Station of Field Crops – University of Anbar/ Abu-Ghraib using six male sterile A-lines (A1 to A6) and three fertility restorer R-lines (R1, R2 and R3) of sunflower (*Helianthus annuus* L.) obtained from Seed Test and Certification Board (STCB)/ Ministry of Agriculture. The used genotypes were crossed according to line X tester mating scheme in the first season to be evaluated in the second. DNA was extracted from seven leaf-stage seedlings in Seed Test and Certificate Lab.-Ministry of Agriculture-Baghdad-Iraq with aid of Genomic DNA Mini Kit-Plant (Geneaid Biotech Ltd., South Korea). The Methylation Sensitive Amplification Polymorphism (MSAP) was accomplished at the Advancement Scientific Office (ASCO).

DNA quantity and quality: Nanodrop was used to check the DNA quality, based on reads that ranged between 1.8-2, according to the following formula:

$$Purity\ of\ DNA = O.D_{260} / O.D_{280} = \geq 1.8$$

Then DNA concentration was adjusted to 50 ng/µl final concentration.

Preparation of MSAP enzymes: Three of MSAP specific restriction enzymes, *EcoRI, HpaII* and *MspI* were supplied by Promega company (Promega-Madison, Wisconsin-USA) and the supplier instruction was followed to restrict the DNA samples as follows:

A mixture of restriction enzymes (*MSPI+ EcoRI*) was prepared by adding 1 µl from each enzyme to 2 µl of its corresponding buffer plus 0.2 bovine serum albumin (BSA), and D.D. water was added in a volume of 7.8 µl. In the same way, the restriction enzymatic mixture of *HpaII+ EcoRI* was prepared. Six µL of DNA sample was added to the restriction mixture, then centrifugated for 5 sec., and finally it was incubated at 37 C° for three hours. T4 DNA ligase was prepared by mixing 6 µl of D.D. water with 1 µl of T4 enzyme and 1 µl of T4 buffer plus 1 µl from each, Eco-adapter ('3-CTCGTAGACTGCG TACC-5') and H/M adapter ('3-GATCATGAGTCCTGCT-5'). Samples were incubated overnight at 37 C° in the incubator. A volume of 5 µl of T4 ligase was added to each of the microfuges. The final mixture was diluted in 1:10 ration by adding 10 µl from mixture to 90 µl of TE buffer in a new microfuge tube, and was centrifuged for 5 sec.

Pre- and final amplification steps: The Pre-amplification step was conducted with aid of two specific primers (Eco Pre-amplification primer '3-GACTGCGTACCAATTCA-5' and H/M Pre-amplification primer '3-ATCATGAGT CCTGCTCGG-5') and the following thermal profile was adopted: Initial denaturation and denaturation at 95ºC for 5 min. and 30 sec. respectively, annealing was at 56ºC for 1 min., and extension and final extension was at 72ºC for 1 and 7 min., respectively.

For the final amplification step, four of the specific primers were used (H/M-1/ '3ATCATGAGTCCTGCT CGGTCT5'; H/M-2 '3ATCATGAGTCCTGCTCGGTCG5'; H/M-3, '3ATCATGAGTCCTGCTCGGTCC5' and '3ATCA TGAGTCCTGCTCGGTTC5') and the following thermal profile was subjected: Initial denaturation and denaturation at 95ºC for 5 min. and 30 sec. respectively, annealing was done at 65ºC for 30 sec., and extension was done at 72ºC for 1 min. for 12 cycles. Then denaturation was set at 95ºC for 5 min. and 30 sec. respectively, annealing was at 56ºC for 30 sec., and extension was done at 72ºC for 1 min. for 23 cycles. Final extension was at 60ºC for 30 sec. for one cycle.

Electrophoresis: Agarose gel prepared by adding 2g of 1:1 standard agarose and metaphor agarose to 100 ml of 1XTAE buffer (Sambrook & Russell, 2001). Ten µL were loaded into the sample wells. Electrophoresis was performed at a voltage of 5 volt cm^{-1} till DNA reached the edge of the gel. Agarose gel was exposed to a UV transilluminator, pictured and documented at 340 nm cannon.

