THE INFLUENCE OF SALICYLIC ACID ON PEARL MILLET (*PENNISETUM GLAUCUM*: *POACEAE*) EPIGENOME DURING HEADMINER (*HELIOCHEILUS ALBIPUNCTELLA*: *LEPIDOPTERA*) INFESTATION AND ALUMINUM TOXICITY STRESSES

BABA NGOM

DOCTOR OF PHILOSOPHY IN MOLECULAR BIOLOGY AND BIOTECHNOLOGY

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MB400-009/15

A thesis submitted to Pan African University Institute of Sciences, Technology and Innovation in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy in Molecular Biology and Biotechnology

April 2018

Declaration

I hereby declare that this is my original work and has not been submitted in any university for the award of degree.

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Dedication

This piece of work is dedicated to my lovely mum, daddy, and wife, Khoudia, Abdoulaye, and Amy respectively. Thank you for your love, support, patience and prayers.

To daddy and mum: You are my world, my inspiration.

To Amy: Thanks for your love and patience my love

To my friends, especially CES3 family: Thank you for your support and advice.

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List of abbreviations and acronyms

5mC: Fifth methyl Cytosine

Al: Aluminum

ARC: Agriculture Research Centre

CCGG: Non-methylation

CmCGG: Internal Cytosine methylation

DAMPs: Damage-Associated Molecular Patterns

DNMTs: DNA Methyltransferases

DRM2: Domains Rearranged Methyltransferases 2

FLS2: Flagellin Sensitive 2

HR: Hypersensitive Response

ICIPE: International Centre of Insect Physiology and Ecology

ICRISAT: International Crop Research Institute of Semi-Arid Tropics

MAMPs: Microbe-Associated Molecular Patterns

mCCGG: External Cytosine methylation

MET1: Methyltransferase 1

MSAP: Methylation Sensitive Amplified Polymorphism

PAMPs: Pathogen-Associated Molecular Patterns

PRR: Pattern Recognition Receptor

RdDM: RNA-directed DNA methylation

ROS1: Repressor Of Silencing 1

SA: Salicylic acid

SABP: Salicylic Acid Binding Protein

SAR: Systemic Acquired Resistance

SARP: Salicylic Acid Repressing Pathway

TEs: Transposable Elements

Abstract

Millets are in the family of cereals grown globally with differential importance across African countries. They are grown in arid and semi-arid areas with a production area of 18.50 million ha by 28 countries covering 30% of the continent. With nine species, pearl millet remains the most important millet crop in term of production and harvested areas in Africa. Pearl millet presents many advantages in responding to the needs and welfare of the poor including food security, nutrition and health, poverty alleviation, potential markets and dry environment enhancement. However, the production faces many constraints in a context of soil degradation and acidity and increase of pest pressure causing yield losses. These include aluminum toxicity and the millet headminer (*Heliocheilus albipunctella*).

Aluminum is widely distributed in tropical and subtropical regions, constituting approximately 30% of the total area of the planet and 50% of the arable land in the world. Aluminum stress is one of abiotic stresses occurring in acidic soils limiting therefore the yield. The main symptom of aluminum toxicity is the inhibition of root growth through plasma membrane damages in the root apex including root cap, meristem, and elongation zone. The millet headminer is one of the most important pest of pearl millet causing important losses about 60% in high infestation during the panicle stage with 73% of incidence. In both stresses, plants respond via cascade of metabolic reactions from signaling to defense compound synthesis through resistance and tolerance genes controlled by epigenetic regulations.

These epigenetic regulations include DNA methylation that is a mechanism of controlling the expression of gene with adding and removal of methyl groups mostly on the fifth cytosine bases through DNA methyltransferases. In plant, “De novo” is the formation of newly methylated patterns, and “maintenance” DNA methyltransferases in which the preexisting methylation patterns

are maintained after DNA replication. These patterns can be changed by external cues including stresses, influencing therefore the resistance gene expression. The external cues may be from biotic release e.g. oral secretion, abiotic elements e.g. Al^{3+} or from internal signals e.g. salicylic acid.

In this study, the aim was to determine the changes of methylation patterns following millet headminer feeding, salicylic acid and aluminum exposure during seedling and panicle development using mainly screening tests and MSAP epigenotyping. The results showed that stresses including *H. albipunctella* and aluminum toxicity increased the DNA methylation level, while application of salicylic acid had reverse effects by decreasing the level. The DNA methylation and demethylation processes occurred mostly at the external cytosine. Moreover, application of salicylic acid inhibited the root growth during seedling development whereas its foliar treatment significantly reduced the larval density during panicle development.

These results demonstrated the importance of salicylic acid in plants. Salicylic acid acts as an elicitor of plant defense mechanism through DNA demethylation pathways during aluminum and millet headminer stresses. That DNA methylation reverse effects caused by salicylic acid during stress could be from the external cytosine of CCGG sites. This may be the key element regulating the defense-related genes during stress. Moreover, increase of DNA methylation following feeding of millet headminer revealed an important strategy of the pest. It seems the oral secretion released by the larvae during feeding could contain suppressors of plant defense mechanisms. This offered new insights on salicylic acid in the epigenetic defense of plants. In a time of climate change, pest pressure increasing and degradation of soils, the use of epigenetic tools like bio-epidesigners having the biological ability to modulate gene expression through the epigenome, could be an important step in overcoming these issues. Additionally, research should be carried out on the secretome of millet headminer that indicates a presence of an unknown bio-epidesigner.

1.0 Chapter 1: Introduction

The regulation of gene expression in time and space is fundamental for normal growth and development in plants. This orchestration of gene expression trajectories is primarily controlled genetically by specific DNA sequences including cis- and trans-acting elements (Bilas *et al.*, 2016). The genetic elements also act either continually or periodically during the different stages of plant development and during environmental threats, producing essential plant enzymes and chemicals for normal growth and defense. However, since the emergence of epigenetics and numerous associated findings, it has been found that epigenetic changes control many genetic functions. By using different mechanisms such as DNA methylation, histone modifications, small RNAs, chromatin modifications, etc., epigenetic changes have the biological power to regulate the expression of genes without any change of DNA sequences.

During plant development, the methylation levels change at the different stages from germination to plant fruit initiation through root growth and leaf formation. In addition, different plant organs do not have the same level of DNA methylation. This indicates the heritable character of epigenetics during meiosis and its deployment during the plant embryogenesis and organogenesis. Similar to plant development, plant stresses change the epigenetic status. In fact, changes have been detected during pest, pathogen or abiotic stresses, suggesting a biological function of epigenetics in plant defense. The DNA methylation level increases during plant stress compared to its normal level during development. These epigenetic changes suggest that plants respond epigenetically to the stressors. During stress, plants deploy many mechanisms from expression of the defense-related genes to production of defense compounds, therefore targeting the bio-invaders. This expression of defense-related genes is usually accompanied by epigenetic regulations including DNA demethylation process in many genes.

Pearl millet presents certain advantages for studying the epigenetics behind crop and yield improvement. Indeed, pearl millet tolerates stress conditions, particularly in arid and semi-arid regions where generally drought stresses occur. In the time of climate change, soil degradation, and, increasing pest and pathogen pressure, pearl millet may remain an excellent biological model to study and to manipulate the gene expression and plant responses. If the DNA sequences cannot be changed to the desired trajectories to control the plant stressors, manipulating the epigenome could provide interesting insight into new crop improvement design and development.

1.1 Background of the study

1.1.1 Pearl millet

Pearl millet is a staple food for many countries in Africa and Asia, especially for the farm households and among the poorest people. Millet is an excellent dietary source of calcium, iron, manganese, and methionine - an amino acid lacking in the diets of hundreds of millions of the poor who live on starchy foods such as cassava, plantain, polished rice, and maize meal. Millet use is diverse, including in cereals (in the form of porridge and kasha), soups, breads and stuffing, fermented beverages, and baby food (ARC, 2006). Furthermore, research into its improvement, nutritional value and use is still new. Crop and production research on pearl millet is being conducted on both the grain and ornamental grass varieties. Millet's resiliency is a reason for its success in arid climates. However, current research efforts are to improve rust resistance (new crop) and other global research addresses resistance to pests and environment (ICIPE, 2002). Some organization such as Agricultural Research Centre (ARC) and (International Crop Research Institute of Semi-Arid Tropics) ICRISAT also conduct research on pearl millet, focusing on pest, environmental stress, weed and disease resistance (ARC, 2006). *Heliocheilus albipunctella* or the

millet headminer represents a major pest of millet, especially in Sahelian regions with incidence rate up to 95% and crop losses of 20% (Gahukar *et al.,* 1986; Bal, 1993).

However, few studies in genetics were performed for pearl millet despite its interesting biology. Recently, the draft genome is promising interesting perspectives in plant breeding and provide resource to improve agronomic traits for food security (Rajeev *et al.,* 2017).

1.1.2 Epigenetic regulations

Epigenetics regulates the expression of genes throughout plant developmental stages and during stresses. It uses different mechanisms including DNA methylation and histone modifications. These do not alter the DNA sequence but play important role between plant and the environmental conditions either positive or negative. These epigenetic mechanisms confer to plants an impressive plasticity to respond to the changing environment by producing plant defense compounds and other chemicals (Kempel *et al.,* 2011). In addition, plants have developed a plethora of memory system under epigenetic control that allow them to adapt to pathogens and pests (Kinoshita & Seki, 2014).

1.1.3 Hormonal signaling under plant development and defense

1.1.3.1 Signaling in plant defense

Salicylic acid, jasmonic acid and ethylene are principal hormones intervening during plant defense. These hormones induce a cascade of metabolic reactions leading to the production of plant defense elements. Jasmonic acid is mainly involved in the production of defenses against insect herbivores Thaler *et al.,* 1996; McConn *et al.,* 1997; Baldwin, 1998; Cipollini & Redman, 1999), whereas SA is involved in plant responses to fungal, bacterial and viral pathogens (Delaney, 1997). However, some recent studies suggest a cross-talk between SA and JA signaling (Kunkel & Brooks, 2002; Spoel *et al.,* 2003; Koornneef & Pieterse, 2008). Moreover, many reports link ethylene to abiotic

stresses such as salinity (Tao *et al.,* 2015), cold (sun *et al.,* 2016), and in drought (Arraes *et al.,* 2015).

1.1.3.2 Signaling in plant development

Hormone signaling plays diverse and critical roles during plant development. Hormone interactions regulate meristem function and therefore control the organogenesis in the plant. Auxin, cytokinin, and brassinosteroid hormones have been recently found to regulate shoot and root apical meristem (Durbak *et al.,* 2012).

The hormonal signals are commonly transmitted through removal of a key transcription factor using an F-box-containing ubiquitin E3 ligase in a hormone-concentration-dependent manner. The transcription factor can be an activator, such as ETHYLENE INSENSITIVE 3 (EIN3) in the ethylene signaling pathway, but more often it is a transcriptional repressor, such as the Aux/IAA, DELLA, and JAZ proteins in the auxin, GA, and JA signaling pathways, respectively. The ubiquitin-related protein degradation machinery targets the transcriptional repressors (Aux/IAA) allowing the auxin response factors to activate a network of genes to regulate plant growth and development (Guilfoyle & Hagen, 2007). Recent reports suggested that the auxin receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1) look like as a ‘‘molecular glue’’ interacting with a complex auxin-Aux/IAA, facilitating the degradation of Aux/IAA proteins (Tan *et al.,* 2007).

1.1.4 Aluminum toxicity

Aluminum is one of the most abundant metals in earth and its toxicity is a major constraint for crop production in acidic soil worldwide. The plant growth, especially the roots are inhibited when the soil pH drops below 4 (phytotoxicity). Although aluminum toxicity has been identified as a problem of acid soils for over 70 years, our knowledge about the chain of events that finally affects

plant growth remains largely speculative (Delhaize & Ryan, 1992). The target of aluminum toxicity is the root tip, in which Al exposure causes inhibition of cell elongation and cell division, leading to root stunting accompanied by reduced water and nutrient uptake (Panda *et al.,* 2009).

1.2 Why research on pearl millet?

Pearl millet, *Pennisetum glaucum*, is widely grown in Sahelian region. It is the most used cereal in rural areas. The cereal is distributed differentially among many African countries; largest producers being in West Africa. Millets are consumed as staple food (78%), drinks and other use (20%). Feed use is still very small (2%). As food, they are nutritionally equivalent or superior to most cereals; containing high levels of methionine, cystine, and other vital amino acids for human health. They are also unique sources of pro-vitamin A (yellow pearl millets) and micronutrients (Zn, Fe and Cu) which are especially high in finger millet (Obilana, 2003).

The plant remains a model to study stresses and climate change. Millets tolerate mostly drought stresses and some diseases. However, *Heliocheilus albipunctella* larvae is the main pest causing damage during panicle stage and reduces yield resulting to economic losses and food insecurity. The damages can reach 85% of production (Payne *et al.,* 2011). Numerous studies have been done to understand the interactions between Pearl millet, *Heliocheilus* larvae and its parasitoids (Payne *et al.,* 2011). Generally, the plant-pest interaction takes place from wounding to the plant responses (Kessler & Baldwin, 2002). Indeed, *H. albipunctella* larvae, as a headminer, feeds on pearl millet grains. This could act and allow Pearl millet defense responses, as other caterpillars (Dudareva, 2006; Louis, 2013).

Today, there are few molecular studies on *P. Glaucum* defense mechanisms. Therefore, the understanding of use and mechanisms of elicitors for resistance gene responses become veritable issues to control the pest (Dilks, 2014). The elicitors may be molecules from pest oral secretion

and can induce a reaction cascade of plant defense against pests or pathogens (Kessler & Baldwin, 2002; Garcia-Brugger *et al.,* 2006; Howe & Jander, 2008). These inducers could permit a preventive control of most damages caused by pests and pathogens preparing the plant ready for prospective pest attacks via different molecular mechanisms such as transgenerational memories that are epigenetic functions. However, in Africa, especially in Sahelian region, there are no pest control methods based on plant defense elicitation. In addition, another important issue in agriculture is metal-based diseases such as aluminum toxicity. Some metals may act as elicitors even though most of them cause abiotic stress. Aluminum ions are reported to be capable to elicit the plant defense system. The level of aluminum producing a plant defense reaction remains uncertain, that's why the aluminum toxicity in plant occurring at low pH is a way of understanding the aluminum effects on plants, especially on the epigenome.

Furthermore, the non-understanding of genes implicating in plant tolerance make it difficult to find and engineer new resistant cultivars to *H. albipunctella* or abiotic tolerance. Most of R genes could work in a pool system that is characterized by several and differential mechanisms and gene products (Xu *et al.,* 1994; Wittstock & Gershenzon, 2002; Lorenzo *et al.,* 2003). Those R genes products imply phytoalexins (Hammerschmidt & Dann, 1999), peroxidases, and volatiles compounds (Pare & Tumlinson 1999). The latter seems important for a biological control, by attracting the natural enemies of *H. albipunctella*. So, how can we engineer a way to cause volatile releases in the agroecosystem, or to engineer new resistant varieties to stresses? This is an important issue for developing integrated pest management (IPM) methods. The plant defense-related genes are affected by hormonal activities. Salicylic acid is seen having that defensive signaling role during stress (War *et al.*, 2011; Park *et al.*, 2016). In plant, a separation between mechanisms intervening in pathogen diseases and in pest defense remains unclear. Indeed,

evidence is growing that there is sharing defense mechanisms and crosstalk. Thus, the role of SA, one of the most important hormones in plant development and defense, could be a key player during pest attacks. Moreover, SA is considered as an elicitor of plant defense (Thakur & Sohal, 2013; Bektas & Eulgem, 2014), affecting some R genes, particularly the PR genes.

The silencing or activation of those genes also remains a critical point for plants during stresses. Epigenetic regulations including cytosine DNA methylation are a way of controlling a gene by adding or removing methyl groups on cytosine bases. Therefore, mapping the whole genome of *P. glaucum* becomes a must to show the degree of methylation during plant development, pest attacks and abiotic stress. In plant, many metabolites and external factors may interact with the epigenome, influencing therefore negatively or positively the gene expression affecting important traits including the productivity. The internal or external cues of plants may have the ability to manipulate the epigenome, and then expressing important proteins for development and defense. In the time of climate change and increase of plant stressors, this would-like manipulators (bio-epidesigner) could help to increase crop production reducing therefore the food insecurity. Salicylic acid, aluminum ions and oral secretion from pest could provide interesting tools for manipulating the epigenome, especially the methylation level in pearl millet. In fact, the levels of methylation are intimately linked to the expression of genes in plants.

1.3 Objectives of the study

Epigenetic mechanisms such as DNA methylation control gene expression of plants, especially during development and stress. Among the plant defense elements, hormones are playing important roles during stresses. Salicylic acid is seen contributing in plant defense, and many reports suggest its action as elicitor. Thus, investigating the biological actions of SA in plant development and defense at epigenetic levels bring questions about the metabolism behind the

plant responses. On other hand, the millet headminer and aluminum toxicity are two models of stress under which studying the epigenetic plant responses could provide a breakthrough in pearl millet biology.

Therefore, the overall objective of this study is to evaluate the influence of salicylic acid on aluminum toxicity and *H. albipunctella* infestation of pearl millet on epigenomic responses. Specifically, the objectives are:

1: to determine the effects of SA on plant development and defense

2: to determine the effects of aluminum on plant development and defense

3: to determine the effects of salicylic acid as elicitor of plant defense during pest stress and aluminum toxicity

4: to determine the methylome changes under stress and salicylic acid signaling

5: to determine the epigenetic diversity during stresses

2.0 Chapter 2: Literature review

2.1 Background of pearl millet

2.1.1 Production and importance

Millets are in the family of cereals grown globally with differential importance across continents and within regions of the world. In Africa, millet cultivation (Figure 1) spreads from Senegal to Eritrea and covering the Sahel and dry Sudano-Guinean agro-climatic zones of West Africa, and the semi-arid zones bordering the Kalahari Desert in the South of Africa.

Figure 1: Pearl millet panicle emergence during field trial in the centre of Senegal (Nioro).

They are grown in arid and semi-arid areas with a production area of 18.50 million ha by 28 countries covering 30% of the continent. There are nine species which form major sources of energy and protein for about 130 million people in Sub-Saharan Africa. Among these, only four are produced significantly in Africa; including pearl millet (the most widely grown in 76% area), finger millet (19% area), tef (9%) and fonio (4%) (Figure 2). Millet production is distributed

differentially among many African countries. The largest producers are in West Africa led by Nigeria (41%), Niger (16%), Burkina Faso (7%), Mali (6.4%), Senegal and Sudan (4.8% each). Finger millet is produced mainly in East and Southern Africa. Worldwide, as a general estimate millet production is broken down into the following percentages (Gramene, n.d.):

- 50% for pearl millet;
- 30% for proso millet;
- 10% for foxtail millet;
- 10% for Finger millet;
- 10% for the others including barnyard and kodo millets.

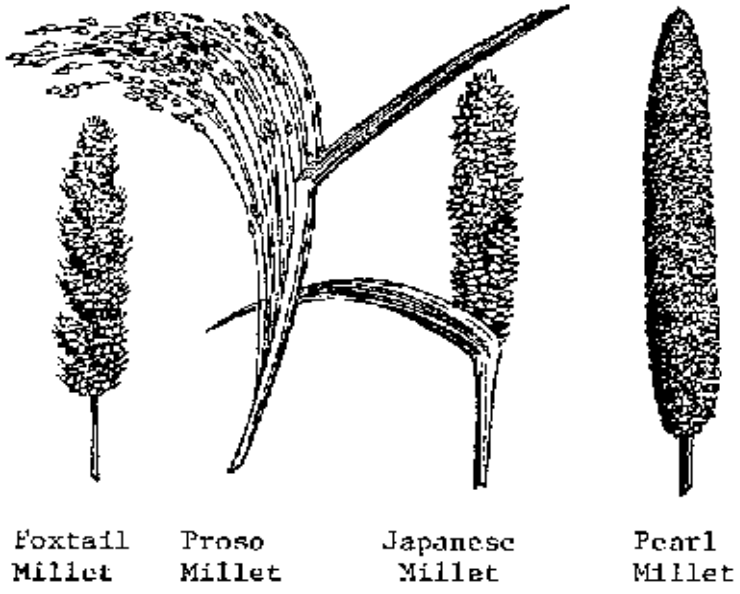

Figure 2: Millet types. Source: Cereal crops versus pulse crops

Millets are important strategic crop for responding to the needs and welfare of the poor including food security, nutrition and health, poverty alleviation, potential markets and dry environment enhancement. Production harvested areas and the yield vary among regions (Table 1 and 3) or continents (Table 2).

Table 1: Millet areas harvested, production and yield in Africa

Regions	Areas harvested (ha)	Production (tons)	Yield (kg/ha)
Eastern Africa	1.437.792	1.751.861	12.184
Middle Africa	1.570.736	933.435	5.943
Northern Africa	3.014.806	1.460.880	4.846
Southern Africa	244.156	51.458	2.108
Western Africa	13.768.300	9.443.999	6.859

The production is dominated by Western Africa, but the yield is still low (Faostat, 2016)

Table 2: Millet areas harvested, production and yield in the world

Regions	Areas harvested (ha)	Production (tonnes)	Yield (kg/ha)
Africa	20.035.790	13.641.632	6.809
USA	167.140	284.810	17.040
China	746.445	1.996.378	26.745
India	8.840.000	10.280.000	11.629

The production dominated by Africa with lower yield (Faostat, 2016)

Table 3: Relative importance of millet species cultivated in Africa

Regions	Pearl millet (%)	Finger millet (%)	Proso millet (%)	Foxtail millet (%)	Teff millet (%)	Fonio millet (%)	Other (%)
North Africa	98	2	0	0	0	0	0
Western Africa	95	0	0	0	0	4	1
Central Africa	87	13	0	0	0	0	0
Eastern Africa	35	50	0	0	9	0	6
Southern Africa	65	30	0	0	0	0	5
Africa	**76**	**19**	**0**	**0**	**2**	**1**	**3**

Pearl millet represents the most cultivated millet crops (source: ICRISAT)

As food, they are nutritionally equivalent or superior to most cereals; containing high levels of methionine, cystine, and other vital amino acids for human health (Table 4 and 5). Millets also contain high protein (up to 9.5 g/100g for teff and fonio), ash, calcium (up to 344 mg/100g for finger millet), phosphorus and potassium (up to 250 mg/100 g and 314 mg/100 g for finger millet).

Figure 3: Pearl millet in grain (a) and in powder (b)
Sources: Jivabhumi.com & timbaktu-organic.org

They are also unique sources of pro-vitamin A (yellow pearl millets) and micronutrients (Zn, Fe and Cu) which are especially high in finger millet. The high levels of methionine and good digestibility make the millets valuable food for humans and other monogastric animals. Millets (together with sorghum) provide 75% of total caloric intake for the poor people living in the semi-arid tropics and sub-humid drought-prone areas. Millets alone provide 13.40 kg/yr per capita food use.

Animal feed as forage, grain and residue is still insignificant, with about 7% (< 2 million tons) of total production going into animal feed. Malting and brewing local beers using millets is significant in Uganda, Zimbabwe, Zambia and Namibia. Non-alcoholic local beverages are commonly made from millets in West Africa. The stalks of the long season, late maturing pearl millet types (called Maiwa in Nigeria) are used in roofing, fencing and as firewood.

