RESEARCH ARTICLE

ALUMINUM TOXICITY VS SALICYLIC ACID EFFECTS IN PEARL MILLET METHYLOME.

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Manuscript Info	*Abstract*
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Aluminum toxicity is one of most distributed plant abiotic stress in the world, causing root inhibition and therefore crop losses. Plants continuously adapt its defense to abiotic stresses through different mechanisms including DNA methylation. The methylome variation is influenced by external cues from environment or by hormonal signals. Salicylic acid is one of the most important hormones in plants, directing growth and defense. Its application is seen having the capacity to elicit plant defense mechanisms. In this study, the effects of aluminum toxicity and salicylic acid on DNA methylation were carried out in pearl millet using MSAP epigenotyping and ELISA test. The methylation analysis revealed aluminum treatment increased the global and CCGG methylation level whereas application of salicylic acid had reverse effects. Moreover, most of the methylation or demethylation due respectively to aluminum and salicylic acid, occurred at the external cytosine. This finding reveals that salicylic acid could overcome the negative effects of aluminum toxicity through DNA demethylation pathways.

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Introduction:-

Aluminum (Al) is the most abundant metal in the earth's crust, comprising about 7% of its mass. They are widely distributed in tropical and subtropical regions, constituting approximately 30% of the total area of the planet and 50% of the arable lands in the world, as well as providing between 25 and 80% of vegetable production (Sade *et al.,* 2016). It becomes phytotoxic when the soil pH drops below 4 and therefore limits plant production by reducing root growth (Kopittke *et al.,* 2015). The root apex including root cap, meristem, and elongation zone, accumulates more Al and attracts greater physical damage than the mature root tissues (Bennet & Breen, 1991; Ryan *et al.,* 1993). It is implicated in oxidative stress (Zheng & Yang, 2005), and has effects on cell wall, plasma membrane and nutrient unbalances (Schmohl & Horst, 2000), induction of callose (Budikova & Durcekova, 2004), and disturbance of cytoplasmic Ca^{2+} homeostasis (Rengel & Zhang, 2003). Additionally, Al toxicity mostly affects the plasma membrane H^+-ATPase by downregulating its expression (Shen *et al.,* 2005; Chen *et al.,* 2013; Guo *et al.,* 2013; Zhang *et al.,* 2017).

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However, although A1 toxicity has been identified as a problem of acid soils for over 70 years, its effects on epigenetic regulations, especially genomic methylation, remain largely speculative. Auxin, important hormone of root growth is seen as the activator of the plasma membrane H^+-ATPase by phosphorylation during hypocotyl elongation in *Arabidopsis* (Takahashi *et al.,* 2012). Recent study suggested that auxin could be influenced by a salicylic acid-repressing pathway through DNA demethylation mechanism at seedling development (Ngom *et al.,* 2017). Furthermore, application of aluminum increased the methylated loci in *Zea mays* seedlings (Taspinar *et al.,* 2018), whereas application of salicylic acid on *Pennisetum glaucum* seedlings caused DNA demethylation (Ngom *et al.,* 2017).

In this study, the effects of aluminum toxicity and salicylic acid were carried on pearl millet (*P. glaucum*) methylome using methylation-sensitive amplified polymorphism epigenotyping.

Materials and methods:-
Plant materials:-
Four genotypes of pearl millet were used in the study: Souna 3 (PMS3), IBV 8004 (PMI8), Gawane (PMG), and Thialack 2 (PMT2). These are early-flowering photosensitive varieties with a growth cycle of 85–95 days. The seeds were obtained from the Senegalese Agricultural Research Institute of Bambey (Senegal). Seeds were disinfected using 1% of calcium hypochlorite $Ca(ClO)_2$ for 10min and then washed five times with sterile deionized water to remove any remaining chlorine.

Plant growth conditions:-
Murashige and Skoog was used as a medium to sow the pearl millet seeds. The media were supplemented with 0.5mM of salicylic acid and 400µM of aluminum (Table 1). After preparation, the media pH was adjusted to 4 and autoclaved with 121°C for 20min. After autoclaving, the media were cooled down in room temperature during 48hrs to check any traces of contamination. The contaminated media were discarded.