Statistical analysis

A matrix was created based on the final PCR results, in which the presence and the absence of DNA fragment were allotted (1) and (0), respectively. Which was later analyzed using R (Version 3.3.2) with MSAP Package (Version 1.1.9).The principal trends (PCoA), Shannon's Index, and cluster analysis of MSAP results were estimated using Euclidean Distance according to the Nearest Neighbor method. Analysis of Molecular Variance was estimated (Excoffier, 2001). Heterobeltiosis was calculated as stated by Laosuwan & Atkins (1997) using the following formula:

$$\text{Heterobeltiosis (H)\%} = \frac{\overline{F1} - \overline{BP}}{\overline{BP}} \times 100$$

Results and Discussion

Molecular analysis of amplified restriction sites: The results of the molecular analysis indicated that the used two restriction enzymes (HpaII and MspI) with the aid of four specific primers succeeded in diagnosing 36 specific loci across the entire genomes of sunflower genotypes (Table 1). Methylation-Susceptible Loci (MSL) reached 35 loci, while only one locus was found to be Non-Methylated (NML). Seventy-two out of the total Methylated Sensitive Loci (MSL) were polymorphic, hence the estimated percentage was of 77%. Shannon's index that reached 0.52, indicated a modest variation in the value of MSL sites. These results were in agreement with those of Hladni *et al*., (2018).

The primers differed in the number of restricted loci that had been amplified across the studied sunflower genomes (Table 1). The first primer H/M-1 had amplified 9 fragments (Fig. 1), all of which were MSL. Six out of these fragments characterized as polymorphic witha percentage of 67%. The second primer H/M-2 (Fig. 1) was more efficient than the first primer as it succeeded in amplifying 11 restriction loci (Table 1), all of which were methylation-susceptible. The total number of polymorphic methylation-susceptible loci was 8, whereby the polymorphism percentage reached 71%. The third primer H/M-3 (Fig. 2) succeeded in amplifying 8 restriction loci, seven (7) of which were methylation-susceptible loci (Table 1). All the detected MSL were different from one genotype to another, hence the polymorphism percentage was 100%. The fourth primer H/M-4 (Fig. 2) produced the same number of restricted loci (8) produced by the previous primer (Table 1), but all were MSL, hence the scored polymorphism was 75%. The four used primers showed different ability to distinguish between the studied genotypes and the second primer H/M-2 was in the lead with unique fragmentation pattern and the highest number of fragments was 11.

Analysis of methylation status: According to MSAP technique, cytosine methylation can occur in 4 different patterns. These states referred to the external, internal, double and single stranded methylation. The difference was detected between the parental lines (A and R) and their F1's in the differentially methylated regions (DMRs). The comparison between the parental populations against their line x tester hybrids (Table 2) referred to considerable convergence between the A-lines and their hybrids population in the unmethylated status which amounted 23% and 22%, respectively. However, R-lines distinctiveness was comprehensible when it gained a higher percentage of unmethylated status (31%). This

performance was approved by the hemimethylated status that reached its minimum value in the hybrid population (0.08), meanwhile, it was 0.10 and 0.13 in the corresponded parental lines (A- and R-lines, respectively). However, A-lines revealed less Internal cytosine methylation (0.10) compared to the restorer fertility inbreds (R-Lines), (0.14) and F1 hybrids (0.13). Status of full methylation or absence of target approved higher percentage in the A-line genomes (0.57) compared to R-lines (0.46) and F1 hybrids (0.50).

Principal coordinates (PCoA) & cluster analysis: Results of Principal Coordinates Analysis (Fig. 3) confirmed that there was no apparent homogeneity in the pattern of DNA methylation even among individuals that belonged to the same population. Hybridization process had played an efficient role in re-distributing methyl groups along with F1's genomes. Accordingly, hybrids (Pop3) showed the most scattered pattern of DNA methylation compared to the parental populations A-(Pop1) and R-lines (Pop2). Variation at the first coordinate (C1) reached 23.5%, while it was 13.69% at the second coordinate (C2).

In the present investigation, cluster analysis of MSLs results showed that the descended hybrids had no completely distinct epigenetic architecture compared to their parental population, whereby an obvious similarity in their epigenetic attitude was observed.