Table 4: Nutritional constituents of the major millets

Millets	**Protein (g)**	**Fiber (g)**	**Mineral (g)**	**Iron (mg)**	**Calcium (mg)**
Pearl millet	10.6	1.3	2.3	16.9	38
Finger millet	7.3	3.6	2.7	3.9	344
Foxtail millet	12.3	8.0	3.3	2.8	31
Proso millet	12.5	2.2	1.9	0.8	14

The nutritional contents vary from species to another. Data taken from 100g for each cereal (Singh, 2016)

Table 5: Important amino acids in millets

Amino acids ($g16g^{-1}N$)	Pearl millet	Finger millet	Teff	Fonio
Cystine	1.6-1.8	1.7	1.9	2.2-2.5
Isoleucine	3.9-4.6	4.0	3.2	4.0-4.3
Leucine	9.5-12.4	7.8	6.0	10.5-11.8
Lysine	2.8-3.2	2.5	2.3	1.9-2.5
Methionine	1.8-2.6	2.9	2.1	3.0-4.5
Phenylalanine	4.1	4.1	4.0	5.7-6.8
Threonine	3.3-4.1	3.1	2.8	3.3-3.7
Tryptophan	1.4-1.5	1.3	1.2	1.6
Tyrosine	3.0	4.1	1.7	3.5
Valine	4.9-6.0	6.4	4.1	5.2-5.5

2.1.2 Millet processing and research

The biggest constraint in the realization of importance of millets is in their handling and limited use by the producers, processors and consumers. Men and women mainly do respectively the harvesting, threshing, and processing for food at the household level. However, there are medium-large scale industrial levels of processing and brewing for malts and drinks even though 80% of the commercialization are carried out by informal trading. The remaining 20% are in form of thin and thick porridges (in west, east and southern Africa) and flat breads (mainly in Ethiopia using teff, and Sudan using pearl millet) (Obilana, 2003). A study of the West and Central African Millet

Research Network revealed that the most preferred staple in sub-saharian countries is millets which are used for breakfast, lunch and dinner, and its market demand is very high; 62-84% eat it in the form of thin (furra) and thick (tuwo/tộ); porridges upto 80% consume it as couscous in Senegal (Ndjeunga & Nelson, 2001). Similar levels can be found for teff in Ethiopia, as flat bread (injera). In southern Africa, commercialization of millet products is more advanced than in West Africa. In Eastern, Central and Southern Africa, traditional beer brewing from finger millet and pearl millet has long been a large-scale commercial enterprise. It is used in the form of malted or not mixture. In Zimbabwe, small quantities of pearl millet and finger millet are used in commercial opaque beer brewing. In Kenya, finished millet products in the form of 'Uji Mixes' are processed in large and small scale private firms; and demand for these products are high.

Africa is excluded in the international trade for millets that is estimated between 200,000 and 300,000 tons, representing approximately 0.1% of world cereal trade equivalent to 1% of world millet production (FAO). However, what is important in Africa is internal national and subregional trading of millets. Substantial quantities of millets are traded within African countries and the subregions. Grains move from surplus to deficit areas, within countries and across borders. Two broad types of grain marketing are identified in West Africa. These include the long-distance exchange from grain involving more than one country in the Western Africa sub-market (Senegal, Mali, Niger and Nigeria). The short distance trading is mainly internal within country grain marketing system. Grain trade in this sub-sector is estimated at 15-20% of domestically produced grains, and the rest consumed locally (Obilana, 2003).

Generally, research has focused on the most important species, pearl millet, which originated in West Africa and evolved through the harsh environments of that area, especially the Sahelian region. Adaptation and improvement of local varieties and local variety derived materials have

been the forms of research. Finger millet (next most important species) research has focused on processing and end-use in thin and thick porridges as composite flour, in eastern and central Africa; and for malting and brewing in southern Africa. In addition, pearl millet represents an important model to study stresses and climate change. Its ability to overcome many pests, pathogens and abiotic stress confers it an advantage in genetic research, the IPM and breeding programs. Genetic improvement of pearl millet has been done in India (Yadav & Rai, 2013) even though few studies were performed, despite its interesting biology. Recently, the sequenced genome draft is promising interesting perspectives in plant breeding and provide a resource to improve agronomic traits for food security (Rajeev *et al.*, 2017).

2.1.2 Ecology and Biology of pearl millet

2.1.2.1 Distribution

Pearl millet is widely distributed in the world (Figure 4). In Africa, the wild taxa are from the Sahelo-Sudanian (350-600 mm rainfall) zone of West Africa to the Sudanian (600-800 mm) bioclimatic zone through coastal Senegal and Mauritania to north-eastern Ethiopia (Brunken, 1977). The wild taxa easily colonize the habitats and are common weeds around the villages (Clayton, 1972).

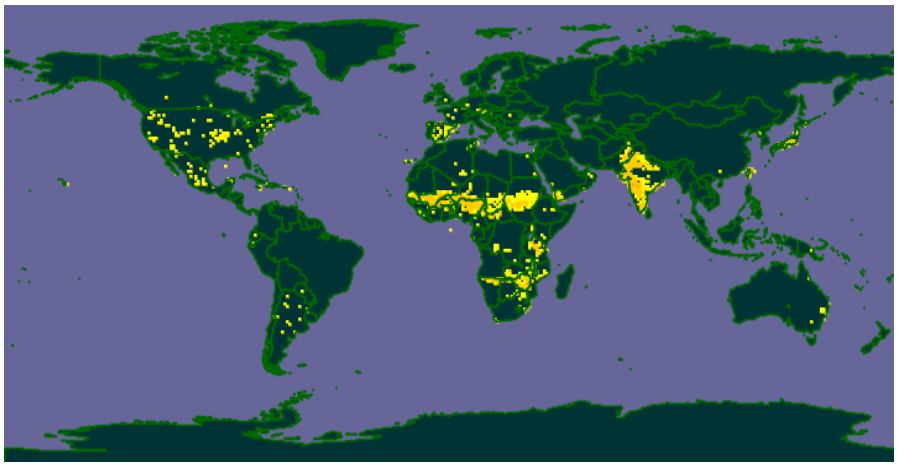

Figure 4: Distribution of pearl millet in the world.
Yellow areas indicate zones of pearl millet culture. Source: Global Biodiversity Information Facility Network.

2.1.3 Morphology and taxonomy

The species is characterized by involucres composed of bristles that each enclose one to nine spikelets. The agricultural weeds known as shibras in West Africa often resemble cultivated pearl millet except for their ability of natural seed dispersal, the involucres of weeds being disarticulated at maturity. In the cultivated pearl millet, inflorescences range from cylindrical to broadly elliptic, and from 5 to 200 cm long. Large inflorescences are commonly produced on plants 16^{th} single culms, while small- to medium size inflorescences are produced on plants that tiller (Brunken, 1977; Clement, 1985; Tostain, 1994). Four races have been identified even though the race typhoides is the most widely distributed (Africa and Indian subcontinent) and characterized by obovate caryopses and variable in length. Pearl millet range height varies from 1.5 to 3 meters (5 to 10 feet) tall and about 2.5 cm (1 inch) thick. The inflorescences may be spikes or racemes, in which the flowers are borne on stalks of about equal length along an elongated axis, or panicles with dense clusters of small florets.

Pearl millet, *Pennisetum glaucum*, has the following summarized classification:

Class: Liliopsida - Monocotyledons

Subclass: Commelinidae

Order: Cyperales

Family: Poaceae - Grass family

Genus: Pennisetum – fountaingrass (USDA & NRCS, 2006).

Its synonyms are numerous and include *Pennisetum typhoides, Pennisetum typhoidis, Pennisetum typhoideum, Pennisetum americanum, Setaria glauca, Setaria lutescens, Panicum americanum,* and *Panicum glaucum* but *Pennisetum glaucum* is the current officially accepted name. It has common names worldwide: bulrush millet, cattail millet, candle millet, mil à chandelles or petit-mil.

2.1.4 Stages of development

Millet is grown in dry areas of tropical Africa, India, China, the United States, Russia, etc. (Thiam, 1979). As annual species, *Pennisetum glaucum* cultivation is suitable for a rainfall of between 150 and 700 mm. Millet (L.) R. Br. The life cycle of the of most varieties from sowing to harvest is 85 to 95 days for pearl millet (Table 6).

Table 6: Duration of each phase of development of millets

Major Growth phase	Short duration	Long duration
Growth phase 1	22	28
Growth phase 2	18	25
Growth phase 3	25	22

According to Maiti & Bidinger (1981), this cycle can be divided into three major phases of development (Figure 5).

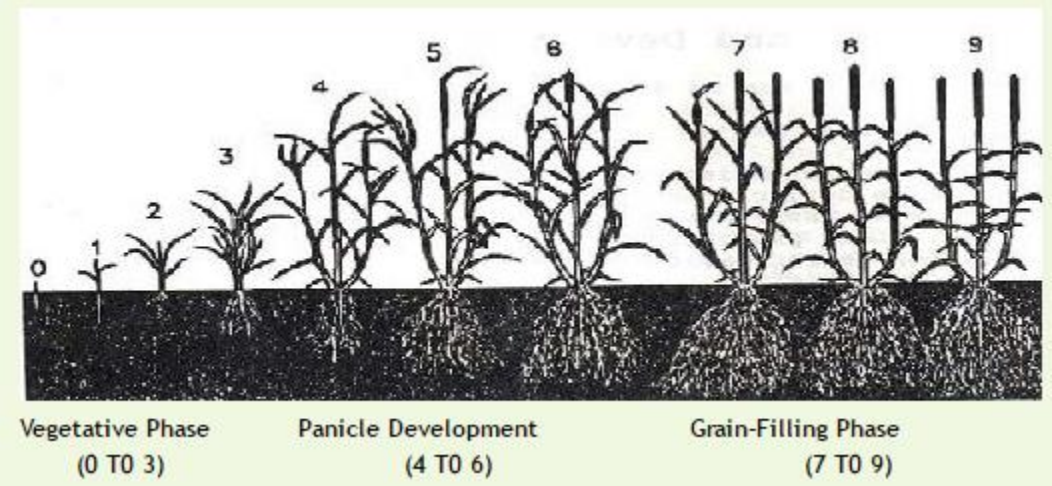

Figure 5: Phases of pearl millet development.
Source: agropedia

The vegetative development phase which begins with the germination until the initiation of the panicle. During this phase, the leaves form and the stems begin to tiller. After tillering, the succession follows with the elongation of the stalks of millet which is accompanied by the formation of the ears or beginning of heading within the sheaths (nested leaves). Leaf growth depends on the temperature with the maximum achieved between 32-35°C. These high leaf growth rates are important adaptations in overcoming early seedling death. In addition, there is a strong correlation between the rate of germination and the rate of leaf production (Mohamed *et al.,* 1988). Another adaptation is the tillering that compensate the seedling mortality or loss of production due to water stress (Mahalakshmi *et al.,* 1987). The secondary root system becomes effective after 20 days after emergence. Root penetration is rapid and by 33 days after emergence roots reached a depth of 1m (Chopart, 1983). Some reports mentioned maximum rooting depth of 3m in mature pearl millet allowing water harvest and extraction of deep accumulations of moisture and nitrate nitrogen under conditions of terminal drought stress.

The fruiting phase which starts during the vegetative development and goes until the end of the flowering marked by the fertilization of female flowers and the beginning of the filling of the grains. This phase is characterized by growth in length and volume of ears, but also by the formation of glumes, spikelets, stigmas, and anthers. This phase is under the control of the photoperiod. Indeed, early flowering increases the risk of poor seed yield, insect attack and bird damage. Late flowering increases the risk of drought and, heat stress during grain filling (Ong & Everard, 1979). The flowering period often corresponds to the end of the rain in pearl millet development. According to Mahalakshmi *et al.* (1987), early flowering limits the ability of a genotype to adjust to the effects of possible early season droughts. The temperature controls the spikelet development. Biomass compensation is possible flowering. During this stage, stress and lack of available moisture remain a serious limitation to grain yield.

The filling phase of the grains which starts from fertilization and goes on until the physiological maturation of millet. This corresponds to the filling of the grains. This phase also coincides with the senescence of the leaves. The grain filling period normally is accomplished in about 20 days at an average (25°C) temperature. Under stress conditions, pearl millet has adapted to produce large number of viable seeds with large size.

2.1.5 Phytosanitary constraints

2.1.5.1 Introduction

Pearl millet production is subjected to many abiotic and biotic limiting factors. The pests of millet are many and belong to several different biological groups: vertebrates, weeds, mushrooms, insects, etc. The parasitic weed *Striga hermonthica* is a major biotic constraint to pearl millet production in Sahelian Africa (Boukar *et al.,* 1996). The major diseases of pearl millet are the rust (*Puccinia substrata*), the smut (*Moesziomyces penicillariae*), the ergot (*Claviceps fusiformis*), the

leaf blast (*Pyricularia grisea*) (Lubadde *et al.,* 2014). For insect pests, about a hundred species have been recorded on millet. These major insect pests of millet include stem borers (*Coniesta ignefusalis*), midge (*Geromyia penniseti*), grasshoppers (*Oedalus senegalensis*), meloids (*Psalydolita spp*.), Aphids (*Rhopalosiphum maidis*), beetles (*Lema planifrons*), etc. However, the incidence of attacks differs from one area to another, and from one species to another (Gahukar *et al.,* 1986). Moreover, since the droughts of the years 1972-74, some Lepidopteran noctuids especially the millet headminer, *Heliocheilus albipunctella* De Joannis has become the most devastating pests of pearl millet (Guèvremont, 1983; Ndoye, 1988).

2.1.5.2 *Heliocheilus albipunctella*: the millet headminer

2.1.5.2.1 Morphology

Heliocheilus albipunctella is a lepidopteran that is heteronous (normal wing venation only in the anterior) and heterocerous (antennas of various forms). It belongs to the family of *Noctuidae* and subfamily *Heliothinae* which contains most harmful species to crops (Ndoye, 1988). The adult measures about 23 to 27 mm wingspan. The sexual dimorphism is well marked between the male and the female, with a distinct ovipositor in the female. The fore wings are red-brown in the female and a thickening of the costal margin is noted in the male. The egg is oval, yellowish-white to dark brown in color typical to the advanced embryonic development. The older larva is stocky and has several colors depending on the stage of development. It is characterized by clear bands visible on the sides. The chrysalis is light brown in color and about 12-13 mm in size (Ndoye, 1988).

2.1.5.2.2 Life cycle

The species *H. albipunctella* remains a specialized pest of pearl millet. Its development cycle cannot be completed if its emergence is asynchronous at the panicle initiation stage of pearl millet.

The life cycle was first described by Guèvremont (1983) in Niger. Emergence of the pest appear one month after the first significant rain, of 20 mm (Ndoye, 1988). As soon as they emerge, the males and females mate and go in search of panicles of millet favorable to the laying, those whose female flowers are only present. Females will begin to lay on the panicle, on the upper third with 80% of the eggs (Guèvremont, 1983). Adult lifespan can last up to 4 days and eggs are incubated for about 3-4 days. After hatching, the young larvae begin to consume the ovaries and cut the spikelets at the bases which are lifted helically from top to bottom. This gives a characteristic aspect of the symptomatic attacks with more important galleries as the larvae grow up to the L4 stage (Figure 6).

Figure 6: *Heliocheilus albipunctella* feeding (a) and symptomatic infestation (b)
Source: GIMEM III.

Four larval stages are distinguished and classified into L1, L2, L3 and L4 depending on the size of the cephalic capsule and the length of the larva among others (Sarr, 1998). Others classify them as

eight larval stages (Eisa *et al.,* 2007). Several larvae can be found on the same spike generating then several galleries and quite significant damage that could compromise the production of millet grains. According to Sarr (1998), the damage depends on the coincidence between the emergence and the phase of panicle of the millet, but also on the capacity of the varieties to compensate for early damages. Losses due to larvae can reach up to 15% (Bal, 1993) or even 85% of production (Payne *et al.,* 2011). According to Badiane (1999), the incidence of *H. albipunctella* can be up to 73% with an estimated severity of 20% *i.e.* level of panicle destruction.

During the last larval stage coinciding with the maturation of pearl millet, the L4 larvae enter in the prenymphal stage and pupate in the soil. Pupae are usually found between 5 and 20 cm depth (Eisa *et al.,* 2007). Pupation of the chrysalid is accompanied by a diapause that can last up to 10-11 months (Green *et al.,* 2004). The mortality can be high during warmer conditions with soil temperatures reaching 50-55°C (Nwanze, 1992). Diapause break would be achieved in part by a sufficient energy accumulation (degree days) by the chrysalid on the one hand and the favorable conditions of temperature and rainfall on the other hand.

2.1.5.2.3 Pest controls

One of the cultural control strategies is usually ploughing the soil before sowing to expose pupae to open air and to sun burn. Resistant / tolerant varieties to the larvae feeding can be used to minimize damage. The latter remains problematic as there are no clearly identified varieties for its resistance to this pest (Payne *et al.,* 2011). Chemical control can be efficiently used for the newly larvae before they enter the spikelets of the panicles.

This method, however, remains problematic because of the bioecology of the pest (Nwanze *et al.,* 1995). In addition, use of insecticides has adverse effects on the environment, the human and animal health including the natural enemies of *H. albipunctella*. The use of natural enemies such

as the larval parasitoids, *B. hebetor* have been utilized in many Sahelian countries although the approaches required some improvements. For egg parasitoids, more research and development need to be done especially on the promising *Trichogrammatoidea armigera*., *Trichogramma sp*. And eventually on *Copidosoma (Lithomastix) sp.* for the residual soil pest population.

2.2 Epigenetic regulations of growth and defense

2.2.1 Definitions

Epigenetics is the study of heritable changes in gene expression (active versus inactive genes) that do not involve changes to the underlying DNA sequence — a change in phenotype without a change in genotype — which in turn affects how cells read the genes.

The word epigenetics was defined in early 1940s by Conrad Waddington (Waddington, 1942). According to him, "epigenetics is the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being" (Waddington, 1968). This definition referred to all molecular pathways modulating the expression of a genotype into a phenotype. Over the following years, the definition has evolved and now epigenetics is generally considered as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence'' (Wu & Morris, 2001). Thus, that changes may be due to factors from the environment such as biotic and abiotic stresses, this can be heritable during meiosis stage. However, the validity of the current definition of epigenetics should be seriously questioned because the previously mentioned epigenetic modifications also have a crucial role in the silencing and expression of noncoding sequences.

Epigenetic marks have a crucial role in guaranteeing genomic stability. Indeed, the silencing of centromeres, telomeres, and transposable elements (TEs) ensures the correct attachment of microtubules to centromeres, reduces excessive recombination between repetitive elements, and

prevents transposition of TEs and resulting insertional mutagenesis (Dodge *et al.,* 2005; Daskalos *et al.,* 2009). Today, the epigenetic modifications generally comprise histone variants, posttranslational modifications of amino acids on the amino-terminal tail of histones, and covalent modifications of DNA bases. All these epigenetic mechanisms are influenced by the environment and its changes.

2.2.2 Influence of the environment on the epigenome

Plants as living organisms are influenced by many factors from the environment. They are external factors affecting, negatively or positively, the growth and the development of plants. Thus, unfavorable conditions can alter expression of genes. Plants have developed a plethora of survival strategies including developmental and morphological adaptations, specific signaling and defense pathways as well as innate and acquired immunity (Gutzat *et al.,* 2012).

Over the past few decades, knowledge of how plants perceive environmental stimuli has increased considerably from the mechanisms for transducing environmental stress stimuli into cellular signaling cascades to gene transcription networks. In addition, it has recently been shown that plants can remember past environmental events and can use these memories to aid the responses when these events recur (Kinoshita & Seki, 2014). These mechanisms are under epigenetic control which enhances the tolerance to stress (Qiao & Fan, 2011).

As a biological mechanism, epigenetic status may be modified the climate elements and abiotic factors (temperature, sunlight, soil) as well as the biotic factors such as pathogens, insects, nematodes, fungi, and other herbivorous animals that can interact with the genome to influence epigenetic changes. These changes may be reflected at various stages throughout a plant life cycle and even in the offspring. From embryo to adult stage, plants face enormous unfavorable conditions affecting its growth through gene expression regulated by epigenetic changes. Recently,

numerous studies have provided new insights into the epigenetic control of environmental stress adaptation. Epigenetic control of stress-induced phenotypic response of plants involves gene regulation (Sahu *et al.,* 2013). The changes in environmental conditions show that plants have different performances, due partly to its genetic variation and also to its epigenetic variation (Grativol *et al.,* 2012).

2.2.2 Epigenetic mechanisms

The epigenetic mechanisms are many and include DNA methylation, noncoding RNA silencing, and Histone modifications. The latter, especially the histone methylation plays a fundamental role in regulating diverse developmental processes and is also involved in silencing repetitive sequences to maintain genome stability. In time of stress, the molecular mechanisms driving the responses of plants depend on nucleosome histone post-translational modifications including histone acetylation, methylation, ubiquitination, and phosphorylation, playing therefore essential role in the regulation of stress responsive gene expression (Yuan *et al.,* 2013). The noncoding RNAs plays important role in the specificity determination of various physiological processes (Ramirez-Prado *et al.,* 2017). They can direct post-transcriptional regulation of gene expression or guide RNA modifications (Eddy, 2001) and have key regulatory functions in plant stress responses. Furthermore, DNA methylation represents the most widely epigenetic mechanisms in plants. Adenine and cytosine methylation are the two types of DNA methylation, the latter occurring more frequently.

2.2.3.1 Cytosine DNA methylation

Cytosine DNA methylation is the most common DNA methylation in plants. Zilberman (2008) stated the DNA primary role of cytosine methylation is a system of genome defense protecting genomes against both endogenous selfish DNA elements (predominantly transposable elements or

TEs) and exogenous virus invasions. In addition, TEs as well as DNA transposons and retransposons are heavily methylated and loss of methylation at these elements may lead to their transcriptional activation. Moreover, cytosine methylation has also been implicated in regulating gene expression across plant development and in times of stress (Zilberman *et al.*, 2007). The level of methylation modification ranges from 6% to 30% in plants (Chen & Li, 2004).

In plant, the methylation process must be established ("De novo" methylation) and maintained ("maintenance methylation") in order to regulate the cytosine DNA methylation (Chen & Li, 2004). The DNA methyltransferases (DNMTs) allow the transfer of methyl group to the C-5 position group of the cytosine ring of DNA (Robertson, 2005). In plant, two kinds of cytosine methylation occur, symmetrical methylation (CG or CHG) and asymmetrical (CHH), where H designates A, T or C. The CpG islands are DNA sites where a cytosine (C) occurs next to a guanine (G) in the linear sequence bases, in which the "p" designates one phosphate. Therefore, in human genome, around 98% of DNA methylation intervene in a CpG dinucleotide in somatic cells, while as much as a quarter of all methylation appears in a non-CpG context in embryonic stem cells (Lister *et al.*, 2009).

Methylation of cytosine in the promoter region may repress gene expression by preventing the binding of specific transcription factors (Watt & Molloy, 1988) or may attract mediators of chromatin remodeling, such as histone-modifying enzymes or other repressors of gene expression (Boyes & Bird, 1991; Hendrich & Bird, 1998; Jones *et al.*, 1998; Nan *et al.*, 1998). The promoter regions of silenced genes possess significantly more methylated cytosines in comparison with actively transcribed genes indicating this implication in transcriptional repression (Naveh-Many & Cedar 1981; Waechter & Baserga, 1982).