Table 1:-Combined treatments of aluminum and salicylic acid

	Aluminum and salicylic acid applications		
	Control	Al	Al+SA
Aluminum chloride	0 µM	400 µM	400 µM
Salicylic acid	0 mM	0 mM	0.5 mM

15 seeds were sown per medium and for each treatment with three replicates. The seeds were incubated in darkness for 48hrs. The germinated seedlings were kept in a growth chamber set at 8 hrs. of darkness and a 16 hrs. of light and a temperature of 25°C during ten days of development for methylation analysis.

MSAP epigenotyping:-
The DNA from roots were extracted using ZR plant/seed DNA minipreps (Cat No. D6020) following the company extraction. The eluted DNA was subjected to genomic DNA methylation analysis. The extracted DNA was subjected to a Methylation-Sensitive Amplified Polymorphism (MSAP) analysis using two isoschizomers MspI and HpaII targeting the CCGG motifs, and EcoRI targeting GAATTC sites (Al-Lawati *et al.,* 2016). This method is based primarily on Amplified Fragments Length Polymorphism (AFLP) analysis. It uses two different reactions with MspI/EcoRI, and HpaII/EcoRI. The isoschizomers recognize the same DNA site 5'-CCGG-3' with different sensitivity to methylation (Table 2).

Table 2:- Sensitivity of the isoschizomers HpaII and MspI to methylation of CCGG sites

HpaII pattern	MspI pattern	Epigenetic status	Motifs
1	1	Unmethylation	CCGG
1	0	Methylation of external cytosine	mCCGG
0	1	Methylation of internal cytosine	CmCGG
0	0	Mutation/Non-target	-

100 ng DNA samples were digested with NEB EcoRI-10 U at 37°C for 2 hrs., before deactivation by heating at 65°C for 20 min. Then, the digested DNA fragments were subjected to NEB HpaII-10 U and NEB MspI-10 U digestion into two separated series at 37°C overnight. The restriction enzymes were deactivated by heating at 80°C for 15 min. Then, each MSAP series was subjected to ligation reactions (NEB T4 DNA ligase-10 U) with EcoRI adaptors (10 mM) and MspI/HpaII adaptor (10 mM) (Table 3). The ligation mix was incubated overnight at room temperature. Pre-selective amplification was performed in a 50 mL reaction volume with EcoRI primer (10 mM), and MspI/HpaII primer (10 mM) (Table 3), diluted restriction-ligation DNA and One Taq standard buffer.

The pre-selective amplification was realized with the following temperature cycling conditions: one cycle at 94C for 30 s; 30 cycles at 94C for 30 s, 51C for 30 s, and 72C for 60 s, and finally one cycle at 72C for 2 min. A 10-mL aliquot of the pre-selective amplification products was run on a 1.5% agarose gel with a 1 kb DNA ladder to validate the pre-amplification step. Finally, a second amplification was done by selectively amplifying methylated DNA fragments using different primer combinations (Table 3) to generate an MSAP fingerprint. The PCR conditions were as follows: 94°C for 30 s, 12 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s, 23 cycles at 94°C for 30 s, 51°C for 30 s and 72°C for 60 s, and finally one cycle at 72°C for 60 s. The PCR products were then run on a gel, and the MSAP profile was used for data scoring.

Table 3:-Primers and adaptors of MSAP epigenotyping

Epigenotyping steps	Adaptors and Primers	Sequences (5'-3')
Ligation	EcoRI adaptors	CTCGTAGACTGCGTACC
		AATTGGTACGCAGTCTAC
	MspI/HpaII adaptor	CGAGCAGGACTCATGA
Pre-selective amplification	EcoRI primer	GACTGCGTACCAATTC
	MspI/HpaII primer	ATCATGAGTCCTGCTCGG
Selective amplification	EcoRI selective primers	GACTGCGTACCAATTC-AAC
		GACTGCGTACCAATTC-ACC
		GACTGCGTACCAATTC-ACA
		GACTGCGTACCAATTC-AAG
	MspI selective primer	ATCATGAGTCCTGCTCGGTCCA
	HpaII selective primer	ATCATGAGTCCTGCTCGGTCAA

The MSAP profile was then transformed into a binary matrix, with 1 as a presence of loci and 0 the absence of loci (Table 2). Only 50-bp or longer PCR products were considered for analysis. The internal cytosine methylation and the external cytosine methylation (hemimethylation) were considered in this study. The absence of both bands (0, 0) is considered as mutation or non-target regions.