The entire genotypes separated into three main clusters, the first was composed of a single genotype (A3XR2), while the second cluster had three members A2XR1, A2XR3 and A1XR1. The third main cluster enclosed the rest 23 genotypes, which in turn separated into three subclusters. Inbreds A4 and A5 tended to act in a different epigenetic way compared to the other inbreds (A1, A2, A3 and A6). Also, the three testers belonged to different methylation level, as R3 showed discrete methylation status.

The hybrids scattered in their MSL values and hybrid A3XR2 approved its special pattern of DNA methylation as it occupied single cluster. It was safe to conclude that, the hybrids A4XR2, A6XR1 and A6XR2 were the most epigenetically divergent hybrids and demonstrated a unique methylation status.

Hybrid vigor

Number of seeds per head: The number of seeds per head is one of the important traits that have a large role in determining the final seed yield (Valkova *et al*., 2016). The exposed heterosis varied according to genetic and epigenetic differences existed in the crossed parents. The hybrid vigor was positive and significant for five hybrids ranging from 43.22% to 2.85% for R1×A2 and R2×A5 hybrids, respectively (Table 3). Likewise, heterosis was insignificant negative values and ranged between -53.38% for A5×R3 hybrid and -3.01% for R1×A6 hybrid. These results indicated a clear effect of over-dominance gene action in hybrids with positive heterosis, while hybrids with negative heterosis revealed partial-dominance gene action. This results are in agreement with (Iqbal *et al*., 2018).

The weight of 100 seeds: The grain weight expresses the efficiency of grain filling process, which is one of the major components of the crop (Valkova *et al*., 2018). It can be noted that hybrids were in different heterosis estimates (Table 3). Hybrids A2×R1, A4×R2, and A6× R1 showed positive and significant heterosis values (11.69%, 4.42% and 33.13%, respectively). Hybrid A2×R2 acted in different ways as it showed the lowest negative heterosis (-47.89%). These results were in consistency with what Kanwal *et al*., (2015), Depar *et al*., (2017) and Iqbal *et al*., (2018) stated.

Seeds yield: The heterotic performance of some hybrids against their best parent was insignificant positive and negative values for seed yield trait (Table 3). Hybrids A2×R1, A4×R2, and A6×R1 exhibited a positive value, and A2×R1 was in the lead (59.98%), where as, A6×R1 hybrid expressed the minimum positive value (29.06%). These hybrids had a distinct methylation pattern, that widened the genetic gap and created more opportunities to produce hybrid vigor. On the other side, significant negative values ranged between -68.54% for hybrid A1×R2 and -3.59% for hybrid A5×R1. In addition to the epigenetic role, such results indicated the presence of varied gene action, between over-dominance in hybrids with positive heterosis values and partial-dominance in hybrids with negative heterosis values. Such results were in accordance with those documented by Kanwal *et al*., (2015), Thakare *et al*., (2016), Depar *et al*., (2017) and Valkova *et al*., (2018).

Table 1. PCR results of the four used MSAP primers.

Primer	Loci/Primer	Loci type		Polymorphic loci		Polymorphism%		Shannon's index	
		MSL	NML	MSL	NML	MSL	NML	MSL	NML
H/M1	9	9	0	6	0	67	0	0.53	---
H/M2	11	11	0	8	0	73	0	0.60	---
H/M3	8	7	1	7	0	100	0	0.45	---
H/M4	8	8	0	6	0	75	0	0.48	---
Total	*36*	*35*	*1*	*27*	*0*	*77*	*0*	*0.52*	---

Table 2. Methylation status of A- and R-lines and their F1 hybrids using MSAP technique in sunflower.