2.2.3.2 DNA methylation and demethylation pathways

2.2.3.2.1 DNA methylation pathways

In Arabidopsis model, cytosine methylation is mediated by DNA methyltransferases that are guided to their targets by methylation patterns, histone marks, small RNAs, or non-coding scaffold transcripts. The cytosine DNA methylation is found in CpG islands under control of maintenance DNA methyltransferase1 (MET1). The analysis of distinct genomic loci has helped to establish mechanistic models that allocate specific functions to the different DNA methyltransferases. MET1 has mainly been discussed in the context of its maintenance function for CG methylation marks, providing more stable epigenetic patterns than the target loci of the RdDM pathway, which show a higher level of epigenetic variation in *Arabidopsis* accessions (Schmitz *et al.,* 2013). The role of MET1, however, is not strictly limited to maintenance of CG methylation. At least at some target regions, MET1 has been shown to affect non-CG methylation as well, for example as a coordinator of methylation of stem–loop structures (Gentry & Meyer, 2013). An indirect MET1-specific effect on non-CG methylation has also been observed at certain loci with CMT2-controlled CHH and CMT3-controlled CHG methylation, which derive from Gypsy elements. These loci lose their H3K9 methylation in a met1 mutant, which results in a loss of CHG and CHH methylation marks (Stroud *et al.,* 2013). Loss of MET1 can generate hypomethylated, active epi-alleles, which are stably transmitted to the next generation (Watson *et al.,* 2014).

The CHH methylation context is regulated by the RNA- directed DNA methylation (RdDM) pathway with 24 nucleotides small interfering RNAs (siRNAs) which guide the de novo DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2). In addition, the RdDM predominantly regulates the repeats and related sequences in heterochromatin and euchromatin regions respectively (Matzke *et al.,* 2009).

MET1 and DRM2 are assisted to the identification of the methylation targets through the non-coding (nc) RNA and histone mark pathways. The DNA polymerases (Pol) IV and V which initiates the biogenesis of small RNAs and assists in targeting of the siRNA complex respectively, found only in plants, are involved in the DRM2-mediated de novo methylation. A dual lysine methyl reader protein, DNA-BINDING TRANSCRIPTION FACTOR 1/SAWADEE HOMEODOMAIN HOMOLOG 1 (DTF1/SHH1) guide the Pol IV to its targets (Zhang *et al.*, 2013). The DDR chromatin-remodeling complex consisting of DEFECTIVE IN MERISTEM SILENCING 3 (DMS3), DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1), and RNA-DIRECTED DNA METHYLATION 1 (RDM1) (Zhong *et al.*, 2012) and by two homologues of the histone lysine methyltransferase, suppressor of variegation [SU(VAR)], SUVH2 and SUVH9, with SRA (SET-and RING-ASSOCIATED) domains that bind methylated DNA guides the Pol V to its target sequences (Johnson *et al.*, 2014). In addition, Pol V assists in the recruitment of DRM2 as part of ARGONAUTE4 (AGO4) effector complexes by producing a non-coding scaffold transcript.

The CHG methylation, found in plants (Jackson *et al.*, 2002), is controlled by the CHROMOMETHY LASE3 (CMT3) which contains a chromodomain that binds methylated H3K9 marks (Cao *et al.*, 2003). CHG methylation is therefore maintained by a self-enforcing loop of cytosine methylation enzymes (Johnson *et al.*, 2002).

2.2.3.2.2 DNA demethylation pathways

The balance of the de novo and maintenance methylation is under the control of the cytosine demethylation of base excision repair pathways involving the 5-methylcytosine DNA glycosylase REPRESSOR OF SILENCING 1 (ROS1) and its homologues DEMETER (DME), DEMETER-LIKE 2 (DML2), and DML3 which are accompanied by the downregulation of the RNA directed

DNA methylation. In many reports, DNA methylation is associated with gene repression context, which does not consider the complex interaction between the different methylation and demethylation systems.

However, the activation of the RNA directed DNA methylation (RdDM) in a MET1 is accompanied by the repression of the of the ROS1 and downregulation of DML2 and DML3 transcripts (Mathieu *et al.,* 2007). The RdDM pathway can also have an activating role through maintenance of ROS1 expression as mutated RdDM pathways reduce ROS1 activity. After demethylation through 5-mC removal, the unmethylated cytosine is restored following 3′ phosphate removal, DNA polymerization, and DNA ligation (Penterman *et al.,* 2007).

Moreover, DNA demethylation is linked to histone modifications as in DNA methylation. The recruitment of demethylation and inhibition of de novo methylation functions are allowed by changes in histones marks. The histone acetylase Increase DNA Methylation 1 (IDM1) binds to methylated loci with low lysine (H3K4) and arginine (H3R2) methylation levels and acetylates H3K18 and H3K23 sites to recruit DNA demethylases (Qian *et al.,* 2012). The chromodomain of CMT3's binding targets is removed by the histone demethylase allowing therefore selective loss of CHG methylation marks that are no longer restored after replication (Inagaki *et al.,* 2010).

2.2.4 Techniques of cytosine DNA methylation analysis

Currently, DNA methylation is one of the most broadly studied and well-characterized epigenetic modifications dating back to studies done by Griffith and Mahler in 1969 which suggested that DNA methylation may be important in long term memory function (Holliday, 2006). Methylation of C as 5mC in CpG dinucleotides in the promoter region of a gene has been associated with transcriptional silencing and plays a central role in epigenetics. In the recent years, high-throughput methodologies have been developed to the point that genome-wide mapping of DNA cytosine

methylation has become feasible and sufficient in term of resolution (Zhu, 2008). Numerous studies have mapped the methylome of *Arabidopsis* and the high-throughput sequencing of bisulfate-converted DNA is used achieving single base pair resolution (Lister *et al.*, 2008). In addition, methylation-sensitive restriction enzymes technique is also used to analyze DNA methylation. In Bisulfite reaction analysis (Hayatsu *et al.*, 1970), the uracil replaces the non-methylated cytosine while the methylated cytosine does not react to the uracil.

However, the methylation sensitive amplified polymorphism (MSAP) is restriction-enzyme based. It aims at identifying molecular markers linked to DNA methylation and identifying genomic regions with differential methylation patterns in relation to biological processes e.g. development and stress (Baurens *et al.*, 2008). The MSAP is used to assess either the CpG islands with the restriction enzymes HpaII / MspI (CCGG methylation) or the CpNpG methylation with EcoRII / PspGI (CCWGG methylation). In CCGG MSAP reaction, two separated reactions series are performed on each sample using as a frequent cutter enzyme a pair of isoschizomers with differential methylation sensitivity. The HpaII /MspI isoschizomer pair recognizes the same DNA sequence (5' CCGG 3'). Conversely to MspI, HpaII cannot cut the internally methylated CmCGG sequence. Comparison between DNA fragments issued from EcoRI /HpaII and EcoRI/ MspI restrictions allows for identification of the methylation status of the CCGG site. The presence of bands in both patterns reflects an unmethylated site, presence in the EcoRI / MspI lane only, reflects an internally methylated site and, finally, the presence of bands in the EcoRI / HpaII lane only reflects a hemimethylated site (Reyna-López *et al.*, 1997; Xiong *et al.*, 1999; Baurens *et al.*, 2008).

2.3 Plant defense and development signaling

2.3.1 Plant development signals

As sessile organisms, plants initiate numerous mechanisms resulting in hormonal synthesis during its development, from germination to senescence, through vegetative and reproductive phases. In the recent years, numerous studies have been done to increase the understanding of plant signals during development, especially the identification of phytohormone receptors. The signaling mechanisms that direct plant development include long-range effectors, such as phytohormones, and molecules with a local intra-organ range, such as peptides, transcription factors and some small RNAs (Sparks *et al.,* 2013). After germination, the physical location of a plant does not normally change during its life cycle. Therefore, the development of a plant both at the cellular level and at the organismal level must be tightly coordinated with external conditions. Plant organogenesis is from two different stem cells, namely as meristems that include the organizing centre in the shoot and the quiescent centre in the root. The different organs (root, leaves, stems, etc.) channel information between each other to perceive environmental cues. Every organ can perceive, integrate, and transmit external or internal signals, emphasizing the need for an efficient communication system between the different tissues.

In plants, there are short range signalers, including peptides and the movement of transcription factors and non-coding small RNAs (sRNAs). The receptors for some signaling peptides are receptor-like kinases (RLKs) which count more than 600 (Shiu & Bleecker, 2001). The movement of transcription factors and sRNAs mostly pass through plasmodesmata, as the disruption of plasmodesmata inhibits movement. However, this is thought to require cofactor association, but no such cofactors have yet been identified and no sequence has been identified that predicts which sRNAs are mobile (Vatén *et al.,* 2011).

In addition to short-range molecules, there are long-range signalers which include the phytohormones like auxins. The auxin receptor is the F-box protein TRANSPORT INHIBITOR RESPONSE 1 (TIR1), which is a part of the SCF^{TIR1} complex. Auxin perception occurs by binding of AUX/IAA proteins to TIR1, targeting them for degradation by the proteasome and releasing AUXIN RESPONSE FACTOR (ARF) transcription regulators from inhibition by AUX/IAA (Sparks *et al.*, 2013). Auxin modulates diverse plant developmental pathways through direct transcriptional regulation and cooperative signaling with other plant hormones like salicylic acid.

However, auxin can be negatively regulated by AUXIN/INDOLE-3-ACETIC ACID repressors (Seo *et al.*, 2011). Armstrong *et al.* (2004) found three compounds interfering with auxin-regulated proteolysis of an auxin/indole-3-acetic acid transcription factor, and two impart phenotypes indicative of an altered auxin response, including impaired root development. Furthermore, some reports suggest that epigenetic marks of DNA methylation regulate de novo shoot regeneration of *Arabidopsis* through modulating auxin signaling, an increase of AUXIN RESPONSE FACTOR3 (ARF3) in MET1 due to DNA demethylation (Li *et al.*, 2011; Yamamuro *et al.*, 2016).

2.3.2 Plant defense signals

2.3.2.1 Introduction

Plants represent a rich source of nutrients for many organisms including bacteria and insects. They have developed a stunning array of structural, chemical, and protein-based defenses designed to detect invading organisms and stop them before they are able to cause extensive damage (Freeman & Beattie, 2008). Many defense layers of sophisticated surveillance mechanisms have been developed by plants to recognize the microbe-associated molecular patterns (MAMPs), damage-associated molecular patterns (DAMPs) or oral secretion from pathogens and insect respectively through the pattern recognition receptors (PRRs). Furthermore, the PRRs detect salicylic acid

when it is applied on plant tissues. The detection of these external molecules the PRRs triggers a cascade of metabolic reactions constituting the plant defense mechanisms.

Jasmonic acid, ethylene and salicylic acid ensure the intercellular transduction during stress. Jasmonic acid and its derivate methyl jasmonates are fatty acid synthesized from linonelic acid (Parchman *et al.*, 1997). Jasmonic acid and its derivatives can modulate aspects of fruit ripening, production of viable pollen, root growth, tendril coiling, and plant resistance to insects and pathogens (Creelman & Mullet, 1997). Jasmonic acid activates genes encoding protease inhibitors (Johnson *et al.,* 1989) and induces genes involved in phytoalexin biosynthesis (Choi, 1994) and phenolics like polyphenol oxidase (Doares *et al.,* 1995) that are involved in plant defense. Ethylene is linked to many abiotic stresses such as pH and temperature, flooding, drought, high salt, both organic and inorganic contaminants (Kazan, 2015; Tao *et al.,* 2015). Salicylic acid, a multifaceted plant hormone, plays important role in defense during stresses (Pieterse & van Loon, 1999; Loake & Grant, 2007; War *et al.,* 2011). These three molecules crosstalk in positive versus negative interactions allowing the plant to fine-tune its defense against specific aggressors (Yang *et al.,* 2015).

2.3.2.2 Focus on Salicylic acid

2.3.2.2.1 Biosynthesis pathways

Produced by plants, SA is shown having important regulatory functions by numerous studies. Recently, two SA biosynthetic pathways have been reported in plants (Vlot *et al.,* 2009; Dempsey *et al.,* 2011): SA biosynthesis from cinnamate catalyzed by phenylalanine ammonialyase (PAL), a key element of the phenylpropanoid pathway (Metraux, 2002; Chen *et al.,* 2009) and SA biosynthesis from chorismate catalyzed by isochorismate synthase from the chloroplast (Dempsey et al., 2011) (Figure 7). Other metabolites such as flavonoids, coumarins or lignans may be

synthesized from the phenylalanine pathway (Fraser & Chapple, 2011). In young pea plants, SA is synthesized from the shikimate pathway (Szalai *et al.,* 2011) that has a link between metabolism of carbohydrates and biosynthesis of aromatic compounds (Herman & Weaver, 1999). The isochorismate synthase (ICS) and the toxin extrusion transporter ENHANCED DISEASE SUSCEPTIBILITY (EDS) genes are among the key regulators of the SA biosynthesis (Table 7).

Table 7: SA-biosynthesis genes with potential functions

Genes	**Functions**	**References**
ICS1 and ICS2	SA Synthesis	Dempsey *et al.* 2011; Vlot *et al.,* 2009; Dempsey *et al.,* 2011
EDS5	SA exporter	Nawrath & Metraux, 1999; Nawrath *et al*. 2002; Serrano *et al.* 2013
EDS1 and PAD4	Function upstream of ICS1 and EDS5	Zhou *et al.* 1998 ; Falk *et al.* 1999 ; Feys *et al.* 2001
CBP60 and SARD1	TFs for ICS1 promoter	Zhang *et al.* 2010

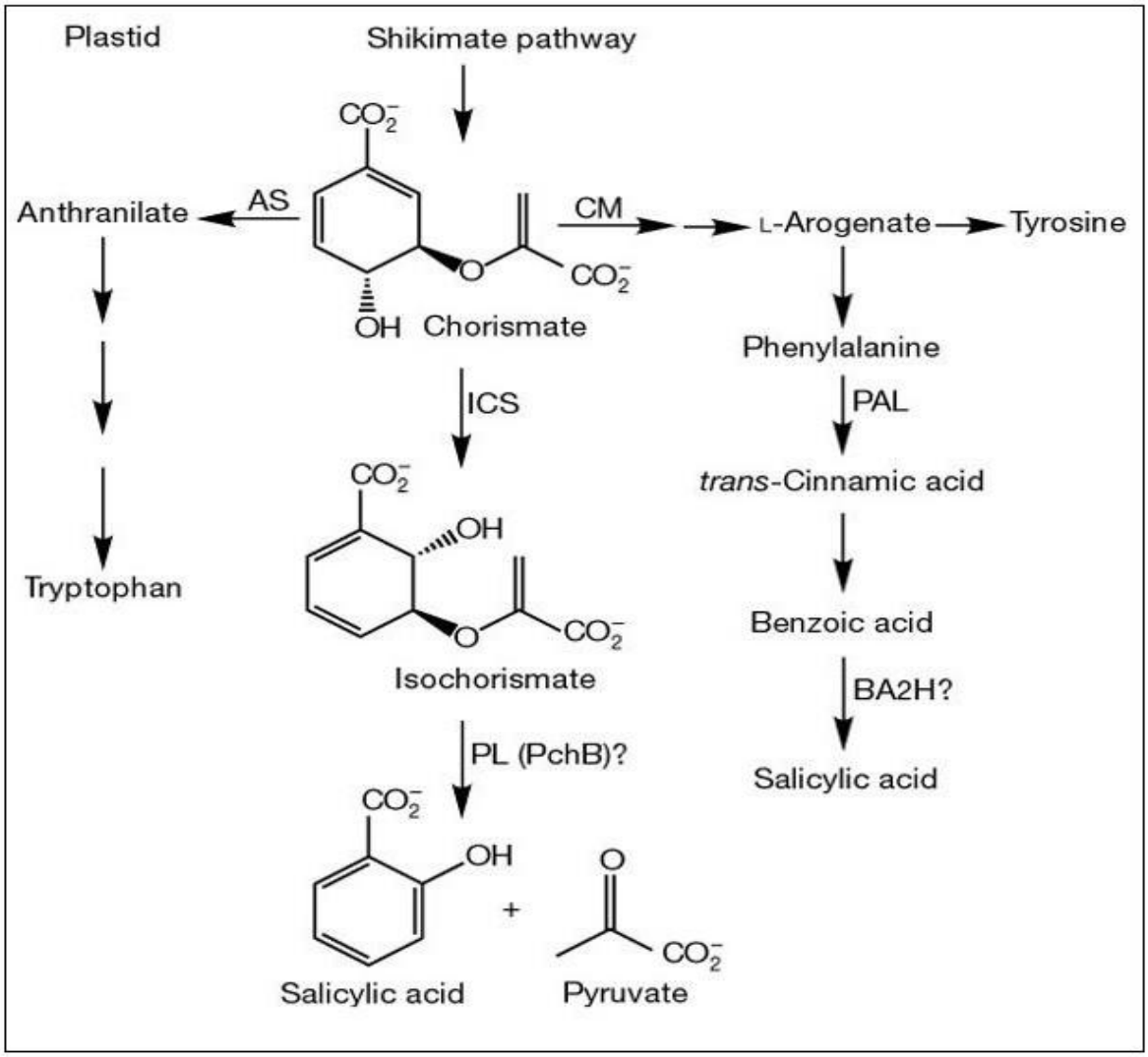

Figure 7: Salicylic acid biosynthesis pathways in plants. (Dempsey *et al.*, 2011)

2.3.2.2.2 Roles of salicylic acid in plants

Salicylic acid is induced under a range of biotic stress conditions. Its level increases during pest attacks or pathogen infection, triggering the hypersensitive response (HR) and the systemic acquired resistance (SAR) (Malamy, 1990). Blocking SA increase through expression of a bacterial salicylate hydroxylase gene in transgenic tobacco nahG plants compromises TMV-induced HR and abolishes SAR (Gaffney *et al.*, 1993). In plants, SA interacts with salicylic acid-binding proteins (SABPs) which include the transcription cofactor nonexpresser of PR genes

(NPR) 1, 3 and 4. Most of the SABPs are catalases that convert H_2O_2 to H_2O and O_2. The Mitogen-Activated Protein Kinases is found in salicylic acid pathway. Sequence analysis of a cDNA clone encoding this SA-induced protein kinase (SIPK) revealed that it is a member of the MAP kinase family. The discovery that SIPK encodes a 48-kDa kinase that is strongly activated by wounding raised questions as to the identity of the wounding-induced protein kinase gene product (Klessig *et al.,* 2000), that is shown its transcripts accumulate after wounding stress. Systemic acquired resistance (SAR) and hypersensitive response (HR) are two plant defense mechanisms under salicylic acid control. The HR is a type of programmed cell death (PCD) during biotic stress that is mediated by salicylic acid (Brodersen *et al*., 2005; Gust & Nürnberger, 2012). Salicylic acid levels increase during stress in response to PCD-inducing infections with association of proteolytic activity in the root tissues, and down regulation of proteases inhibitors (Kovács *et al.,* 2016). The nuclear translocation of the transcriptional cofactor non-expresser of pathogenesis-related (PR) 1 (NPR1) genes controlled by salicylic acid is required by the SAR. The degradation of NPR3-4 accompanies the PCD in infected cells with accumulation of NPR3-4 in the nucleus (Fu *et al.,* 2012).

Moreover, salicylic acid regulates the major components of induced plant defense against biotic and abiotic stresses such as peroxidase, polyphenol oxidase, superoxide dismutase, and phenylalanine amonialyase antioxidative enzymes. Indeed, peroxidase constitute an important group of defense enzymes that defend plants against various stresses while polyphenol oxidase also plays a pivotal role in plant defense (War *et al.*, 2011; War *et al.*, 2012).

2.3.2.2.3 Effects of treatments of salicylic acid in plants

Exogenous salicylic acid has many effects in plant development. In *Brosimum alicastrum* seedlings, 1µM of SA increases the root and shoot lengths (Loría & Larqué-Saavedra, 2012). A

low salicylic acid concentration (0.5mM) increases plant height and protein yield in optimum conditions (Sadeghipour & Aghaei, 2012). Foliar applications of salicylic acid (10^{-2}mM) increase the amount of vitamin C, lycopene, diameter of fruit skin (Javaheri *et al.,* 2012).

However, negative effects have been reported in other studies, and could be related to the SA concentrations employed. In *Arabidopsis*, salicylic acid doses more than 1mm delay or even inhibit germination (Rajjou *et al.,* 2006). In barley, doses more to 0.250mM inhibit seed germination (Xie *et al.,* 2007), while maize germination is completely inhibited by SA doses ranging from 3 mM to 5 mM (Guan & Scandalios, 1995). Attempts have been made to explain the negative effects of SA on seed germination and it is presumably due to an SA-induced oxidative stress. In *Arabidopsis* plants treated with SA (1–5 mM), hydrogen peroxide (H_2O_2) levels increase up to 3-fold because of increased activities of Cu, Zn-superoxide dismutase and inactivation of the H_2O_2-degrading enzymes, catalase and ascorbate peroxidase (Rao *et al.,* 1997).

As in plant development, salicylic acid, a phenolic compound, play an important role on plant defense against insect herbivores. The inhibition of growth (root and germination) could be from the oxidative stress which is implicated in primary plant defense system. The catalase activity (CA) implicated in hydrogen peroxide decomposition in Tobacco is inhibited by salicylic acid, and higher concentrations (from 10 to 104 µM) results in CA inhibition (Conrath *et al.,* 1995). In saline conditions, (100–150 mM NaCl) only 50% of *Arabidopsis* seeds germinate, but in the presence of SA (0.05–0.5 mM) seed germination increases to 80% (Vicente & Plasencia, 2011). Similar results were reported under abiotic stress by other studies (Rajjou *et al.,* 2006; Alonso-Ramirez *et al.,* 2009). In fact, overexpression of pathogenesis-related genes has been reported following treatment of salicylic acid activating therefore the plant defense mechanisms (Ward *et al.,* 1991) which

induce higher activities of POD, PPO and amounts of total phenols, H_2O_2 and protein content (War *et al.,* 2011).

2.3.3 Concept of insect elicitors and suppressors

The agroecosystem is characterized by the biotic interaction between plants, animals, microbes and the abiotic environment. This long-term interactive equilibrium is currently being impacted by human population pressure related activities. Plant/pathogen-insect interaction is an example of that evolution where each of the agents develop strategies to either feed on the plant or the plants to protect itselves. Plants develop a recognition system that allows them to defend their integrity against pests and pathogens. Many insects release molecules through oral secretion or oviposition fluids recognized by the PRRs. These cues from insect are called elicitors and could trigger plant defense mechanisms (Mithöfer & Boland, 2008; War *et al.,* 2012).

In recent years, the understanding of elicitors' action permits to develop and to improve the integrated pest management methods against pest and pathogens preparing the plant ready to a prospective bioagressor attacks. In research work already done, there are few elicitors discovered yet, and its metabolism remains little known. Most of elicitors are contained in insect oral secretion and the first biotic elicitor was discovered in 1968. They are classified into chemical and physical as well as divided into biotic and abiotic. The first ones are from insect and the second ones from environmental factors (Radman *et al.,* 2003). Furthermore, based on plant elicitor interaction, there are specific and general elicitors (Vasconsuelo & Boland, 2007).