The raw data from the MSAP profile were analyzed using RMSAP 1.1.8 for classification of methylation-susceptible locus or non-methylated locus. The outputs generated by the software were the percentage of methylated and unmethylated fragments and the types of methylation (Perez-Figueroa, 2013).

Global methylation estimation:-
To estimate the relative global cytosine DNA methylation, the enzyme-linked immunosorbent assay technique using an Imprint® Methylated DNA Quantification Kit (MDQ1, Sigma) was used with equal amount of DNA (100ng) following the company instructions.

Nei epigenetic distance:-
Following aluminum and salicylic acid treatments, the Nei genetic distance was determined using DNA methylation data and GenAlex version 6.5 (Peakall & Smouse, 2012) for all pearl millet varieties.

Statistical analysis:-
All statistical analysis was performed using R 3.2.5. Each data set was tested for normality and homogeneity of the variance. The data was analyzed using ANOVA to compare methylation level difference and LSD for mean separation.

# Results:-

CCGG DNA methylation level:-
The methylation analysis was carried following aluminum and salicylic acid treatments. The results showed a significant difference between the treatments for all varieties (P<0.05). The control plants (without aluminum and salicylic acid) had lowest methylation level with plants treated with combined aluminum and salicylic acid. The highest methylation level was found during aluminum treatment while adding of salicylic acid diminished it (Figure 1).
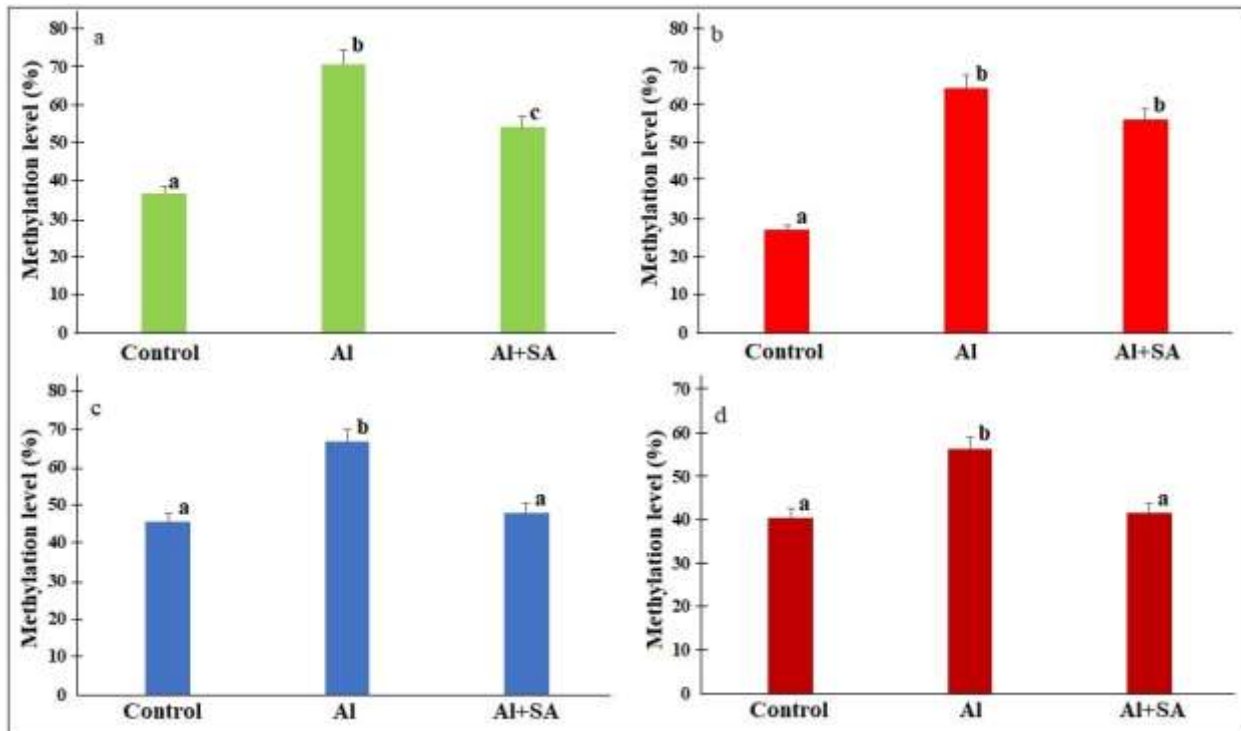
Figure 1:- Methylation level after aluminum and salicylic acid treatments. Highest methylation level found during aluminum application. Al: aluminum, SA: salicylic acid, control: without SA and Al. CCGG: non-methylation, mCCGG: hemimethylation, CmCGG: internal cytosine methylation. a: PMS3; b: PMG; c: PMI8; d: PMT2