Population	Methylation status %			
	Unmethylated	Hemimethylated	Internal cytosine methylation	Full methylation or absence of target
	HPA+/MSP+	HPA+/MSP-	HPA-/MSP+	HPA-/MSP-
A-Lines	0.23	0.10	0.10	0.57
R-Lines	0.31	0.08	0.14	0.46
F1 Hybrids	0.22	0.13	0.13	0.50

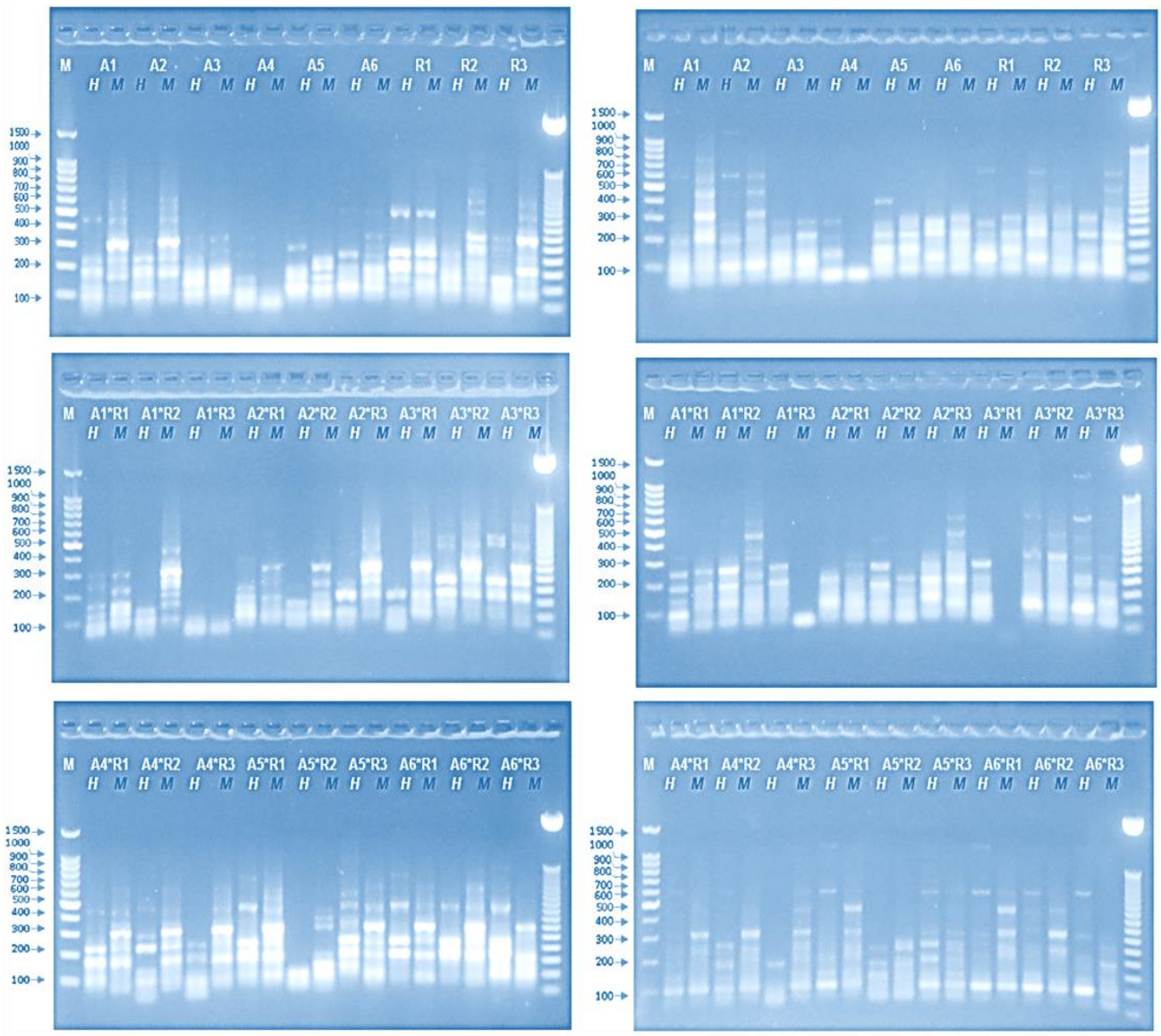

Fig. 1. Restricted loci generated by *MspI* and *HpaII* specific enzymes in the genomes of A-lines (A1 to A6), Testers (R1 to R3) and their F1 hybrids amplified with H/M-1 primer (on the left) and H/M-2 primer (on the right) in MSAP technique in sunflower.

Table 3. Estimated hybrid vigor (%) of the studied traits in CMS sunflower.