The method of elicitor-induced resistance to disease in plants is characterized by many essential advantages. According to Patel & Krishnamurthy (2013), first, elicitor uses are ecological safety, because the method is based on induction of the native immune potential of genes. Second, it allows a systemic and prolonged protective effect. Third, it involves multiple defense systems in

induced resistance which makes inefficient the attacks of the pathogens and herbivores to protected plants nearly impossible. Finally, the last argument is the Induction of nonspecific resistance to the number of fungi, bacteria, viruses, nematodes, etc.

Insect-specific elicitors, found in oral secretions or oviposition fluids, are frequently responsible for the specificity of the plant responses. Many of them are found in l*epidopterian* oral secretions. Mattiacci *et al.* (1995) found the B-Glucosidase from the oral secretions of white cabbage butterfly (*Pieris brassicae*) larvae can elicit production of volatiles from cabbage plants. These volatiles attract parasitic wasps like *Cotesia glomerata* to feeding larvae. Other peptides like inceptins from armyworm's oral secretions also play as elicitors (Schmelz *et al.,* 2006, 2007). Fatty acid-amino acid conjugates (FACs) composed of either linolenic acid or linoleic acid and their derivatives and an amino acid moiety, Gln or Glu have the eliciting capacity (Spiteller & Boland, 2003; Maffei *et al.,* 2004). A hydroxyl FAC (N-17-hydroxylinolenoyl-LGln) called Volicitin from armyworm (*Spodoptera exigua*) induces volatile release in maize seedlings (Alborn *et al.,* 1997). Since then, different forms of FACs have been found in another insect species (Mori *et al.*, 2001; Spiteller & Boland, 2003).

However, growing evidence has been reported recently that some insects could avoid or inhibit the plant defense mechanisms through its oral secretion, a mixture of secretions from the labial and mandibular salivary glands and regurgitant (Vadassery *et al.,* 2012). In contrast to elicitors, there are molecules called suppressors that inhibit plant defense at translational, posttranslational or epigenetic levels. A suppressor of plant defense responses was isolated *Helicoverpa zea* oral secretion (Eichenseer *et al.,* 1999; Musser *et al.,* 2005). The Colorado popato beetle (*Leptinotarsa decemlineata*) uses bacterial symbionts in its oral secretions belonging to the genera *Stenotrophomonas*, *Pseudomonas*, and Enterobacter to suppress the plant defense mechanisms,

which are accompanied by an accumulation of SA (Chung *et al.,* 2013). Similar results were found in mite species (Villarroel *et al.,* 2016).

2.4 Aluminum toxicity

Aluminum is the most abundant metal in the earth's crust, comprising about 7% of its mass. Al is present in water, soil and air but most of it is incorporated into aluminosilicate soil minerals and only very small quantities (at submicromolar levels) appear in soluble forms capable of influencing biological systems (May & Nordstrom, 1991). Different forms of aluminum occur in soil solution: $Al(OH)^{2+}$ and $Al(OH)^{2+}$ at pH 4–5, Al^{3+} at pH 5.5–7, and $Al(OH)_4$ at pH 7–8. Other complex ions $AlO_4Al_{12}(OH)_{24}(H2O)^{127+}(Al_{13})$ and Al^{3+} are almost certainly toxic, but no phytotoxicity has been detected for $AlSO^{4+}$ and $Al(SO4)_2$ or Al-F e.g. AlF^{2+}. They are widely distributed in tropical and subtropical regions, constituting approximately 30% of the total area of the planet (Figure 8) and 50% of the arable land in the world, as well as providing between 25 and 80% of vegetable production (Sade *et al.,* 2016).

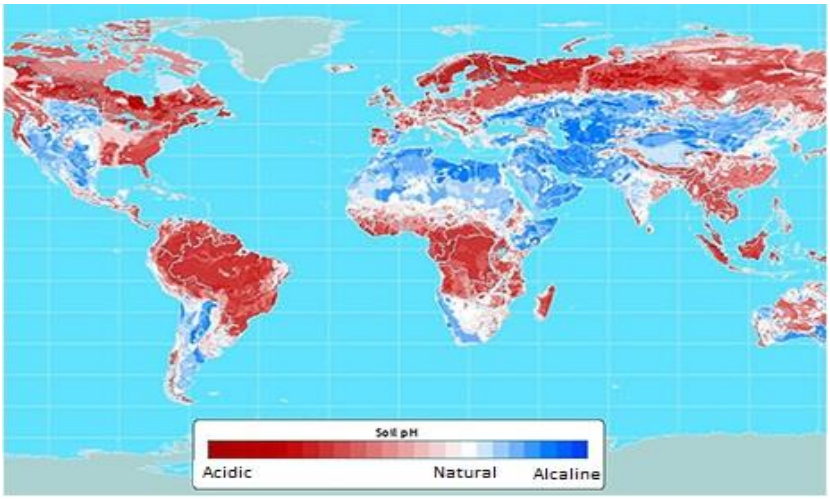

Figure 8: Aluminum toxicity occurrence in the world. Source: Cornell Chronicle.

Aluminum stress is one of abiotic stresses occurring in soil at low pH. The natural forms of Al do not interact with physiological processes of plants. Therefore, when the soil pH drops below 5, the

aluminum ions solubilize and become toxic forms. A1 toxicity is a major factor limiting plant production on acid soils, reducing root growth and yield. The aluminum toxicity is also implicated in oxidative stress (Zheng & Yang, 2005), and has effects on cell wall, plasma membrane and nutrient unbalances (Schmohl & Horst, 2000), induction of callose, and disturbance of cytoplasmic Ca^{2+} homeostasis (Rengel & Zhang, 2003). Although A1 toxicity has been identified as a problem of acid soils for over 70 years, our knowledge about the primary sites of toxicity and the chain of events that finally affects plant growth remains largely speculative.

2.4.1 Effects of aluminum toxicity on plant development

Around 50% of the arable soils are unusable because of aluminum toxicity. Most of the cereals are toxic to Al^{3+} at micromolar concentrations. It was recognized for the first time, over 100 years ago, that concentrations of soluble Al increase in acid soils (Veitch, 1904) and that this soluble Al is toxic for plant growth, the main effect of Al toxicity being inhibition of root growth (Kopittke *et al.,* 2015). Root growth inhibition was detected 2–4 days after the initiation of seed germination (Bennet & Breen, 1991). Indeed, the root apex including root cap, meristem, and elongation zone, accumulates more Al and attracts greater physical damage than the mature root tissues. The exposure of 2-3µM of the apical (root cap and meristem) of maize roots to aluminum causes growth inhibition (Ryan *et al.,* 1993). Bennet & Breen (1991) described several changes to the ultrastructure of cap cells in maize roots after two hours. treatment with Al and suggested that Al might inhibit root growth indirectly, via a signal-response pathway involving the root cap, hormones, and secondary messengers, but the root cap focuses on and highlights largely the importance of meristem.

2.4.2 Plant responses to aluminum toxicity

It has been indicated that Al tolerance in wheat populations is controlled by one major dominant or single gene located on chromosome-4, or single locus by multiple allelic series (Kerridge & Kronstad, 1968). Campbell & Lafever (1981) stated that Al tolerance in wheat was not simply inherited and that the expression of Al tolerance was additive with high values of heritability. In trifoliate orange, application of boron alleviates the toxicity of aluminum by regulating the activities of antioxidant enzymes, proline, secondary metabolites (phenylalanine ammonia lyase and polyphenol oxidase) contents, stabilized integrity of proteins, and reducing reactive oxygen species and Al concentrations (Riaz *et al.*, 2018). The plasma membrane H^+-ATPase plays a central role in all plant physiological processes. Changes in the activity of the plasma membrane H^+-ATPase through regulating the expression and phosphorylation of this enzyme are also involved in many plant responses to Al toxicity (Zhang *et al.*, 2017). In fact, aluminum toxicity affects the expression and post-translation of the plasma membrane H+-ATPase in some plant species (Guo *et al.*, 2013). Moreover, some plants have adapted to aluminum toxicity by tolerance or avoidance. Tolerant plants, such as buckwheat, hydrangea, melastoma, and tea allow Al accumulation in plant tissues, using Al sequestration in the vacuole and/or Al detoxification via Al binding to organic acid anions or proteins as the tolerance mechanisms (Morita *et al.*, 2008). In contrast, plants with the avoidance mechanisms decrease Al accumulation in roots via cell wall polysaccharide modifications (Schmohl *et al.*, 2000) or exudation of organic acid anions from root tips (Chen & Liao, 2017).

Recently, a discovery of a gene – and the protein it expresses – that play a major role in allowing rice to tolerate the toxic metal in acid soils, has given insight into plant tolerance to aluminum. In that study, the authors explain how the so-called NRAT1 gene in rice expresses a transport protein

that move aluminum away from the root cell walls (Li *et al.,* 2014). In addition, plants respond epigenetically to the changes induced by the aluminum. In *Zea mays*, the DNA methylation increased following aluminum exposure (Taspinar *et al.,* 2018). Similar results were found in tropical and temperate maize (Kimatu *et al.,* 2013). Plants increase the level of salicylic acid through overexpression of the SA-responsive gene GmNPR1 and the activities of phenylalanine ammonialyase in the root apex (Liu *et al.,* 2017). Exogenous salicylic acid has the same alleviation effects in rice (Pandey *et al.,* 2013) and tomato seedlings (Surapu *et al.,* 2014).

3.0 Chapter 3: Effects of salicylic acid and aluminum on growth

3.1 Introduction

The germination percentage and delay as well as the root growth remain important biological factors indicating the health of early plant development. All these factors could be affected by stressors from the environment like aluminum or internal signals from the plant metabolism including salicylic acid treatments.

Salicylic acid has differential effects in plants depending on the species or the genotypes and the applied doses (Vicente & Plasencia, 2011; Khan *et al.,* 2015). Germination and root growth have been affected by salicylic acid. In aluminum toxicity, root growth is reported to be inhibited in acidic soils. The aim of this study was to determine the effects of salicylic acid or aluminum on early pearl millet development.

3.2 Materials and methods

3.2.1 Plant materials

Four genotypes of pearl millet, Souna 3 (PMS3), IBV 8004 (PMI8), Gawane (PMG), and Thialack 2 (PMT2) were used in this study. They 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).

3.2.2 Seed disinfection

Seeds of four pearl millet varieties were disinfected using 1% of calcium hypochlorite $Ca(ClO)_2$ to obtain maximum germination and to avoid any contamination during the germination on the medium. Prior to germination, 15 seeds were used per treatment and soaked in $Ca(ClO)_2$ for 10min

with agitation and then washed five times with sterile deionized water to remove any remaining chlorine.

3.2.3 Preparation of medium

Murashige and Skoog medium was used in sowing the pearl millet seeds. The composition of the medium is given below (Table 8). The media were separated into two groups; the first one for salicylic acid test and the second one for the aluminum test. They are supplemented with different concentrations of salicylic acid and aluminum chloride (Table 9). A combination of aluminum concentration (400µm) and salicylic acid (0.5mm) was realized (Table 10).

After preparation, the pH of the medium was adjusted to 5.7 for those supplemented with salicylic acid and to 4.0 for those supplemented with aluminum chloride. All media were autoclaved with 121°C for 20min for sterilization.

After autoclaving, the media were cooled down at room temperature and left for 48hrs to check if there was any contamination. The contaminated media were discarded (Figure 9).

Table 8: Chemical composition of Murashige and Skoog medium

Components	Doses (mg/L)	Components	Doses (mg/L)
Ammonium nitrate	1650.0	Magnesium sulfate	180.7
Boric acid	6.2	Manganese sulfate • H_2O	16.9
Calcium chloride anhydrous	332.2	Molybdic acid • $2H_2O$	0.25
Cobalt chloride • $6H_2O$	0.025	Potassium iodide	0.83
Cupric sulfate • $5H_2O$	0.025	Potassium nitrate	1900.0
Na_2-EDTA	37.26	Potassium phosphate monobasic	170.0
Ferrous sulfate • $7H_2O$	27.8	Zinc sulfate • $7H_2O$	8.6
myo-Inositol	100.0	Nictotinic acid (free acid)	0.5
Pyridoxine • HCl	0.5	Sucrose	30,000
Thiamine • HCl	0.1	Agar	8000.0

Table 9: Different separated treatments of salicylic acid and aluminum

Treatments	Block 1	Block 2	Block 3	Block 4	Block 5
Salicylic acid (mm)	0	0.5	1.0	2.0	3.0
Aluminum chloride	0	200	400	600	-

Table 10: Combined treatments of aluminum and salicylic acid

	Aluminum and salicylic acid applications		
	Control	Al	Al+SA
Aluminum chloride	0µm	400µm	400µm
Salicylic acid	0mm	0mm	0.5mm

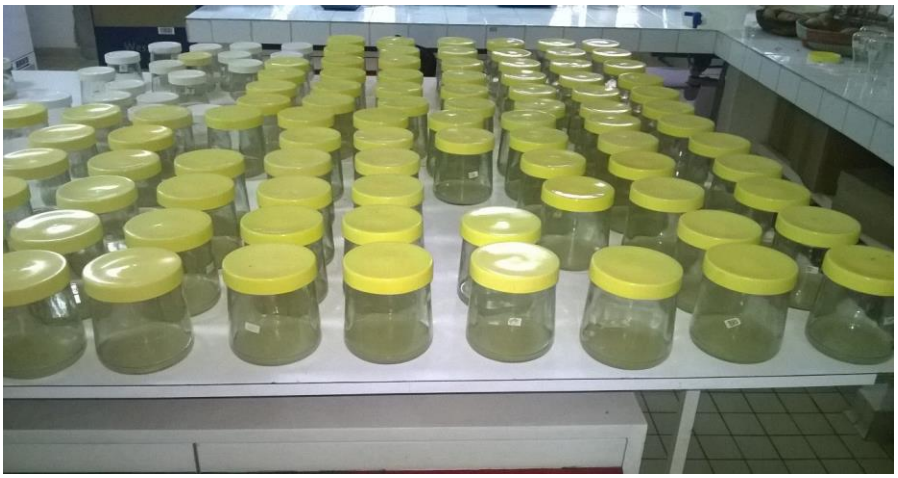

Figure 9: Cooling media after autoclaving

3.2.4 Growth conditions and morphological measures

The disinfected pearl millet seeds were sown on the media in the laminar flow hood to avoid any external contamination. 15 seeds were sown in each plate and incubated in the darkness for 48 hrs. at room temperature to allow germination.

After 48 hrs., the germination, delay germination and root length were recorded. The seedlings which had root lengths measuring less than 1 mm were not considered as germinated but were recorded as seedlings with delayed germination. In total, 900 seedlings were harvested for morphological measures, 225 seedlings for each variety.

After salicylic acid and aluminum screening test, the media with 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 molecular analysis.

3.2.4 Data 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 with Root growth, germination rate and germination delay data, and LSD for mean separation.

3.3 Results

3.3.1 Effects of salicylic acid on development

3.3.1.1 Germination

The significant difference among doses of salicylic acid ($P<0.05$) indicated that the percentage of germination varied from dose to another one and from variety to another one. In fact, for all varieties in general, more the salicylic acid concentration, the greater the percentage of seed germination decreased. The doses 2mm and 3mm had more inhibiting effects on germination. At high dose (3mm), salicylic acid completely suppressed the germination of all varieties, excepted the PMG (Table 11).

Table 11: Germination (%) of four pearl millet varieties under salicylic acid treatments

Varieties	Salicylic acid doses (mm)				
	0	**0.5**	**1**	**2**	**3**
PMS3	37 a	40 a	33 a	0 b	0 b
PMG	53 a	30 b	46 a	13 c	23 c
PMI8	97 a	87 a	20 b	23 b	0 c
PMT2	87 a	27 b	93 a	0 c	0 c

Four different varieties have been used. Same letter on the same row means significant difference at 0.05 level.

Furthermore, the delay was measured after 48 hrs. of germination with significant difference between treatments($P<0.05$). The germination delay was mostly important for the 0.5mm, even though PMT2 and PMG were more sensitive to salicylic acid (Table 12).

Table 12: Germination delay of pearl millet varieties under different levels of Salicylic acid

Varieties	Salicylic acid doses (mm)				
	0	**0.5**	**1**	**2**	**3**
PMS3	0 a	33 b	7 a	0 a	0 a
PMG	13 a	67 b	10.25 a	17 a	7 a
PMI8	0 a	7 a	10.25 a	10.25 a	3 a
PMT2	0 a	17 b	3 a	7 a	7 a

Four different Pearl varieties have been used. Same letter on the same row means significant difference at 0.05 level.

3.3.1.2 Root growth

The significant difference ($P<0.05$) indicated that the root growth decreased when the concentrations of SA increased. Additionally, the effects varied from variety to another one. Generally, high concentration of salicylic acid (3mM) completely inhibited the root growth except for PMG variety. PMG, PMS3 and PMI8 had more root growth than PMT2, suggesting genotype and dose-dependence at 0.5mM. Furthermore, only the root growth of the variety PMG was increased after the treatment of 0.5mM of salicylic acid (Figure 10).

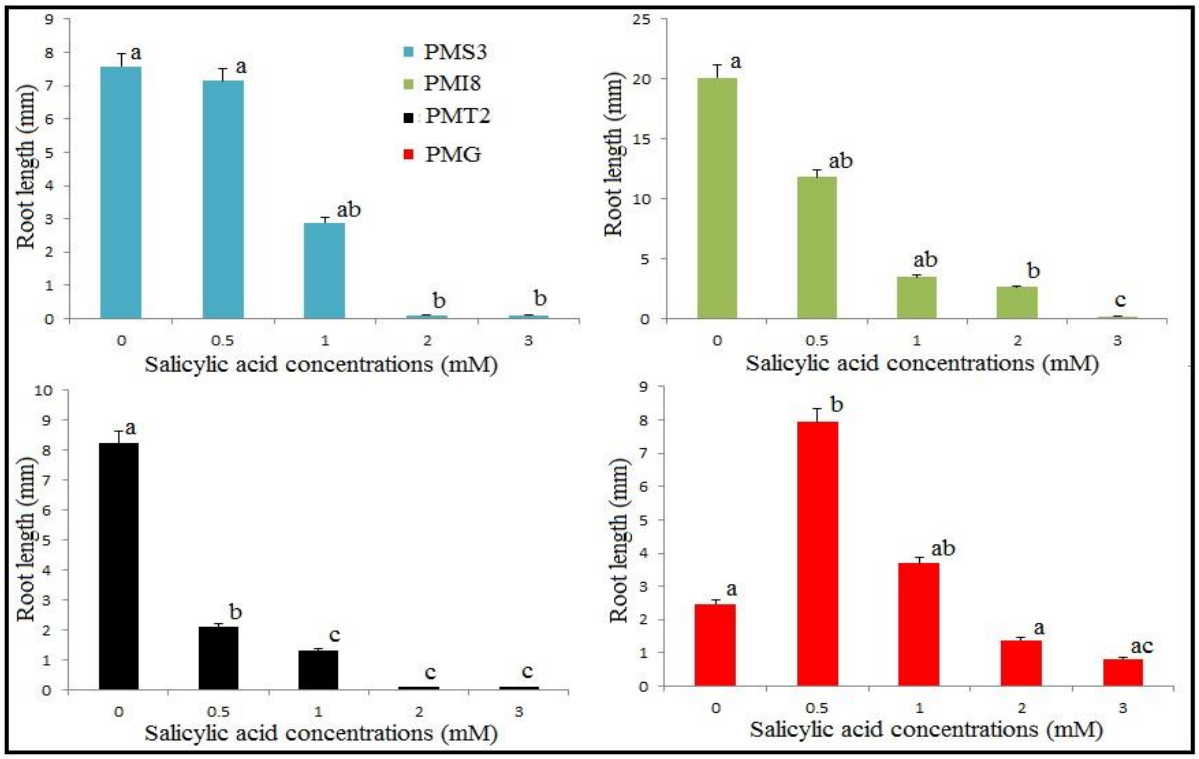

Figure 10: Root length after salicylic acid treatments.
High salicylic acid concentrations inhibited root growth. Error bars indicate s.d. The results are a representative of three biological repetitions with 180 seedlings for each line. Same letter means no significant difference at 0.05 level.

3.3.2 Effects of aluminum on development

The effects of aluminum were evaluated during seedling development such as germination percent, germination delay, and root growth with different doses of aluminum (Al^{3+}). The results showed a significant difference between the doses and the varieties ($P<0.05$).

3.3.2.1 Germination

The results revealed variation among doses and varieties with significant difference ($P<0.05$). There were two groups such low Al dose group (0 and 200µM) and high Al dose group (400 and 600µM). In the first group, the rate of germination was high except for PMT2 which had lower germination at control level (33%). However, the germination percent dropped at high level at 400 and 600µM of Al even though a tolerance seemed to be seen for PMI8 (400µM) and for PMG (600µM). Therefore, good germination percentage was noticed for all varieties only at 200µM Al (Table 13).

Table 13: Germination rate of four pearl millet varieties under aluminum treatments.

Varieties	Aluminum doses (µM)			
	0	**200**	**400**	**600**
PMS3	86 a	73 b	66 b	66 b
PMG	100 a	100 a	53 b	93 a
PMI8	93 a	100 a	93 a	20 b
PMT2	33 a	80 b	66 c	66 c

Same letter means no significant difference at 5% level.

The germination delay was variable, depending on the genotypes and the doses used. However, high Al doses such as 400µm and 600µm caused more delay than the other doses for all varieties

except the genotype PMT2 which had a decrease of germination delay when the concentration of Al was increasing (Table 14).

Table 14: Germination delay of pearl after aluminum treatments.

Varieties	Aluminum doses (µm)			
	0	**200**	**400**	**600**
PMS3	3 a	20 b	13 b	20 b
PMG	7 a	7 a	20 b	17 b
PMI8	0 a	7 a	20 b	57 c
PMT2	23 a	17 a	7 b	7 b

Results shown as percentage. Same letter means no significant difference at 5% level.

3.3.2.2 Root growth

The results showed a significant difference between doses and varieties ($P<0.05$), indicating genotype-dose dependence. The root length was shorter in high aluminum dose (600µM) than in the others, especially for PMS3 and PMI8. In PMG variety, only the dose 400µM negatively influenced the root growth compared to the rest that were quietly the same. However, the doses 200 and 400µM seemed to positively increase the root length in PMT2 and PMI8 genotypes respectively (Figure 11).