CCGG methylation mapping:-
The type and the occurrence of methylation have been determined. The percentage of unmethylated cytosine (CCGG) was very low in all varieties without aluminum and salicylic acid treatments (controls). However, it increased with aluminum and salicylic acid applications. Furthermore, most of the methylation occurred at the external cytosine while the level of methylation was low at the internal cytosine. The hemimethylation was higher during aluminum treatment and decreased a bit after combination of aluminum and salicylic acid (Figure 2).
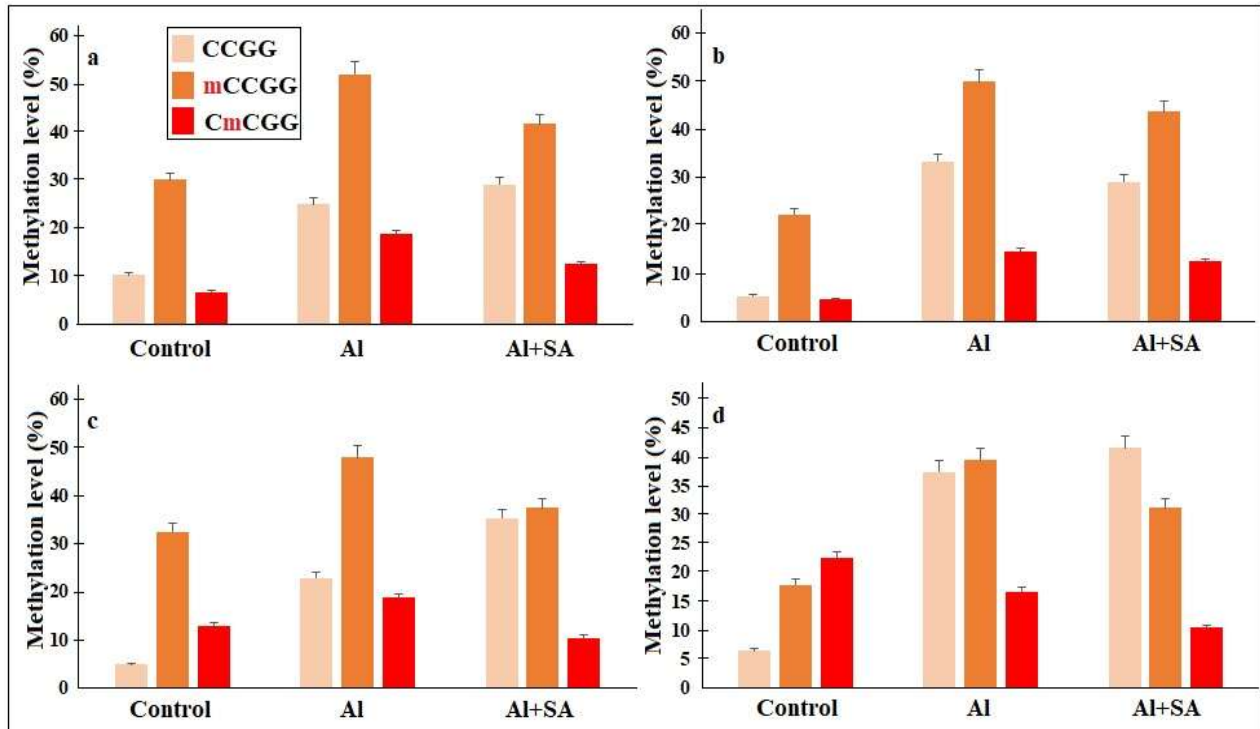
Figure 2:-Types and occurrence of methylation following aluminum and salicylic acid applications. Most of the methylation occurred at external cytosine. Four varieties: a PMS3, b PMG, c PMI8, and d PMT2. Al: aluminum, SA: salicylic acid, control: without Al and SA. CCGG: non-methylation, mCCGG: external cytosine methylation (hemimethylation), and CmCGG: internal cytosine methylation