Studied traits	Number of seeds per head	Weight of 100 seeds	Seeds yield
A_1 x R_1	-23.46	-8.83	-30.27
A_1 x R_2	-48.6	-39.39	-68.54
A_1 x R_3	-34.33	-39.93	-60.6
A_2 x R_1	43.22	11.69	59.98
A_2 x R_2	-21.85	-47.89	-59.28
A_2 x R_3	16.43	-25.91	-13.61
A_3 x R_1	-3.91	-22.95	-26.09
A_3 x R_2	-29.48	-18.66	-42.53
A_3 x R_3	-42.26	-38.43	-64.5
A_4 x R_1	-5.14	-10.96	-15.61
A_4 x R_2	14.21	4.42	19.35
A_4 x R_3	-22.78	-19.23	-37.64
A_5 x R_1	9.24	-11.76	-3.59
A_5 x R_2	2.85	-17.64	-15.31
A_5 x R_3	-53.38	-9.22	-57.66
A_6 x R_1	-3.01	33.13	29.06
A_6 x R_2	-18.4	-46.19	-56.03
A_6 x R_3	-22.54	-35.77	-50.23

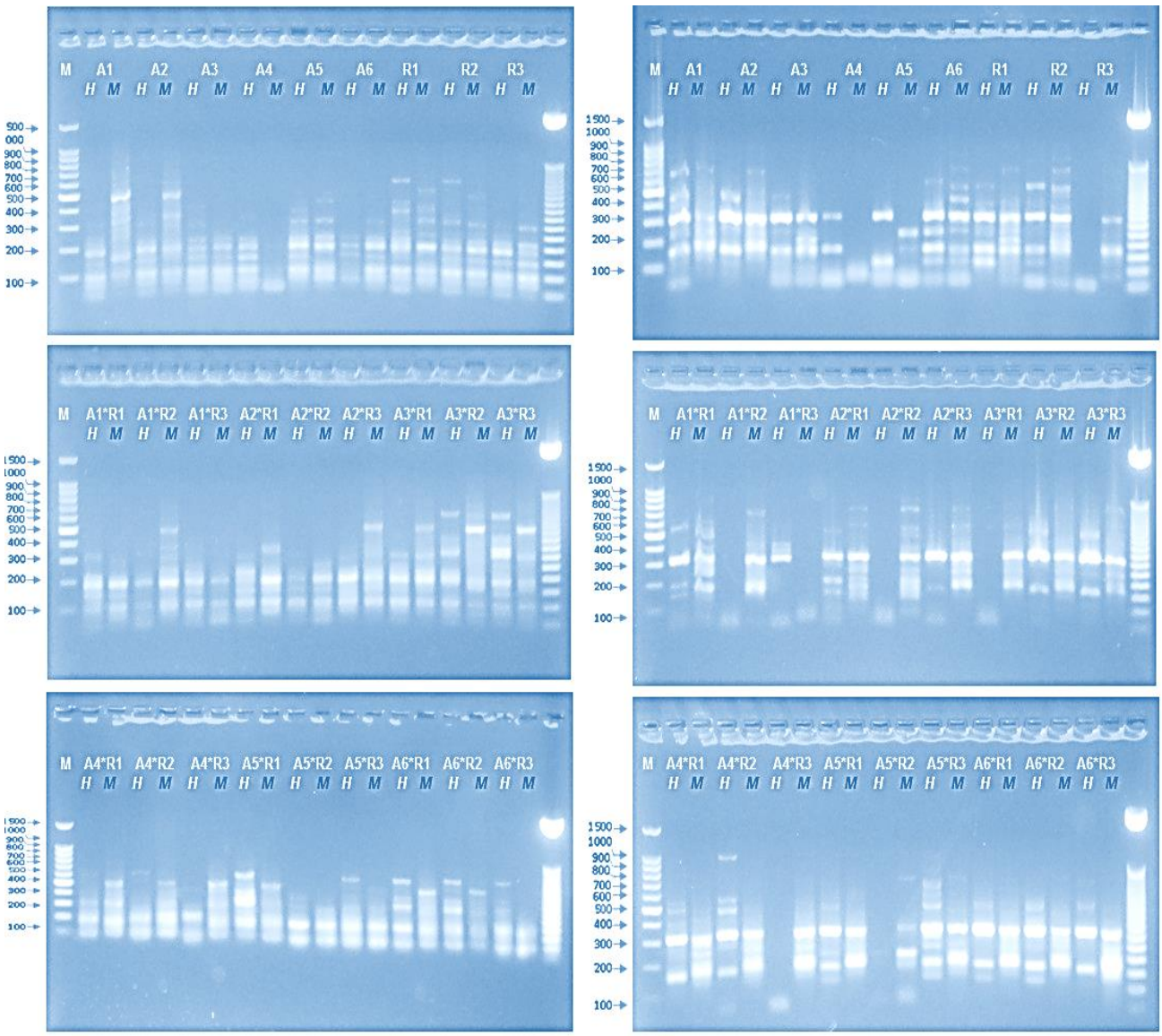

Fig. 2. Restricted loci generated by *MspI* and *HpaII* specific enzymes in the genomes of A-lines (A1 to A6), Testers (R1 to R3) and their F1 hybrids amplified with H/M-3 primer (on the left) and H/M-4 primer (on the right) in MSAP technique in sunflower.