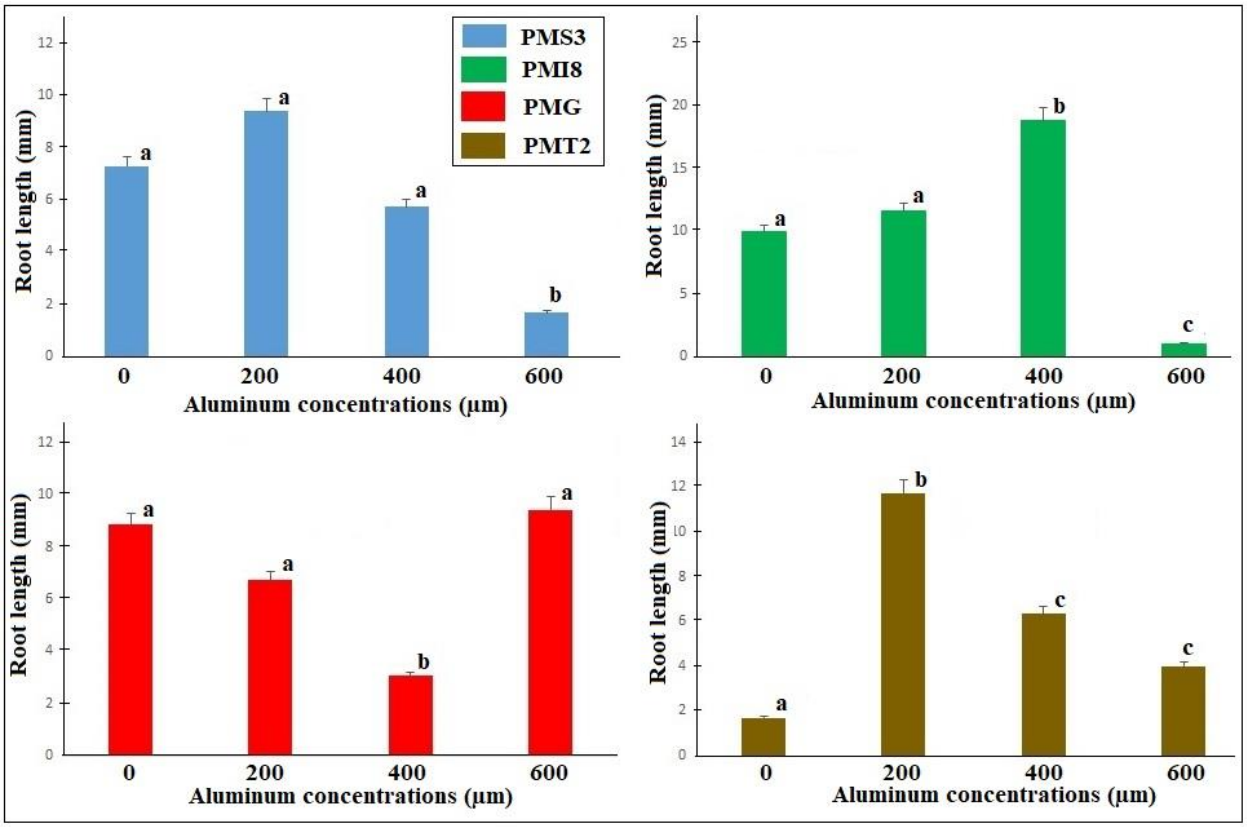

Figure 11: Root growth of pearl millet under aluminum treatments.
Aluminum variably influences the root growth. Results shown as mean ± s.d. Same letter means no significant difference between the doses.

3.4 Discussion

3.4.1 Salicylic acid inhibits plant development

A significant difference ($p<0.05$) between the salicylic acid doses and between the varieties suggests a dose- and genotype-dependence response to salicylic acid. All developmental factors such germination (percentage and delay) and root growth decreased when the concentrations of salicylic acid increased. The screening tests revealed that the seeds did not tolerate salicylic acid as it reduces mostly germination rate and delays it. The same results were found in root growth, especially in high concentrations of salicylic acid.

These results were similar to studies by Guan & Scandalios (1995), Rajjou *et al.*, (2006) and Xie *et al.* (2007). In pepper, this SA inhibiting effect is found for high doses (Jullien *et al.*, 2012). In

Arabidopsis, both germination and growth were inhibited by salicylic acid (Rajjou *et al.*, 2006). These show that salicylic acid may have detrimental effects on early plant development in pearl millet either by disturbing the molecular root growth network involved in posttranslational regulation pathways.

However, other studies showed contradictory results. Lower salicylic acid doses (10^{-5}mM) enhanced germination rate and the root length after 7 days, and 100–500mM of salicylic acid inhibited the plant development (Rao *et al.*, 1997). In wheat, salicylic acid increased root length of the two varieties drought tolerant and drought susceptible (Ward *et al.*, 1991), as well as in fava bean (Malamy *et al.*, 1990). This demonstrated a plant dose-genotype dependence response to salicylic acid treatments.

3.4.2 Effects of different levels of aluminum on plant development

The effect of aluminum on growth of pearl millet was tested. The results showed significant difference among doses ($P<0.05$). Most toxic effects were found in the dose 600µM in all development factors including germination percent and delay, and root growth (Bennet & Breen, 1991; Delhaize & Ryan, 1995; Kochian, 1995) (Figure 12). Reports revealed that the major Al toxicity symptom observed in plants is inhibition of root growth, with greater signs of cellular damage in the roots than other parts of the plant (Rincon & Gonzales, 1992). The toxic effect can be detected from 2-4 days after the initiation of seed germination (Bennet & Breen, 1991). Other studies reported even detection of inhibition within hours in many agriculturally important plant species (Kochian, 1995).

The variety PMG seems to tolerate the aluminum, even at high dose (600µM). Indeed, plant species and ecotypes growing on acid soils have become tolerant to the inhibitory effects of aluminum on root absorption and growth (Van Praag *et al.*, 1985). Moreover, some plants release

organic acid agents through its roots chelating the aluminum. Wheat and maize, that are genetically close to pearl millet detoxify aluminum using the aluminum-activated anion channels in the plasma membrane (Ma *et al.*, 2001).

4.0 Chapter 4: Evaluation of the effects of salicylic acid on infestation of millet headminer

4.1 Introduction

Plants produce different defensive compounds during pest attacks. Insects' feeding on plants is a dynamic ecological interaction, with special relationship between insect and the plant, affecting feeding behavior. During feeding, the pest could release chemical cues that allow plants to detect the attack and mount an efficient defense response (Stahl *et al.,* 2017) through gene-for-gene interaction pathways. Oral secretions or oviposition fluids contain molecules that can be recognized by pattern recognition receptors located on the plant plasma membrane which are the first stress signal transmitters that trigger a cascade of metabolic activities from hormone signaling to defense systems. An example is the case of *Nesidiocoris tenuis* triggering defense response induction in tomatoes (Naselli *et al.,* 2016). The plant defensive compounds could affect feeding, growth, and survival of herbivores and include development of structural barriers, toxic chemicals, and attraction of natural enemies of the target pests (Howe & Jander, 2008; War *et al.,* 2012). However, some insects may develop strategies to avoid or overcome plant defense by suppressing the plant immune responses (Musser *et al.,* 2005).

The interaction between *H. albipunctella* and *P. glaucum* was evaluated through salicylic acid treatments during panicle development. The aim of this work was to determine whether salicylic acid was able to act as an elicitor of pearl millet defense mechanisms by reducing the larval infestation (density per panicle) and therefore giving a temporal tolerance to pearl millet against the headminer. In the evaluations, the number of larvae per panicle (larval density), the relative yield and the absolute yield were determined.

4.2 Materials and methods

4.2.1 Experimental design and sampling

The experimental design was a split plot (Fisher, 1925) with stratification of the field to improve the representativeness of the sample by reducing sampling error as the headminer behavior is aggregative. The experiment was carried out in three replicates. Each main plot corresponded to one variety. Then, the plot was divided into three subplots where the salicylic acid treatments (0 mM, 1.5 mM, and 3 mM) were applied. A random sampling was carried out in each subplot where 15 panicles were harvested to determine the incidences and the larval density per panicle.

4.2.2 Growth conditions

The field was established under natural conditions in the middle of Senegal (Nioro) in a region where there is high infestation of *H. albipunctella*. Sowing was done at the end of June during the rainy season after the first rain of about 20mm. Plowing and weeding were performed several times as necessary during the vegetative phase to ensure good growth of the plants. Fertilizers were applied 14 days after the germination of the seeds as per the recommended practice. Foliar application of SA were performed at the panicle initiation at 56 days after planting and repeated on the third and sixth day.

4.2.3 Entomological studies

4.2.3.1 Incidences

The incidence of *H. albipunctella* was calculated from the number of larval-mined ears relative to the total number of panicles sampled:

$$I = \frac{P_m}{P_t} X\ 100$$

where I is the incidence of the headminer, P_m the number of mined panicles, and P_t the total of panicles in the stratum (Ngom, 2013).

4.2.3.2 Larval density

The larval density is the number of larvae per panicle. It was calculated using the following formula:

$$D = \frac{N_l}{N_p}$$

where D indicates the larval density, N_l the number of larvae, and N_p the number of panicles.

4.2.4 Data analysis

The software R version 3.2.5 was used to perform ANOVA between salicylic acid treatments for each variety. LSD was used for mean separation.

4.3 Results

4.3.1 Pest incidence

The incidence of *H. albipunctella* were calculated during panicle development. The results showed significant difference between the treatments and varieties. The incidence varied from 40 to 100% in control plants. However, it decreased globally in salicylic acid treated plants except for PMG and PMI8 in 1.5mM treatment (Table 15).

Table 15: Incidence (%) of *Heliocheilus albipunctella* after salicylic acid treatments.

Varieties	Salicylic acid concentrations		
	0mM	**1.5mM**	**3mM**
PMS3	40 a	20 b	40 a
PMG	40 a	60 b	20 c
PMI8	60 a	80 b	20 a
PMT2	100 a	40 b	40 b

Same letter means no significant difference at 5% level.

4.3.2 Effects of salicylic acid on larval density

Foliar treatments of SA were carried out before the initiation of the first panicle expansion stage. The results showed a significant difference between the controls (without SA) and the treatments ($p<0.05$). The varieties PMT2, PMI8 and PMS3 remained susceptible to *H. albipunctella*, while a treatment with SA significantly reduced the larval density per panicle. Only the larval density of *H. albipunctella* in PMG variety increased after applications of 1.5 mm SA. Therefore, at the control level, the tolerance of the varieties to the millet headminer was different, with PMS3 and PMT2 being more sensitive. The SA concentrations (1.5 and 3 mM) did not vary significantly, except for PMG variety (Figure 12).

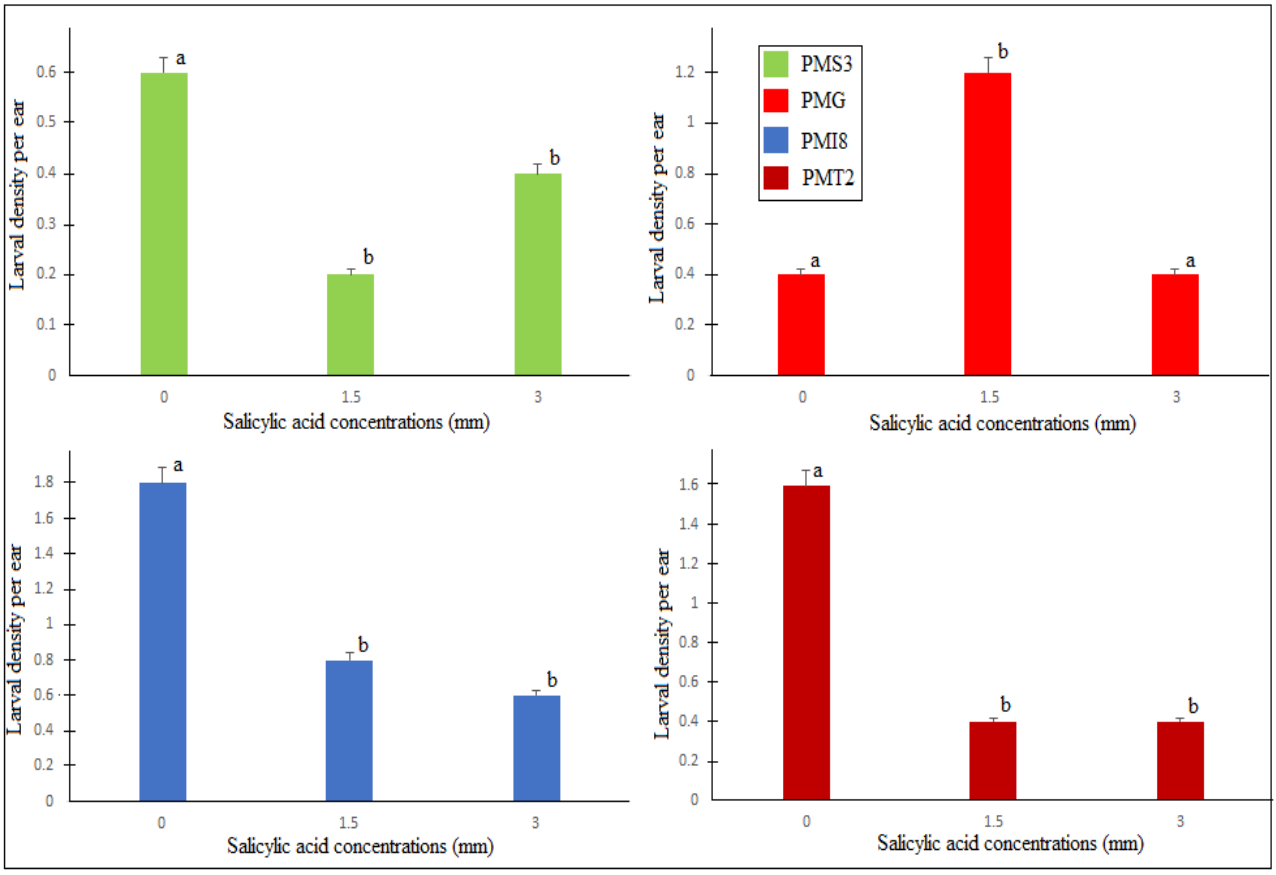

Figure 12: Effects of salicylic acid treatments on the millet headminer.
SA (0 mm, 1.5 mm and, 3 mm) was applied on four pearl millet varieties. Results showed SA act as elicitor by decreasing the larval density for the varieties tested. Means with the same letter are no significant difference at 0.05 probability level. a: PMS3; b: PMG; c: PMI8; d: PMT2

4.4 Discussion

The effects of foliar treatments of salicylic acid on pearl millet and on the millet headminer have been carried out. There was significant difference ($P<0.05$) between control plants (without salicylic acid) and the treated plants (with salicylic acid). This showed salicylic acid reduces larval density per panicle in all varieties even though in PMG genotype, only the concentration 3mm decreased the number of larvae per panicle. Indeed, Fragniere *et al.*, (2011) and Khan *et al.*, (2015) revealed that salicylic acid as an elicitor of plant defense, especially for pathogen and abiotic stresses. It seems now that SA-eliciting pathway is more complex and has a cross-talk with the defensive mechanisms involved during herbivore attacks. In fact, plants use direct and indirect

ways of defense during insect attacks. The first way involves production of compounds and enzymes directly targeting the pest, while the second uses volatile compound organics release to attract its predators and parasitoids (War *et al.,* 2011; Song & Ryu, 2013; Shi *et al.,* 2016). Each mechanism involves complex gene-related defense regulations controlled at epigenetic level which could be blocked by the larvae feeding through molecules released in the plant tissues. This inhibition is overcome by the eliciting effects of SA through methylome regulations. In some plants, SA influences the defense responses following insect elicitor treatments (Engelberth *et al.,* 2011).

5.0 Chapter 5: Effects of stressors on the epigenome

5.1 Introduction

Approximately 70% of soil in world is contaminated with acid, alkali, or heavy metals including aluminum. It has phytotoxic actions in certain conditions to plants, especially inhibiting the root development and therefore limiting the crop yield. The root apex including root cap, meristem and elongation zone is highly sensitive to aluminum and accumulates it very easily. Aluminum inhibits the transcription functions. In the nucleus, binding of aluminum to DNA or to chromatin could condense DNA molecules and inhibit cell division by reducing its capacity to provide a viable template for transcription (Matsumoto, 1988). Aluminum exposure may affect the ROS production in plants and induction of the expression of several genes encoding antioxidative enzymes such as glutathione S-transferase, peroxidase and superoxide dismutase (SOD). Moreover, root development (Cell division and cell differentiation) is under epigenetic control (Takatsuka & Umeda, 2015) including DNA methylation and histone modifications. The latter seems playing key role with the histone acetylation machinery by interacting with the transcriptional coactivator ADA2b, thereby boosting the expression of auxin-responsive genes that control root growth (Weiste & Dröge-Laser, 2014). However, little is known about the effects of aluminum of global and CCGG DNA methylation changes in root. In *Arabidopsis*, all DNA methyltransferases (except for DRM1) are strongly expressed in the root tip (Jullien *et al.,* 2012).

Addition to aluminum stress, pest attacks remain a big problem in agriculture. Plant and insect interaction is very complex and still now remain unclear. Plants have evolved innate immune systems that recognize insects from its oral secretion or oviposition fluids. These mechanisms include damage-associated molecular pattern (DAMPs), or pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (Chisholm *et al.,* 2006; Jones

& Dangl, 2006; Dodds & Rathjen, 2010). PTI is conferred by PRRs that recognize salicylic acid, PAMPs, DAMPs or endogenous elicitors (Zipfel, 2013) which allow plants to mount an efficient defense. However, the oral secretion from insect feeding could play important role in larval survival by downregulating plant defense-related genes which are controlled by a series of coordinated epigenetic events on the nucleosomes, including DNA methylation (Baulcombe & Dean, 2014; Gijzen *et al.,* 2014; Espinas *et al.,* 2016). The methylome variation directs expression of plant defense-related genes. A decrease and increase of methylation level are associated with upregulation and downregulation of genes respectively during stresses. The insect feeding may act as methylating agents on the epigenome, especially on the CpG islands which 95% of CG dinucleotides are disposed throughout 99% of the genome and are typically methylated and found in half of all promoters (Vinson & Chatterjee, 2012).

Finally, this epigenetic-related defense of both stresses may also be influenced by salicylic acid, a multifaceted plant hormone that have eliciting effects in plant defense (Vicente & Plasencia, 2011; Razmi *et al.,* 2017) and in plant development (Xie *et al.,* 2007). SA is mostly linked with abiotic and biotic stresses (Zehra *et al.,* 2017), and not directly to pest stress. Pathogen infection induces accumulation of SA as defense signals (Gao *et al.,* 2015) while its application enhances the production of defense compounds (Zehra *et al.,* 2017). SA-responsive genes induce oxidative stress, increase level of hydrogen peroxide, inhibit catalase activity, affect activation of pathogenesis-related genes (Ananieva *et al.,* 2004; Madhusudhan *et al.,* 2009), hypersensitive response (HR) and the systemic acquired resistance (SAR) during pest attacks and pathogen infection (Hayat *et al.,* 2012; Gao *et al.,* 2015).

In this study, the effects of salicylic acid, millet headminer and aluminum on the methylome variation were evaluated using MSAP epigenotyping.

5.2 Materials and methods

5.2.1 Aluminum and Salicylic acid treatments during seedling development

Leaves and roots from seedlings treated with 1.5mm salicylic acid and 400µm aluminum respectively, and from the foliar treatment of 1.5mm salicylic acid were harvested for DNA extraction, including leaves from the control without treatment of salicylic acid or aluminum. DNA was extracted from leaves of 20 seedlings (4 lines, 5 SA doses) with four replicates using ZR plant/seed DNA miniprep (Zymo Research, Cat No. D6020) following the company protocol and an isoamyl alcohol chloroform step was included to increase DNA yield and quality. DNA samples from SA treatments (20 samples) were considered individually. Additionally, roots treated with combined 400µm aluminum and 0.5mm were harvested for methylation analysis.

5.2.2 Foliar treatment of salicylic acid during panicle development

To study the role of salicylic acid during the millet headminer-pearl millet interaction, salicylic acid was applied at panicle stage. After treatment (1.5mM), leaves from subplots (salicylic acid and controls) were collected for methylome analysis. For control subplots (without salicylic acid treatments), they were divided into positive control (with infested plants) and negative control (healthy plant) plants. The DNA was extracted from 15 leaves for each variety using the ZR plant/seed DNA miniprep (Zymo Research, Cat No. D6020).

5.2.3 DNA extraction protocol

The DNA from leaves and roots were extracted using ZR plant/seed DNA minipreps (Cat No. D6020) following the company extraction. Therefore, the procedure was a little bit modified for more efficiency. An isoamyl alcohol chloroform step was added to the protocol. The eluted DNA was subjected to genomic DNA methylation analysis.

5.2.4 MSAP Epigenotyping

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 17). 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 16). The adaptors were renatured by heating at 98°C for 5 min, cooled down at room temperature in a polystyrene box for 2 hrs., and held at 4°C. The ligation mix was incubated overnight at room temperature. Pre-selective amplification was performed in a 50 µL reaction volume with EcoRI primer (10 mM), and MspI/HpaII primer (10 mM) (Table 16), diluted restriction-ligation DNA and One Taq standard buffer. The pre-selective amplification was realized with the following temperature cycling conditions: one cycle at 94°C for 30 s; 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s, and finally one cycle at 72°C 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 16) 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, 56°C for 30 s and 72°C for 60 s, and finally one cycle at 72°C for 60 s. The size of PCR products was then checked on a agarose gel, and the MSAP profile was used for data scoring.

Table 16: Primers and adaptors used during the epigenotyping

Epigenotyping steps	Adaptors and Primers	Sequences (5'-3')
Ligation	EcoRI adaptors	CTCGTAGACTGCGTACC
		AATTGGTACGCAGTCTAC
Pre-selective amplification	MspI/HpaII adaptor	CGAGCAGGACTCATGA
	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

5.2.5 Data scoring

The MSAP profile was then transformed into a binary matrix, with 1 as a presence of loci and 0 the absence of loci (Table 17). 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.

Table 17: Sensitivity of the isoschizomers HpaII and MspI to different types of methylation of CCGG

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	-

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).

5.3 Results

5.3.1 Effects of salicylic acid on methylome changes

5.3.1.1 CCGG DNA methylation level

The methylation analysis was performed for 0.5 mm SA dose. The effects of SA treatment on the pearl millet methylome using MSAP polymorphism showed, 63% of epiloci are polymorphic. The level of methylation decreased following the application of 0.5 mm of SA for all varieties, except PMG ($p<0.05$). This result was similarly correlated ($R=0.93$) with the root growth (Figure 13).