Global Cytosine DNA methylation:-
The global cytosine DNA methylation was measured after salicylic acid and aluminum application during seedling development. The results showed that aluminum treatment increased the global DNA methylation for all varieties compared to the controls. However, the combination aluminum-salicylic acid decreased the level of DNA methylation even though the changes depended on the variety, PMG being less sensitive to salicylic acid (Figure 3).
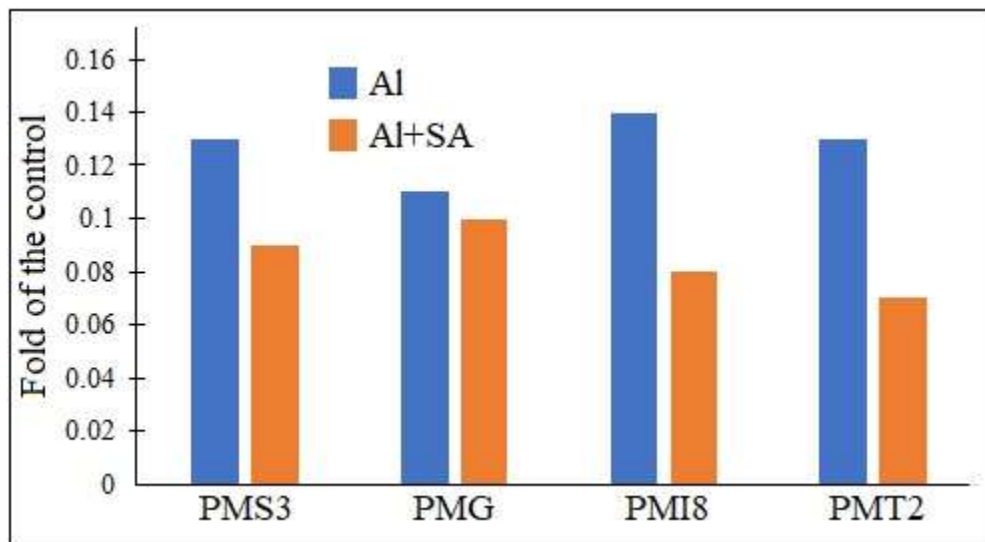
Figure 3:-Global cytosine DNA methylation changes after Al and SA treatments. Aluminum increased the methylation level while SA had reverse effects. Al: aluminum, SA: salicylic acid.

Epigenetic diversity:-
The epigenetic diversity induced by aluminum and salicylic acid treatments was determined with Nei epigenetic distance using GenAlex version 5.3. The results showed the Nei epigenetic distance rate was lower between the control (0mM aluminum) and treated (400µM aluminum) plants. In contrast, the application of salicylic acid (0.5mM) increased the epigenetic variation in all varieties (Figure 4).
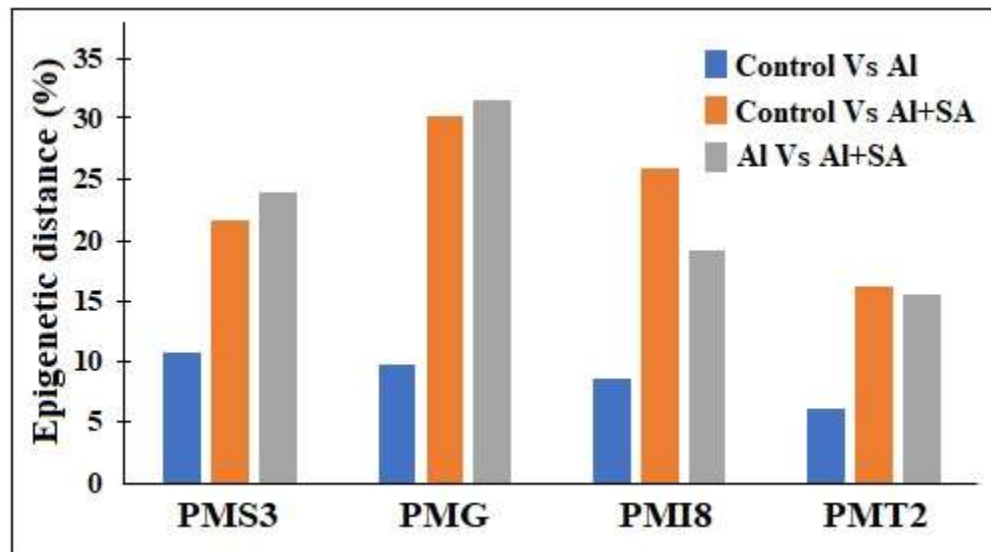
Figure:- Nei epigenetic distance following aluminum stress and application of salicylic acid. Salicylic acid increases the epigenetic variation