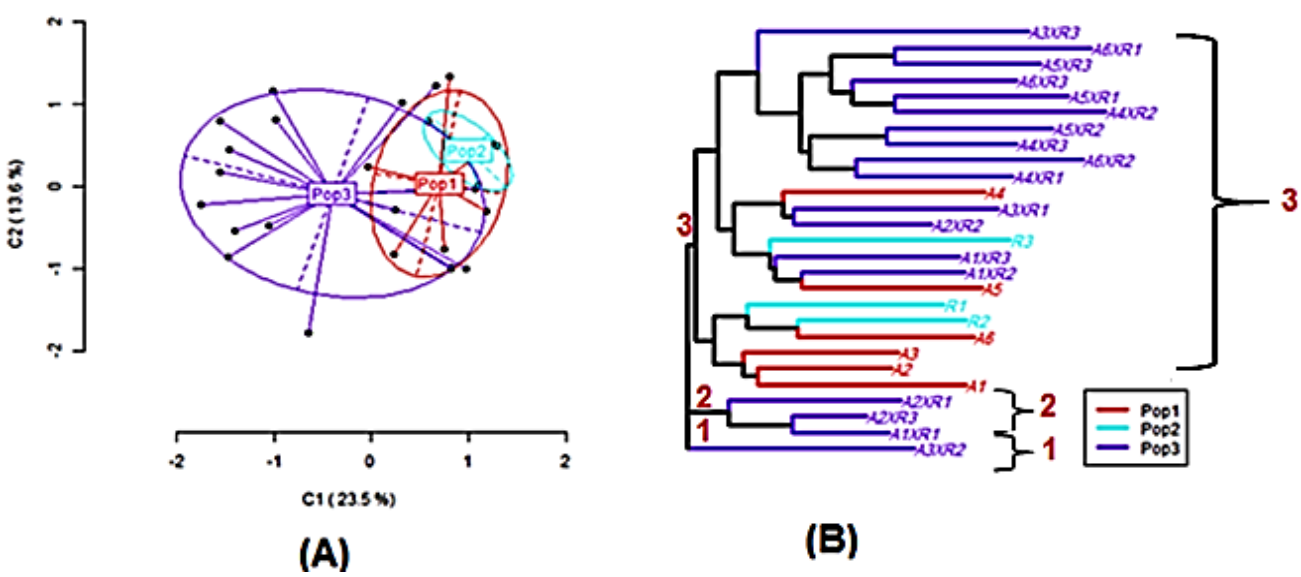

Fig. 3. (A) Principle coordinates analysis (PCoA) and Cluster analysis (B) of MSL results in A-lines (Pop1), R-lines (Pop2) and F1 hybrids (Pop3) of sunflower.

Conclusion

Epigenetic variance in the distribution of methylated cytosine in the genotype's genomes was reflected on the phenotypic traits in these genotypes. Similarly, epigenetic variation increased hybrid vigor as shown by some hybrids, whereby the hybridization process effectively influenced the pattern of DNA methylation and redrawing the total variation. Nevertheless, according to the methylation pattern, parental lines were more heterogeneous than their descended hybrid populations.

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