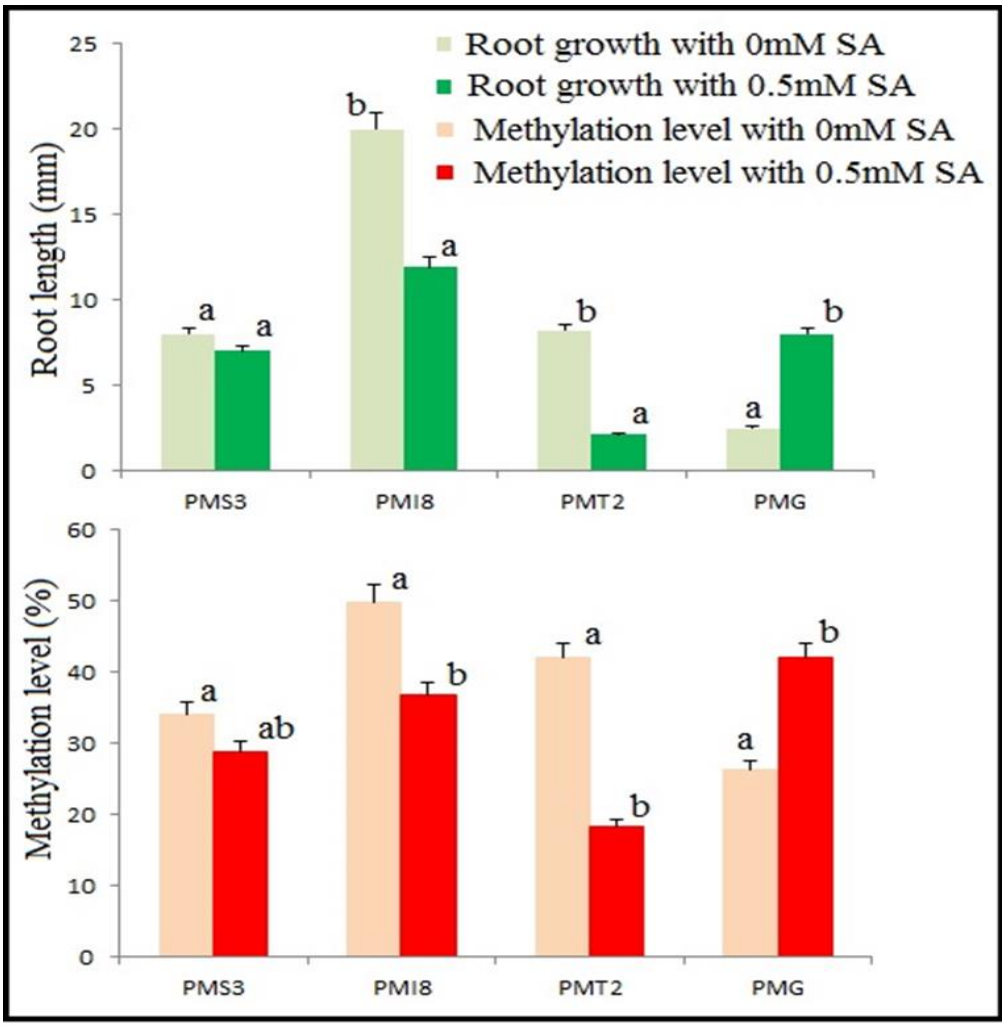

Figure 13: Dynamic correlation between methylation level and root growth under SA treatment. *This showed a positive correlation. The methylation level decreased with the root length for PMS3, PMI8 and PMT2, in contrary for PMG. Same letter means no significant difference at 0.05 level. SA: salicylic acid.*

5.3.1.2 CCGG methylation mapping

All the varieties tested including the controls (without SA) were external cytosine hypermethylated (hemimethylation, mCCGG), except PMT2 where the hypermethylation was internal. In addition, the hypomethylation state mostly occurred in the internal cytosine methylation (PMS3, PMT2 Treated plants, PMG control plants). The varieties are highly hyper-hemimethylated (mCCGG),

while the methylation level is low at the internal cytosine (CmCGG) forPMS3 (Hypomethylation) (Figure 14).

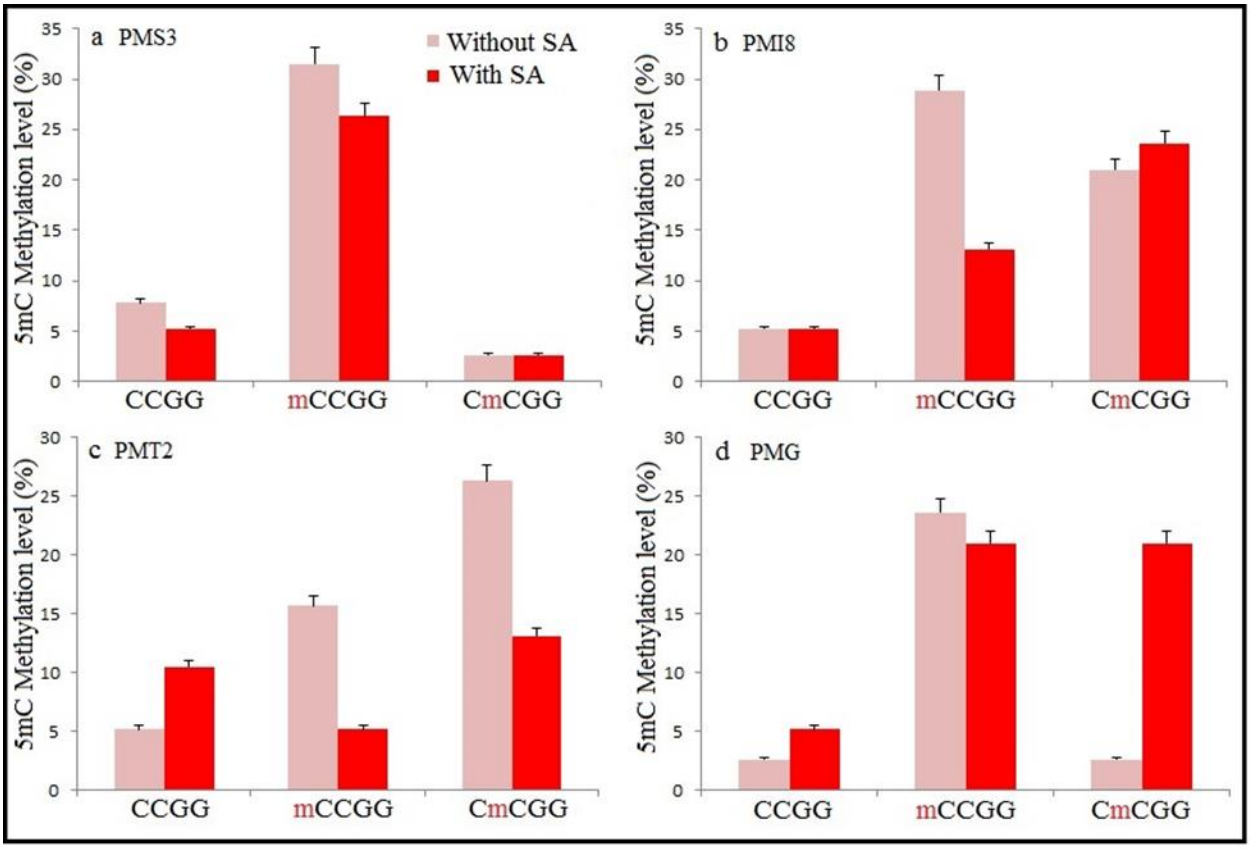

Figure 14: External and Internal cytosine methylation level after SA treatment Hypermethylation occurred mostly at mCCGG for all varieties with and no SA treatment. Data are shown as percentage ± s.d. a: PMS3, b: PMI8, c: PMT2, and d: PMG. SA: salicylic acid.

5.3.1.3 Absence of targets of CCGG

The percentage of mutated and non-targeted CCGG were determined. The results showed that treatment of salicylic acid increased the level of non-target CCGG sites, excepted for PMG where a decrease was noticed (Table 18).

Table 18: Non-targeted CCGG motifs following salicylic acid application

Varieties	Salicylic acid doses (mm)	
	0	**0.5**
PMS3	57.8	65.7
PMG	71	52.6
PMI8	44.7	57.8
PMT2	52.6	71

5.3.2 Effects of aluminum on DNA methylation

5.3.2.1 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 15).

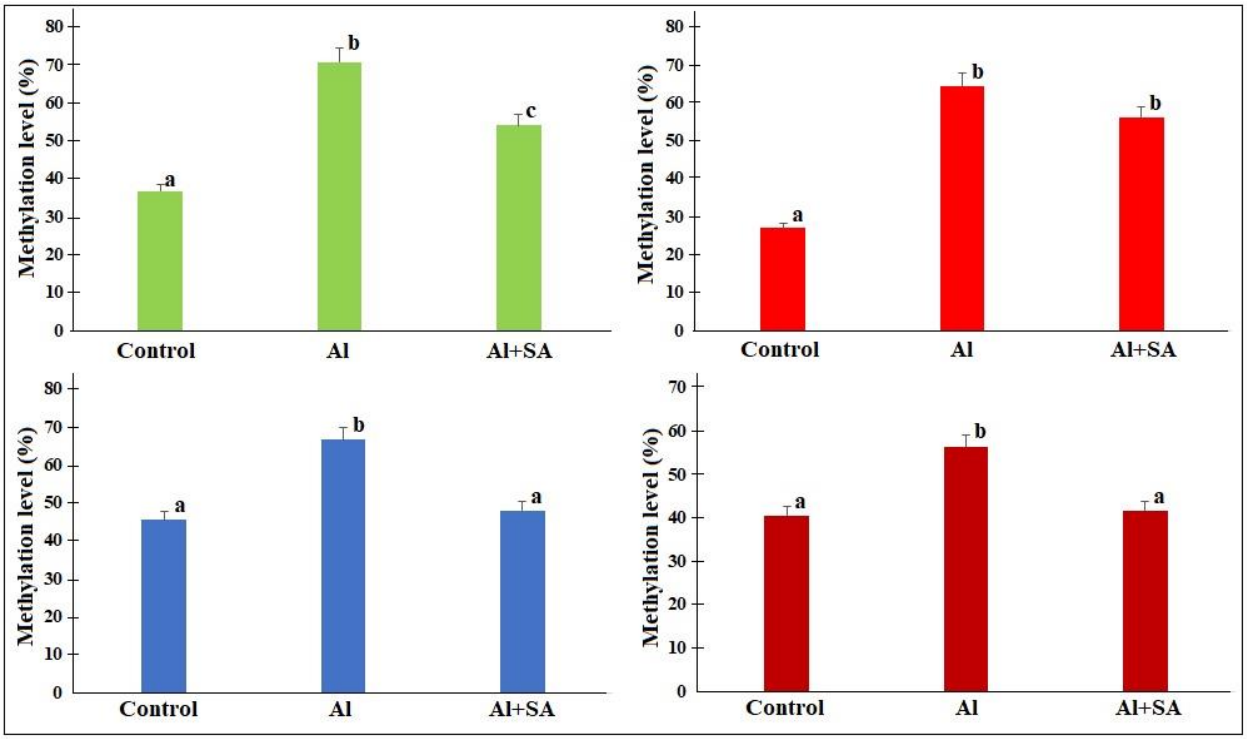

Figure 15: Methylation level after aluminum and salicylic acid treatments
Highest methylation level found during aluminum application. Al: aluminum, SA: salicylic acid, control: without SA and Al. CCGG: non-methylation, mCCGG: hemimethylation, CmCGG: internal cytosine methylation.

5.3.2.2 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 16).

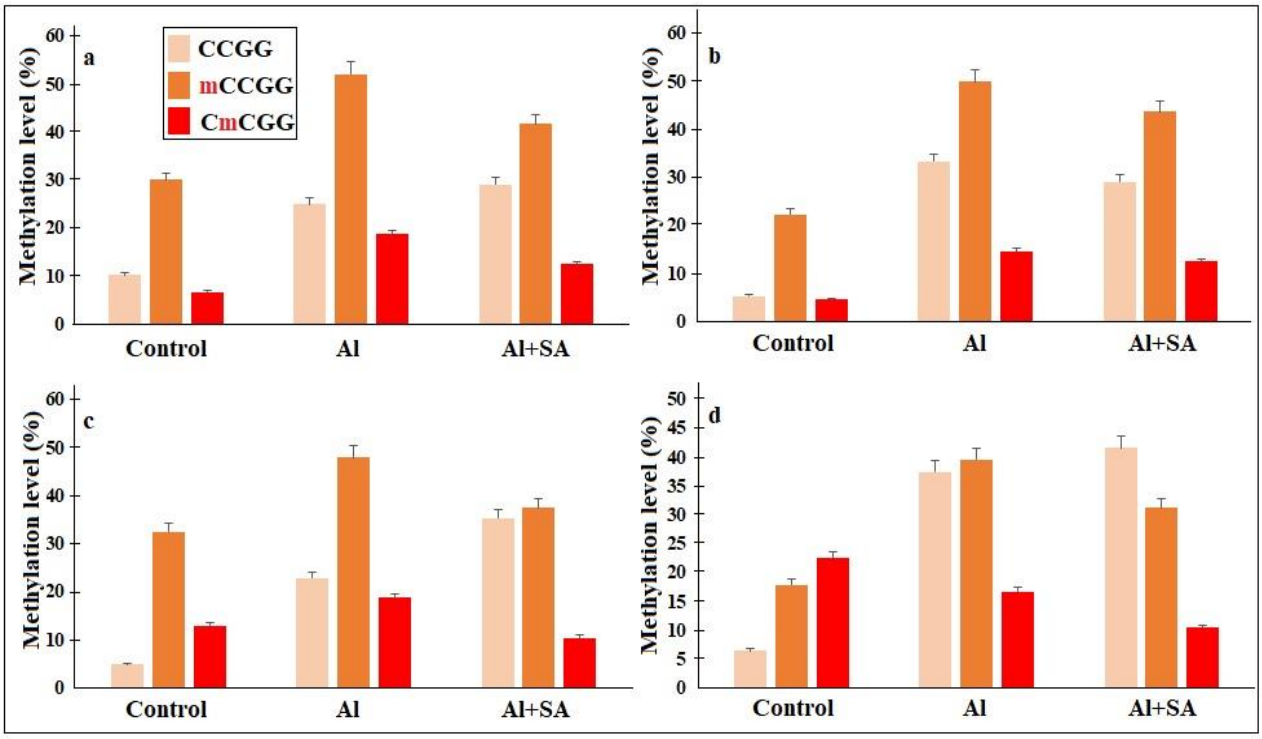

Figure 16: Types and occurrence of methylation following aluminum and salicylic acid applications.

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.

5.3.2.3 Absence of targets of CCGG

The level of non-target elements was highest at control level (without aluminum and salicylic acid). Treatment of aluminum reduced drastically the level of mutation/non-targets while combination of salicylic acid and aluminum increased a bit its level (Table 19).

Table 19: level of non-target and mutations of CCGG sites.

Variety	Aluminum and salicylic acid treatments		
	Control	**Al**	**Al+SA**
PMS3	52.85	4.16	16.67
PMG	67.69	2.08	14.58
PMI8	49.47	10.42	16.67
PMT2	53.00	6.25	16.67

High at control treatment for all varieties. Al: aluminum, SA: salicylic acid.

5.3.3 Effects of *Heliocheilus albipunctella* on DNA methylation variation

5.3.3.1 CCGG DNA methylation level

Analysis was performed to determine the methylation level following the millet headminer feeding (positive control), without feeding (negative control) and salicylic acid treatment at 1.5 mm. The methylation level varied significantly from a variety to another ($p<0.05$). The methylation level was higher during *H. albipunctella* feeding (positive control) and decreased after SA application in all varieties. The average normal methylation level (negative control) for all varieties was 61%, while individually the highest (75.5%) was found in PMS3 (Figure 17).

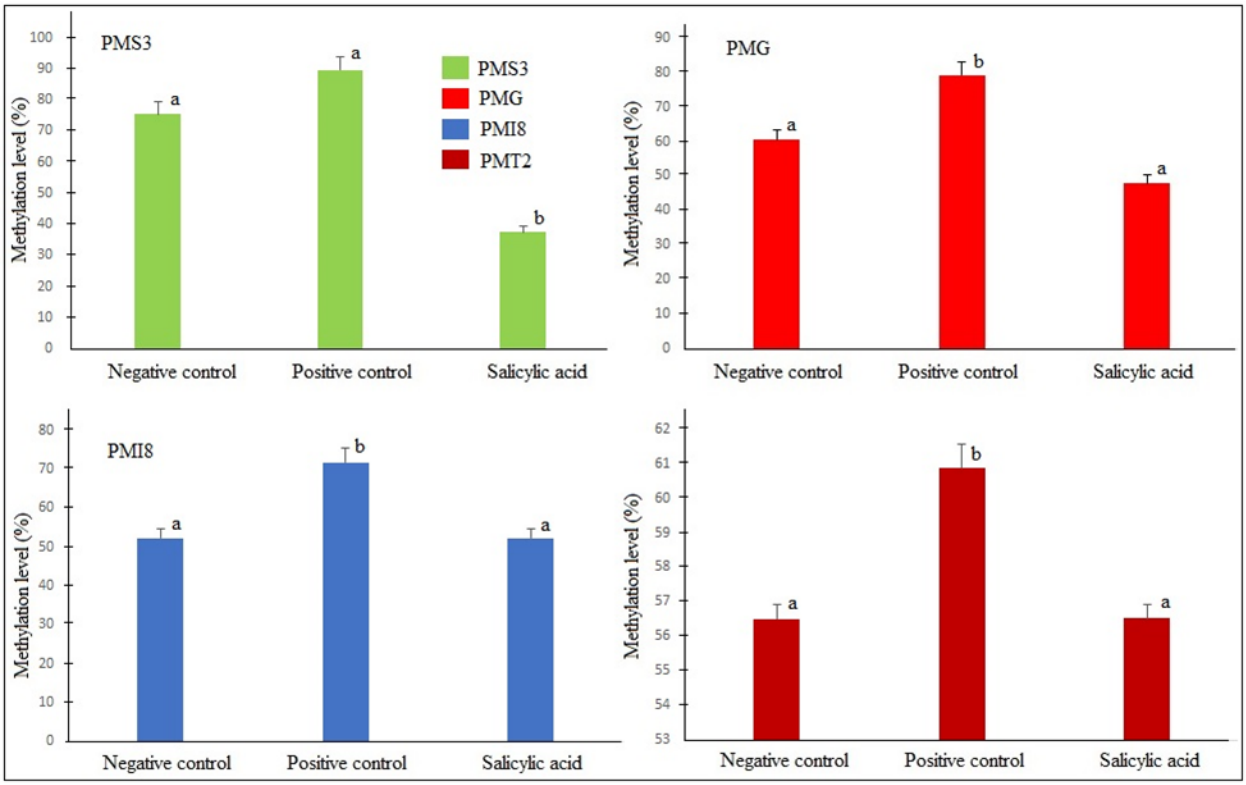

Figure 17: Methylation level of pearl millet following headminer feeding and salicylic acid treatments

Methylation level and mutated CCGG sites of each treatment was determined using MSAP analysis and RMSAP respectively. Millet headminer feeding increased the level of methylation, while SA treatments decreased. The CCGG mutation level was higher in PMG and PMI8 during salicylic acid application. Means with the same letter are no significant difference at 0.05 probability level. a: PMS3; b: PMG; c: PMI8; d: PMT2

5.3.3.2 CCGG methylation mapping

The types of methylation and its occurrence were determined. The results showed that most of the methylation occurred at the external cytosine (hemimethylation, mCCGG) which is higher during insect feeding. Additionally, the hemimethylation level decreased after salicylic acid treatment. In each variety, the level of unmethylated CCGG sites decreased after larvae infestation while application of salicylic acid seemed to reestablish its pattern (Figure 18).

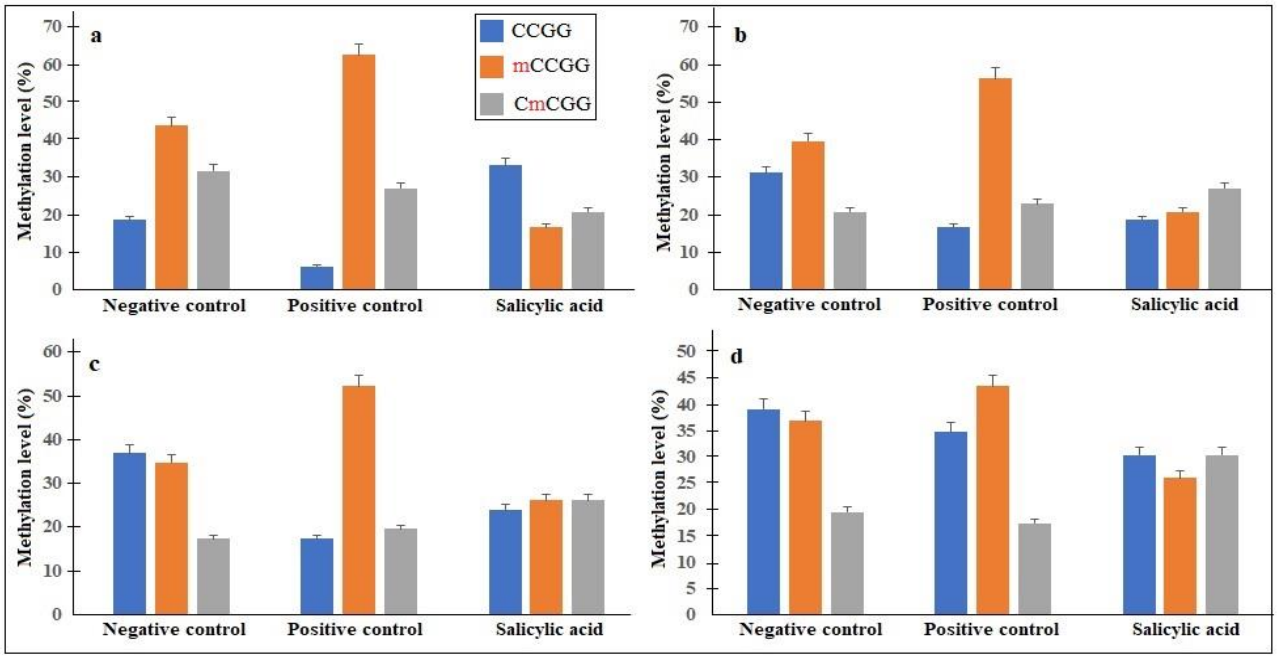

Figure 18: Occurrence of types of methylation during pest stress

Results showed most of the methylation occurred at the external cytosine. Results shown as percentage ± s.d. a: PMS3; b: PMG; c: PMI8 and d: PMT2. CCGG: unmethylation; mCCGG: external cytosine methylation; CmCGG: Internal cytosine methylation

5.3.3.3 Absence of targets of CCGG

The percentage of non-target CCGG sites were very low for PMS3 variety in control and salicylic acid application. However, these increased after salicylic acid treatment for the rest while PMG having the bigger increase (Table 20).

Table 20: Levels of non-target and mutations in CCGG sites

Varieties	Non-target/Mutation status		
	Negative control	**Positive control**	**Salicylic acid**
PMS3	6.25 a	4.16 a	2.91 a
PMG	8.33 a	4.16 a	33.30 b
PMI8	10.87 a	10.87 a	23.91 ab
PMT2	4.34 a	4.34 a	13.40 ab

Same letter means no significant difference at 5% level.

5.4 Discussion

5.4.1 Stressors increase CCGG methylation level

The effects of stressors such as aluminum toxicity and pest stress like *H. albipunctella* on methylome were evaluated on four pearl millet varieties. The results revealed a significant difference ($P<0.05$) between the controls (without stress) and the others (with stress) and revealed an increase of DNA methylation levels during stresses.

The millet headminer increased the DNA methylation level while feeding. This suggests a functional role of larvae oral secretion which is secreted on plants during feeding. The effects of oral secretion may have two trajectories, eliciting or suppressing effects. As, the larval density of *H. albipunctella* is positively correlated with the methylation level, the suppressing effects could be responsible for the increase of the genomic methylation. It seems its oral secretion may contain suppressors playing key roles in downregulating resistance genes through the different layers of the plant defense at translational, posttranslational or methylome levels. Like *H. albipunctella*,

aluminum treatment also increased the genomic DNA methylation in pearl millet. Many studies reported similar results in maize (Kimatu *et al.,* 2013; Taspinar *et al.,* 2018) while Bednarek *et al.,* (2017) reported contradictory results in Triticale with demethylation occurring during aluminum treatments in CG context.