Discussion:-

The effects of aluminum toxicity on epigenome were carried out on four pearl millet varieties. The results revealed a significant difference ($P<0.05$) between the controls (without stress) and the plants treated with aluminum and salicylic acid. Indeed, aluminum stress increased the DNA methylation level. Many studies reported similar results in maize (Kimatu *et al.,* 2013; Taspinar *et al.,* 2018) whereas Bednarek *et al.,* (2017) reported contradictory results in Triticale with demethylation occurred during aluminum treatments in CG context.

The application of salicylic acid decreased the genomic methylation level, even though there was genotype-dependence. It seems that salicylic acid plays important role in the pearl millet tolerance to aluminum toxicity through demethylation pathway. Plants usually use two mechanisms to tolerate aluminum exposure. Some exclude aluminum from the root apex and others accumulate them in the root and shoot symplasm (Panda *et al.,* 2009). Moreover, many studies revealed the strong evidence of aluminum tolerant genotypes of wheat, corn, sunflower, soybean and common bean excluding aluminum from root by exertion of organic acids that chelate aluminum (López-Bucio *et al.,* 2000; Watanabe & Osaki, 2002). However, recent studies showed improvement of aluminum tolerance by salicylic acid through the modulation of the reactive oxygen species (Liu *et al.,* 2017). This is accompanied by higher activities of superoxide dismutase, peroxidase, and ascorbate peroxidase, and lower catalase activity, indicating alleviation of aluminum toxicity.

During both aluminum and salicylic acid treatments, the external cytosine methylation varied more compared to the internal cytosine methylation. The external cytosine methylation (hemimethylation) was higher during aluminum, indicating a functional role of the external cytosine. This high occurrence of the hemimethylation during stress could reveal important role of the external cytosine in plant tolerance. In contrast to aluminum, external cytosine demethylation occurred after salicylic acid application, therefore confirming its importance. It could be a key player for identification of de novo methylation events, following changes in the environment or threats. This hemimethylation is very unstable and mainly due to external cues (aluminum and salicylic acid) affecting the methylome pattern. However, the cellular external cytosine DNA methylation and demethylation is far from being explained. Aluminum and salicylic acid could influence the expression of the DOMAINS REARRANGED METHYLTRANSFERASE 2 and DEMETER or Repressor of Silencing 1, respectively.

Furthermore, the Nei genetic analysis revealed an increase of epigenetic distance following salicylic acid and aluminum treatment. This was more noticed in aluminum and salicylic acid combination than aluminum alone. In fact, the diversity of the epigenetic loci seems to be lowered during stress and normal times in all pearl millet varieties. The epigenetic variation is a mechanism of defense and salicylic acid could confer to the plants more diversity by generating epialleles based-stress. This is used today for crop improvement (Zhang & Hsieh, 2013), plant biomass increases and stability in ecosystem (Latzel *et al.,* 2013).

This finding revealed the importance of salicylic acid in plant defense, especially on the methylome variation during stress. This research showed that the key player regulating that variation could be the external cytosine of the CCGG motifs.

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