Furthermore, the aluminum and pest stresses seem to share similar methylation pattern. Indeed, the external cytosine methylation was higher during aluminum and pest stress, indicating a functional role of the external cytosine. This high occurrence rate of the external cytosine methylation during stress could reveal important role of the external cytosine in plant tolerance to stress. The demethylation occurred after salicylic acid treatment was mostly that of the external cytosine. The external cytosine may be important 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 (pest feeding, aluminum and salicylic acid) could be a chemical player affecting methylation pattern. The cellular external cytosine DNA methylation and demethylation is far from being explained. All these stress elements (oral secretion from larvae and aluminum ions Al^{3+}) could play important role in the induction of the RdDM through downregulation of the DME and ROS1 and activation of the DRM2.

5.4.2 Salicylic acid promotes defense mechanism of pearl millet

5.4.2.1 Inhibition of root growth, a mechanism of plant defense strategy

Methylation analysis revealed similar pattern between the level of methylation and the root growth of the seedlings. In fact, root growth decreased with the treatment of salicylic acid, as well as the methylation level for PMS3, PMI8 and PMT2 varieties. In contrast, when the root growth increased after salicylic acid application, the methylation rate also increased for PMG variety. This indicated that salicylic acid and the DNA methylation may participate to downregulate the root

growth genes. The expression of demethylase increased after salicylic acid treatment in *Vitis amurensis* (Gaffney *et al.,* 1993), suggesting a decrease of methylation level. Hypomethylation often followed salicylic acid treatments, particularly for PMT2, indicating the activation and overexpression of certain R genes. These salicylic acid-demethylation targeted regions may play a dynamic role in plant development and defense. Salicylic acid decreases methylation rate while inhibiting or minimizing the plant development. Indeed, two hypotheses are proposed.

The first hypothesis is the salicylic acid-repressing pathway 1 (SARP). In this case, salicylic acid may downregulate the RNA-directed DNA Methylation (RdDM) pathway, inducing the Repressor of Silencing 1 (ROS1) demethylase activity. The exogenous salicylic acid application is detected by the Flagellin Sensitive 2 (FLS2), one of the PRRs, that initiates the demethylation pathway (Pumplin & Voinnet, 2013). Therefore, a mutated NRPD2 gene encoding DNA-directed RNA polymerases IV and V subunit 2 and responsible for the overexpression of a salicylic acid-Inducible gene procured a functional relationship between stresses signaling and the RNA-directed DNA methylation (RdDM) pathway (Lopez *et al.,* 2011). Moreover, the same results were obtained in mutants partially defective in CG and non-CG methylation, and applications of demethylating agents, such as 5-azadeoxycytidine, reduces plant defense (Akimoto *et al.,* 2007). Enhancing RdDM in ROS1–4 plants leads to lowered resistance to Pst DC3000 (Yu *et al.,* 2013).

The second hypothesis is about the inhibition of plant development, particularly the root growth through SARP2. This SARP2 could be deployed to minimize the vegetative development like root growth and prioritizes the plant defense responses. Some of the R genes after activation by demethylation via SARP1 may control some genes implicated in root growth by downregulating its expression. Auxin is the main hormone regulating root and plant development (Tanimoto, 2005; Overvoorde *et al.,* 2010; Jung & McCouch, 2013). Emerging evidence indicates that auxin is

involved in plant disease susceptibility. GH3.5, a member of the GH3 family of early auxin-responsive genes in *Arabidopsis,* acts as a bifunctional modulator in both salicylic acid and auxin signaling during pathogen infection. Studies showed an upregulation of the GH3.5 gene in an activation-tagged mutant gh3.5–1D led to an elevated accumulation of salicylic acid and increased expression of PR-1 in local and systemic tissues in response to virulent pathogens. Furthermore, 2 T-DNA insertional mutations of GH3.5 partially compromised the SAR with downregulation of PR-1 in systemic tissues (Zhang *et al.*, 2007). The salicylic acid pathway is amplified by GH3.5 through inducing salicylic acid-responsive genes and basal defense components, whereas the auxin pathway is depressed through up-regulating indole-3-acetic acid (IAA) biosynthesis and down-regulating auxin repressor genes (Zhang *et al.*, 2007), causing root growth and plant development inhibition (Figure 19).

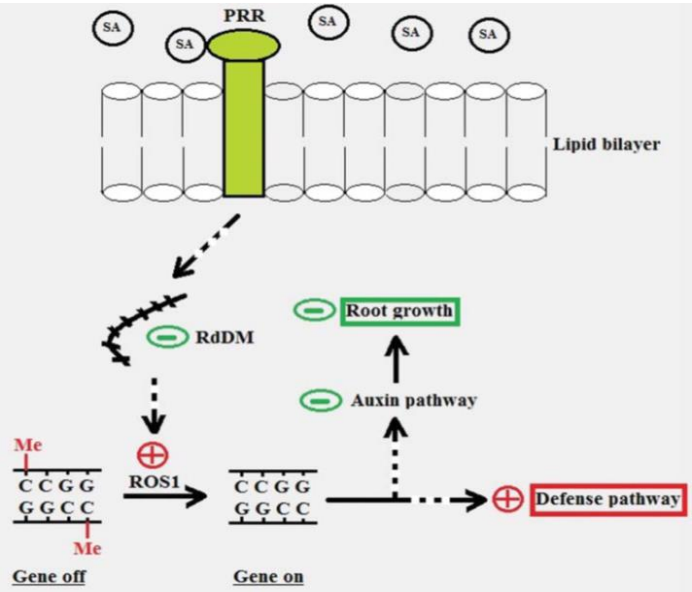

Figure 19: Theoretical actions of exogenous SA on Pearl millet

SA application is detected by Pattern Recognition Receptors (PRRs). This initiates a downregulation of the RNA-directed DNA Methylation (RdDM) and an induction of Repressor of Silencing 1 (ROS1) demethylase activity that activates R genes. Some R genes could control some genes implicated in early root growth (Auxin), minimizing its expression. Me: methyl group; SA: salicylic acid; CCGG: target region.

5.4.2.2 Salicylic acid confers tolerance to pearl millet

Salicylic acid has demonstrated many functional roles in plant development (root growth and germination) and plant defense including abiotic stress (aluminum toxicity) and biotic stress (*H. albipunctella*). In all these cases, pearl millet reacts in the same way by decreasing the level of methylation during either pest attacks or aluminum application.

The negative effects from aluminum treatments also are overcome by salicylic acid application, by causing DNA demethylation. 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.

Additionally, applying salicylic acid removes suppressing effects of *H. albipunctella* through DNA demethylation in pearl millet by reducing the larval density. Many studies revealed the effect of SA as an elicitor of plant defense, especially for pathogen and abiotic stresses (Fragniere *et al.*, 2011; Khan *et al.*, 2015). It seems now that SA-eliciting pathway is more complex and has a cross-talk with the defensive mechanisms involved during herbivore attacks. In fact, plants use direct and indirect ways of defense during insect attacks. The first way involves production of compounds and enzymes directly targeting the pest, while the second uses volatile compound organics release to attract its predators and parasitoids (War *et al.*, 2011; Song & Ryu, 2013; Shi

et al., 2016). Each mechanism involves complex gene-related defense regulations controlled at epigenetic level which could be blocked by the larvae feeding through molecules released in the plant tissues. This inhibition is overcome by the eliciting effects of SA through methylome regulations. In some plants, SA influences the defense responses following insect elicitor treatments (Engelberth *et al.*, 2011).

6.0 Chapter 6: Epigenetic diversity during stress

6.1 Introduction

Biological diversity within species can be an important driver of population and ecosystem functioning. Traditionally, genetic resources of crop plants have been characterized using a combination of morphological and agronomic traits, such as growth habit, earliness, and disease and pest resistance as well as biochemical and molecular markers (Gepts & Clegg, 1989). Although this information is important, evidence is growing that the genetic diversity is not only from mutations, but phenotype variation may be from epigenetic changes including DNA methylation that promotes it (Ashikawa, 2001). Epigenetically diverse populations of *Arabidopsis thaliana* produce up to 40% more biomass than epigenetically uniform populations (Latzel *et al.*, 2013). The positive effects of epigenetic diversity increase within plant community growth with stressor pressure (pathogen, pest, abiotic stress, etc.).

Pearl millet could present all these characters in a changing ecosystem with more stressor pressure. In this chapter, the aim was to determine the epigenetic diversity induced by salicylic acid and stressors such as aluminum toxicity and *H. albipunctella* using DNA methylation analysis.

6.2 Materials and methods

Four pearl millet varieties were used in this study. Aluminum (400µM) and salicylic acid (0.5µM) were applied during seedling development; and 1.5mM salicylic acid during panicle stage. Epigenetic data have been generated from DNA methylation status of the four pearl millet varieties by analyzing EcoRI-MspI and EcoRI-HpaII fragmentation profiles separately and together. Different indices were calculated using R version 3.2.5 and GenAlex version 6.5 (Peakall & Smouse, 2012): Nei epigenetic distance, and Clustering analysis with PcoA was carried out using RMSAP version 1.1.8.

6.3 Results

6.3.1 Effects of aluminum on 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 (0.5) increased the epigenetic variation in all varieties (Figure 20).

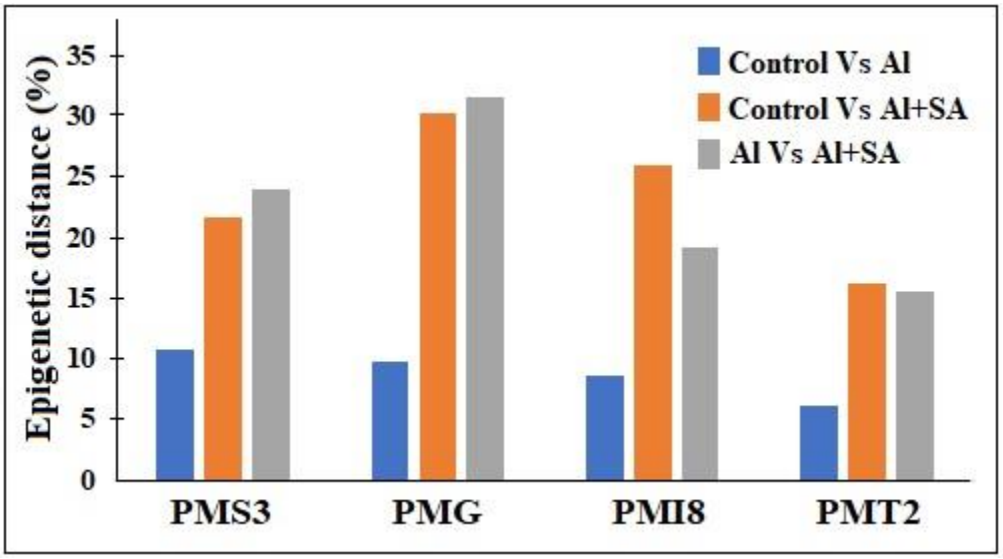

Figure 20: Nei epigenetic distance following Al and SA treatments
Salicylic acid increases the epigenetic variation. Al: aluminum; SA: salicylic acid.

Furthermore, clustering analysis was performed using PcoA. Three clusters were determined. The cluster 1 and cluster 3 gathered all plant controls and plants treated with combination of salicylic acid and aluminum respectively. Finally, the cluster 2 had all aluminum treated plants, except PMS3 treated with combined salicylic acid and aluminum (Figure 22).

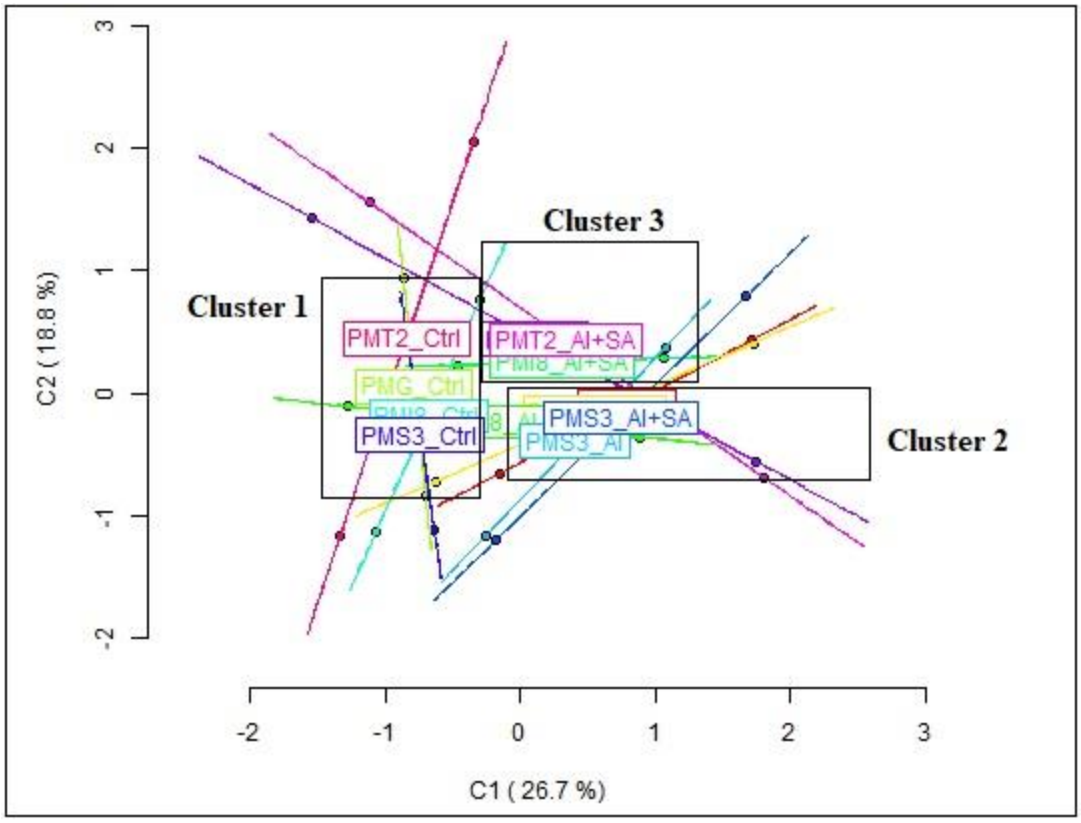

Figure 21: Principal coordinates analysis of methylated loci following aluminum and salicylic acid applications.
Three clusters revealed. SA: salicylic acid; Ctrl: Control; Al: aluminum.

6.3.2 Effects of pest on epigenetic diversity

The effects of pest feeding and salicylic acid applications on epigenetic diversity were determined with Nei epigenetic distance and principal coordinates analysis using GenAlex Version 5.3 and R version 3.2.5, respectively. The results revealed similar patterns with the epigenetic diversity induced by aluminum. The epigenetic diversity and rate was higher with salicylic acid and lower with pest (Figure 22).

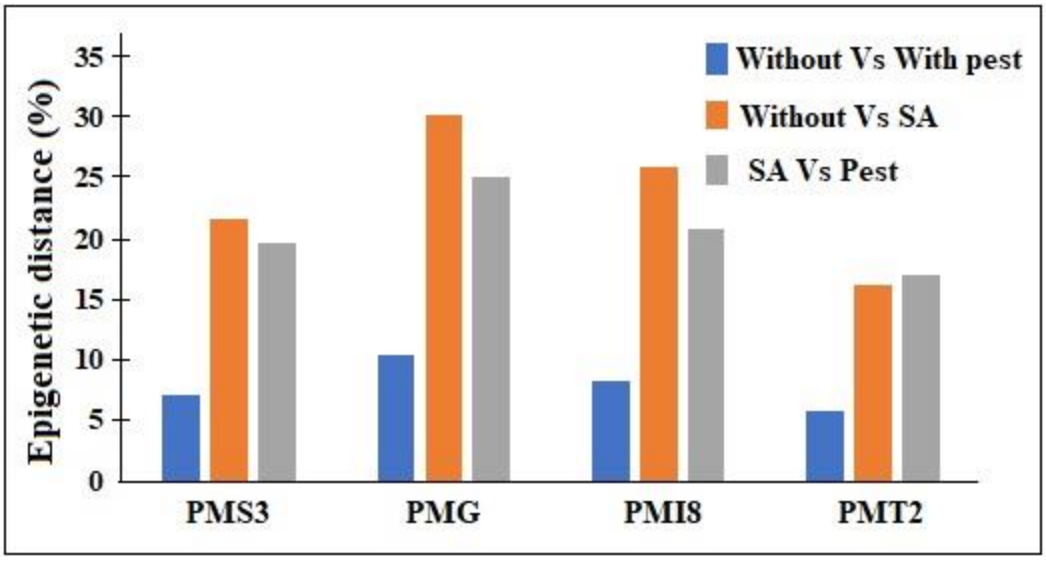

Figure 22: Nei epigenetic distance after millet headminer attacks and salicylic acid treatment
Salicylic acid increases the epigenetic variation. SA: salicylic acid; Pest: millet headminer

Furthermore, the principal coordinate analysis (PcoA) revealed three separated clusters. The larvae feeding (positive control) and the healthy plants (negative control) clustered separately from the others. The SA treatment formed a group with only PMS3, PMT2, and PMI8, with PMG being isolated as a single cluster (Figure 23).

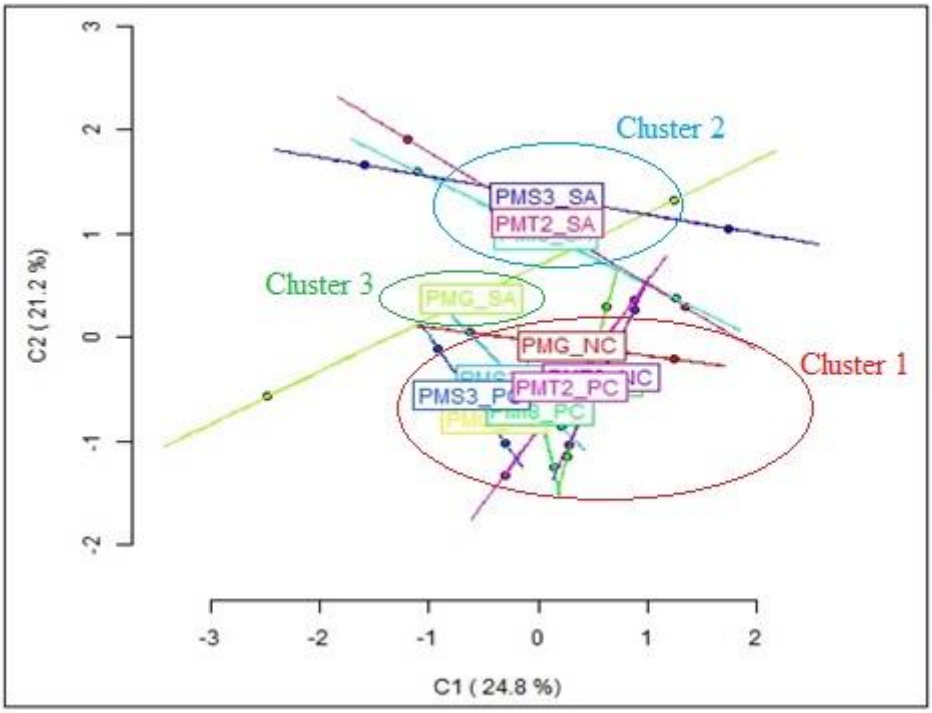

Figure 23: Principal coordinate analysis of methylome loci.
Three groups appeared: the negative and positive controls, the SA group (PMS3_SA, PMT2_SA, and PMI8_SA) and the single PMG_SA group. SA=salicylic acid; NC=negative control; PC=positive control. C1=24.8% and C=21.2%. Cluster 1 gathers the feeding and non-feeding groups; Cluster 2 gathers the SA treatments except for PMG; Cluster 3 with the isolated SA for PMG.

6.4 Discussion

The principal coordinates analysis and Nei epigenetic distance were used to determine the epigenetic diversity following stress and application of salicylic acid. The results showed that epigenetic diversity was lower during stresses including aluminum toxicity and headminer attacks and increased with treatment of salicylic acid. Additionally, plants clustered into different groups according to stress and salicylic acid.

In fact, the diversity of the epigenetic loci seems to be lowered during stress and normal times in all pearl millet varieties. The comparison between plant with and without stress (headminer and aluminum toxicity) revealed a low epigenetic diversity. However, plants subjected to salicylic acid

tend to increase the epigenetic diversity. This could a salicylic acid mechanism conferring to the plants more diversity by generating epialleles based-stress. Today, many studies focus on the use of epialleles for crop improvement (Zhang & Hsieh, 2013). This epigenetic diversity could increase the plant biomass and stability in ecosystem (Latzel *et al.*, 2013).

7.0 Chapter 7: General Discussion

The study of effects of stress on plant epigenome offers important understanding in plant adaptability and plasticity to changing environment and threats. Aluminum toxicity and millet headminer remain two stressors limiting crop yield in Africa. A study was carried out to investigate the effects of those stressors on pearl millet methylome during seedling and panicle development. The results showed that both stressors increased the genomic methylation level. However, the application of salicylic acid diminished that DNA methylation level. During seedling development, root growth was inhibited by salicylic acid treatment corresponding to a decrease of the methylation level, except for PMG variety. This suggests a plant mechanism downregulating hormonal pathways involved in root development like auxin pathways and promotion of plant defense. These SARP 1-2 pathways described early in the text could reveal the important role of the gene family GH3 involving in that defensive mechanism. This argument is supported by the fact of the effects of salicylic acid on both aluminum toxicity and pest attacks. In both cases, salicylic acid promotes defense pathways by decreasing the gene methylation level and the headminer density per panicle.

These defense mechanisms seem to indicate a cascade of metabolism reaction from plant signaling to defensive compound synthesis. Indeed, plants have innate systems of perception surface-localized on plasma membrane. That pattern recognition receptors play important roles in the detection of oral secretion and oviposition fluids from pests, the PAMPs or the MAMPs, the DAMPs. These perceive conserved microbial and pest features resulting during disease or attacks conducting to the deployment of the hypersensitive response (Brodersen *et al.*, 2005; Gust & Nürnberger, 2012; Kovács *et al.*, 2016) and systemic acquired resistance (Luo *et al.*, 2017) through salicylic acid pathways.

Moreover, the growth inhibition and the energy mobilization facts for defense mechanisms could be supported by negative effects of salicylic acid on photosynthesis by decreasing the chlorophyll and carotenoid contents in cowpea, wheat or in sunflower plants (Cag *et al.*, 2009). Additionally, it induced stomatal closure and photoinhibition in *Arabidopsis* (Mateo *et al.*, 2004). Not only chloroplast functioning is affected, the redox cascades in mitochondria generate molecular signals like reactive oxygen, plastoquinone, and thioredoxin, which regulate many aspects of plant such as growth and defense are also influenced. This adjusts energy production to utilization, interfacing with hormone signaling including salicylic acid, to respond to environmental change at every stage of plant development (Foyer & Noctor, 2003).

However, salicylic acid application and use have emerged many questions about the doses and mechanisms on epigenome. According to Khan *et al.*, (2003), its metabolic activities seem to be contradictory in plants, suggesting a bidirectional hypothetical model. In fact, low concentrations of SA enhanced metabolic activities such as plant defense and development while higher concentrations could inhibit them (Figure 25).

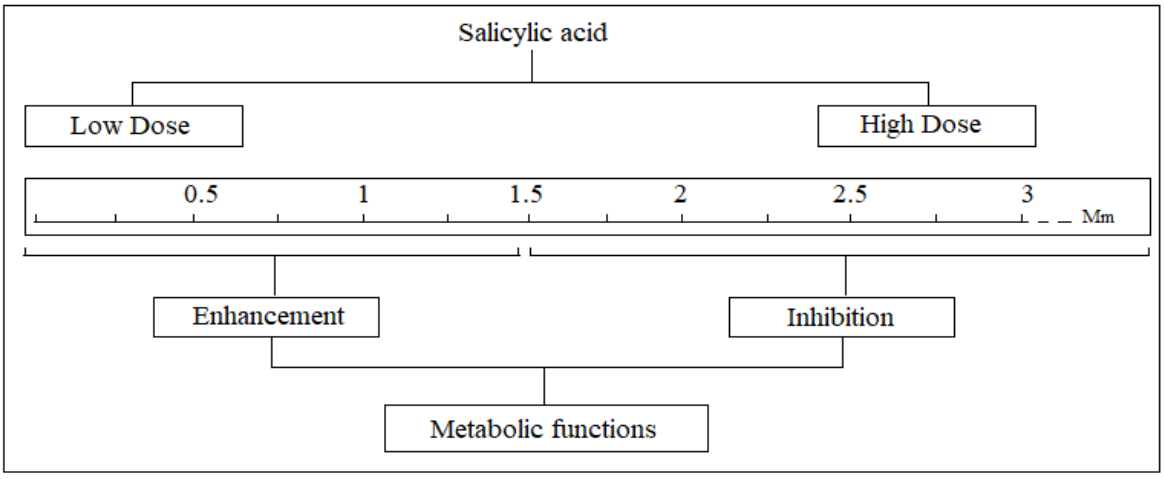

Figure 24: Bidirectional model of actions of SA in pearl millet.
High SA concentrations seem to generally inhibit the metabolic functions such as plant development and defense while low doses enhance them.

Furthermore, that bidirectional model could not explain itself the numerous roles of salicylic acid. It seems the most important role of salicylic acid is the death program in cell and plant levels. Indeed, at cell level, the death program is the programmed cell death. At this level, the damaged cells tend to be eliminated from the plant to avoid the development of the disease by accumulation of SA in the damaged sites (Verberne *et al.,* 2000). This contributes to transmit the stress signal to the whole plant conducting to the deployment of the systemic acquired resistance, as a cogenerational plant defense induction (Cogenerational memory). In contrast, in plant community level, when plants undergo to severe attacks, it could inhibit the plant defense and development to avoid therefore horizontal and vertical infestation or infection of the rest of the community. Meantime, following the effects of stress, plants increase the methylation levels. This methylation increase status could imprint specific epigenetic patterns by coding a methylation memory for prospective infestation or pest attacks, as a transgenerational plant defense induction (Transgenerational memory, Figure 26). This like-methylation memory coding acts as a mechanism imprinting on the genome the traces/marks of previous stresses and is transmitting during meiosis to the next generation. Transgenerational induction has been detected in offspring phenotypes in *Arabidopsis* with priming of state of SA-inducible genes (Luna *et al.,* 2012) or with the feeding of *Pieris rapae* (Rasmann *et al.,* 2012). Herbivory attacks generates trichomes in the next generation in *Mimulus guttatus* (Scoville *et al.,* 2011). Application of salicylic acid in *Taraxacum officinale* seedlings causes methylation patterns (Verhoeven *et al.,* 2010).

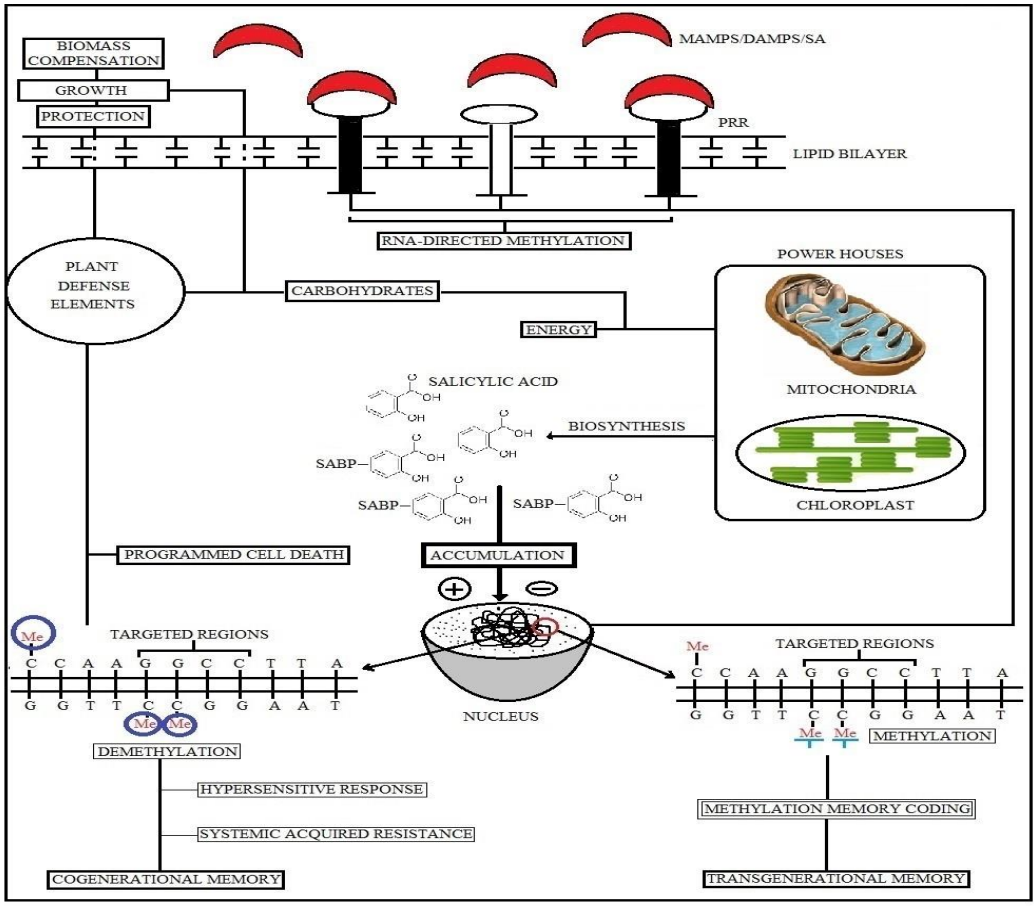

Figure 25: Hypothetical actions of Salicylic acid influencing plant defense strategies
During infection/infestation of pathogen/pest, these produce MAMPs/DAMPs detected by the PRRs. These PRRs initiate a plant response depending on the severity of the attack. Demethylation could be promoted if the attack can be overcoming (low severity). In this case, the hypersensitive response, the systemic acquired resistance and the biomass compensation are deployed to minimize and to restrict the damages (Tolerance/resistance) by producing plant defense elements. These take place at the same generation, starting at the infected/infested sites, then are transmitted to the whole plant (Cogenerational memory). In other hand, when the severity of the attack is high, the methylation increases, and the plant tends to limit its growth by inhibiting the carbohydrates and salicylic acid synthesis. This methylation imprints patterns transmitting through meiosis to the offspring for prospective attacks, as the transgenerational memory. SABP= salicylic acid-binding protein; Me= methyl group; PRR = pattern recognition receptor; MAMP= microbe-associated molecular pattern; DAMP= damage-associated molecular pattern; SA= salicylic acid.

In nature, the plant strategies for cogenerational or transgenerational plant defense induction do not work constantly. Sometimes, pests and pathogens find the way to overcome the defense responses conducting to the failure of the strategies of both trans-GM and Co-GM (Figure 27). This phenomenon is often seen in plant-pest interactions, pest producing in its oviposition fluids or oral

secretion, molecules that are suppressors of plant defense induction. These were isolated from *Helicoverpa zea* oral secretion (Eichenseer *et al.,* 1999; Musser *et al.,* 2005). Oral secretion was found to suppress wound-induced responses in *Arabidopsis* (Consales *et al.*, 2012).

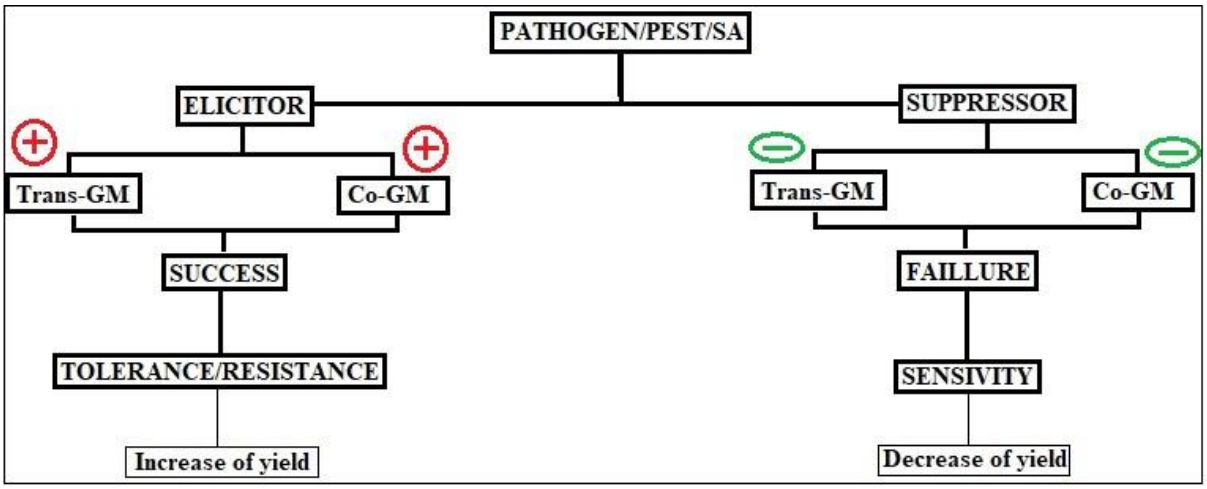

Figure 26: Relationship between elicitors and suppressors, and yield.
Trans-GM: transgenerational memory; Co-GM: Cogenerational memory

Conclusion and Recommendation

The study of the effects of stressors on pearl millet epigenome has offered interesting understanding on plant development and defense pathways. The methylome variation is indeed at the heart of metabolism responses to the changing environment and environmental threats. The plasticity and adaptability of plants like pearl millet depend on its capacity to regularly vary its own methylome that regulate the gene expression. Aluminum ions on acidic soils and oral secretion from pest feeding increase the methylome level corresponding to a downregulation of R genes. The headminer secretome contains suppressors of defense that may be investigated in future research. Research on application of salicylic acid as an elicitor of plant defense mechanism revealed a breakthrough about its action on the epigenome by lifting the defense-related gene inhibition. In both aluminum and headminer stresses, a methylome pattern appeared following applications of salicylic acid. The optimal doses of salicylic acid for effective protection of the plants during seedling and panicle development vary from 0.5 to 1.5mM. That range provides the protective methylome pattern of pearl millet. As the action of salicylic acid is not limited to the developmental stages, only the timing of applications matters. Treatments (seed or foliar) of salicylic acid should be carried out before the period of sensitivity preparing therefore the plants ready for prospective infestation or infection.

Additionally, both salicylic acid and secretome from pest could offer interesting perspectives in epigenetic studies. Both can modify the methylome pattern as "*bio-epidesigners*". This word is the first time employed in this study as a molecule having the biological power to influence the dynamism of gene activities through the epigenome regulation in a biological system. However, much works must be done to clearly identify the mechanisms behind the "bio-epidesigners" in order to implement them in crop improvement.

Finally, the transgenerational and cogenerational memories of plants through salicylic acid offer new perspectives to the researchers in plant breeding and IPM. Many questions and hypotheses are arising from the trans-GM and the Co-GM. The understanding of its epigenetic mechanisms associated with the defense hormones will open new insight into the epigenetic defense, and especially the hypothetical methylation coding.

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Appendices

Appendix 1: DNA extraction Protocol

The DNA from leaves, roots and seeds were extracted using ZR plant/seed DNA minipreps (Cat No. D6020) following the company extraction. Therefore, the procedure was a little bit modified for more efficiency. After harvesting the plant materials, they were hydrated during 10min. A mortar was used to grind the materiel properly with 750µL of lysis solution. Then, the mixture was put in the ZR Bashing Bead Lysis Tube incubated at 74° for 15min. At the end, the incubated tube was secured in a multi-plate shaker at 3200rpm during 20min. Then, the Bashing lysis tube was centrifuged at 10,000 rpm for 1min. After, alcohol isoamyl-chloroform was added to the bashing lysis tube.

350µL of supernatant were transferred to the spin filter in a collection tube and centrifuged at 7,000 rpm for 1min. 1200µL of DNA binding buffer were added to the filtrate in the collection tube. The mixture was mixed before to transfer 750µL to the IIC column in a new collection tube and centrifuged at 10,000 rpm for 1min. The flow-through was discarded and the last operation was repeated. Then, 200µL of DNA pre-wash buffer were added to the IIC column and was centrifuged at 10,000rpm for 1min. After the pre-wash step, 500µL of wash buffer were added to the same column and centrifuged at 10,000 rpm for 1min. The IIC column was transferred to a clean 1.5ml microcentrifuge tube, and 25µL of DNA elution buffer were directly added to the column matrix, and centrifuged at 10,000rpm for 30 seconds to elute the DNA. The eluted DNA was transferred to the IV-HRC spin filter and centrifuged at 8,000 rpm for 1min.

Appendix 2: Global methylation estimation

Global DNA methylation was measured using the enzyme-linked immunosorbent assay technique using an Imprint® Methylated DNA Quantification Kit (MDQ1, Sigma) with equal amount of DNA (100ng). In each well, 30 μL of Binding Solution was added to the diluted DNA. The plates were incubated at 37 °C for 60 minutes. The DNA Binding Solution alone was used as a blank to measure the absorbance. After, 150 μL of Block Solution directly to each well were added directly to each well and incubated at 37 °C for 30 minutes. Then, the DNA and Block solutions were removed from each well and washed three time with 150 μL of 1x Wash Buffer.

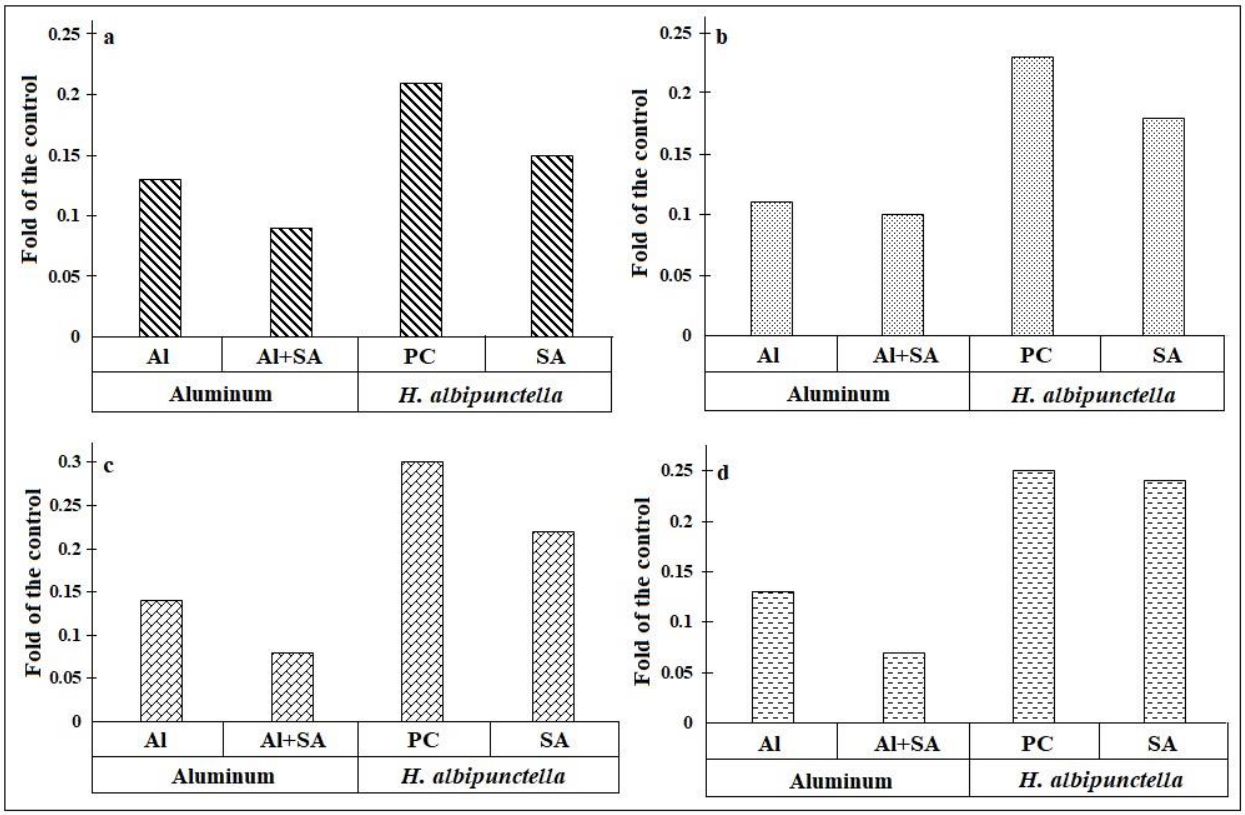
The Capture Antibody was diluted 1:1000 in 1x Wash Buffer, added to each well (50 μL) and incubated at room temperature for 60 minutes. After incubation, the diluted Capture Antibody was removed from wells and washed four times with 150 μL of 1x Wash Buffer. To each well was added 50 μL of Detection Antibody diluted 1:1000 in 1x Wash Buffer and incubated at room temperature for 30 minutes. The diluted Antibody was removed from wells, and then washed five times with 150 μL of 1x Wash Buffer.

100 μL of Developing Solution were added to each well and incubated at room temperature away from light for 1-10 minutes. When the solution turned to blue, 50 μL of Stop Solution were to each well. The new solution turned to yellow. The absorbance was read at 450 nm and the relative global methylation levels was calculated using the formula:

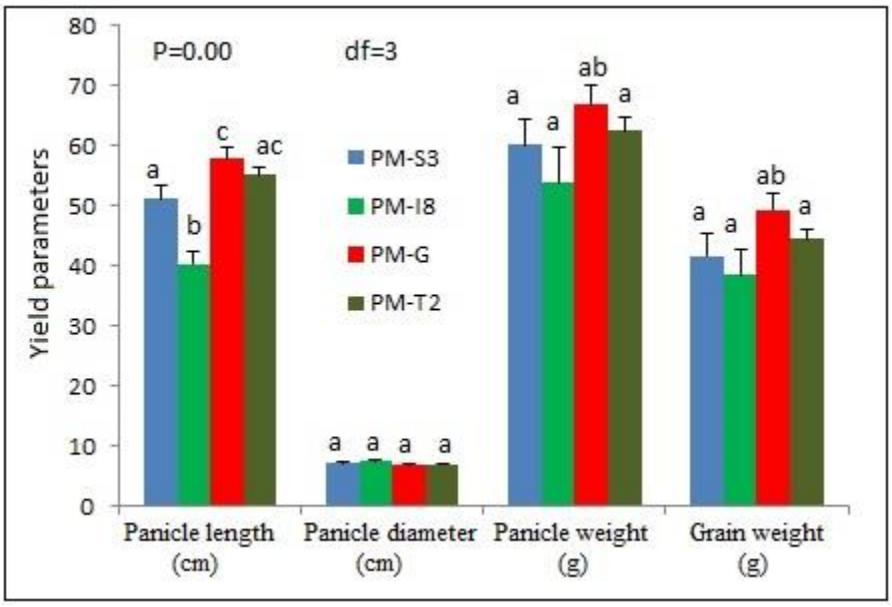
$$Me = \frac{S_{450} - B_{450}}{C_{450} - B_{450}} X\ 100$$

Where Me indicates the relative global methylation; S_450 the average measures of A450 from DNA samples; B_450 the average measures A450 of the blank solution; C_450 the average measures of the A450 of the methylated control DNA.

Appendix 3: Relative 5-methyl cytosine global methylation following aluminum and pest stress *Treatment of salicylic acid reduce the global fifth cytosine methylation. Al: aluminum, SA: salicylic acid, PC: positive control (with larvae feeding). PMS3, PMG, PMI8 and PMT2 represents the letters a, b, c, and d respectively.*



Appendix 4: Pearl millet yield parameters



Appendix 5: Pairwise Population Matrix of Nei Genetic Identity during aluminum treatment

PMS3			**PMG**			**PMI8**			**PMT2**				
Ctrl	Al	Al+SA	Ctrl	Al	Al+SA	Ctrl	Al	Al+SA	Ctrl	Al	Al+SA		
1.000												Ctrl	**PMS3**
0.901	1.000											Al	
0.806	0.788	1.000										Al+SA	
0.970	0.892	0.790	1.000									Ctrl	**PMG**
0.909	0.961	0.747	0.907	1.000								Al	
0.788	0.778	0.955	0.739	0.729	1.000							Al+SA	
0.934	0.849	0.776	0.923	0.881	0.727	1.000						Ctrl	**PMI8**
0.901	0.927	0.822	0.909	0.908	0.795	0.917	1.000					Al	
0.826	0.808	0.976	0.827	0.774	0.954	0.771	0.825	1.000				Al+SA	
0.935	0.843	0.778	0.932	0.891	0.729	0.976	0.902	0.773	1.000			Ctrl	**PMT2**
0.900	0.899	0.814	0.924	0.923	0.780	0.914	0.943	0.817	0.940	1.000		Al	
0.892	0.824	0.896	0.890	0.824	0.849	0.857	0.841	0.927	0.850	0.857	1.000	Al+SA	

Appendix 6: Pairwise Population Matrix of Nei Genetic Identity during pest attacks

PMS3			**PMG**			**PMI8**			**PMT2**				
NC	PC	SA	NC	PC	SA	NC	PC	SA	NC	PC	SA		
1.000												NC	**PMS3**
0.931	1.000											PC	
0.806	0.822	1.000										SA	
0.970	0.912	0.790	1.000									NC	**PMG**
0.919	0.977	0.779	0.900	1.000								PC	
0.788	0.830	0.955	0.739	0.778	1.000							SA	
0.934	0.878	0.776	0.923	0.875	0.727	1.000						NC	**PMI8**
0.905	0.953	0.809	0.904	0.941	0.790	0.922	1.000					PC	
0.826	0.852	0.976	0.827	0.799	0.954	0.771	0.811	1.000				SA	
0.935	0.853	0.778	0.932	0.860	0.729	0.976	0.906	0.773	1.000			NC	**PMT2**
0.904	0.887	0.800	0.919	0.894	0.775	0.918	0.943	0.803	0.944	1.000		PC	
0.892	0.851	0.896	0.890	0.832	0.849	0.857	0.828	0.927	0.850	0.844	1.000	SA	