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Full Length Research Paper

Morphological and cytosine DNA methylation changes induced by a combined effect of boron (B) and salt toxicity in *Sorghum bicolor* inbred line

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Boron (B) toxicity is one of the abiotic stresses limiting plant growth in arid and semi arid regions globally. Although studies have been conducted on the combined effect of B and sodium chloride (NaCl) toxicity on overall plant growth revealing an antagonistic relationship, the morphology and epigenetic interactions have not fully been explained. Germinating seeds of an inbred line of *Sorghum bicolor* (YN267) were subjected to various concentrations of B (10, 50, 100, 200, 300 and 400 mM) in a constant concentration of high NaCl (100 mM). Methylation-sensitive amplification polymorphism (MSAP) was used in the assessment of changes in the methylation levels and patterns. Morphological results show that plants at the B concentration range of 10 to 200 mM were adversely affected by the combined stress application than at 300 and 400 mM. In addition, the cytosine methylation status at 300 mM showed an increased overall hypermethylation, while hypomethylation was induced at 400 mM. These results show that not only did the combined treatment induced cytosine DNA methylation changes which was reflected in the plant morphology, but the alleviating effects of the combination at toxic levels are suggested to be due to the epigenetic alterations and expression/repression of stress responsive genes.

Key words: Cytosine DNA methylation, *Sorghum bicolor* L, boron and sodium chloride toxicity, methylation-sensitive amplification polymorphism (MSAP).

INTRODUCTION

The adaptation of plants to environmental stresses is known to be controlled by a chain of molecular networks (Vinocur and Altman, 2005), which include the triggering of secondary stress signals capable of inducing changes in epigenetic regulators (Chinnusamy and Zhu, 2009). These epigenetic regulators in turn modify molecular mechanisms such as DNA methylation (Chinnusamy and Zhu, 2005). DNA cytosine methylation is a very important epigenetic phenomenon in gene regulation and in controlling the effects of many abiotic stress factors on the DNA. Studies have shown that when there is hypermethylation of promoter sequences, transcription is silenced, while the hypermethylation of coding or transcribed regions leads to post-transcriptional gene silencing (Paszkowski and Whitman, 2001).

DNA methylation can be altered directly by the environment through abiotic stress factors (Richards, 2006), but the response of plants depends on the kind of stress (Peng and Zhang, 2009). Studies show that abiotic stresses in plants can either up-regulate or down-regulate the expression of certain genes causing hypermethylation or hypomethylation of DNA (Kovarik et al., 1997; Steward

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et al., 2002; Chinnusamy and Zhu, 2009). Abiotic stresses inhibit growth and limit productivity in many crop species by more than 50% from their potential yield (Gao et al., 2007) such as sorghum. They can occur indivi-dually, but have more harmful effects when they occur in combinations (Mittler, 2006).

A combination of abiotic stresses or one resulting in the occurrence of the other is boron (B) toxicity in the presence of soil salinity. B toxicity and salinity are closely associated, occur simultaneously and are found mostly in the arid or semi arid regions of the world (Gupta et al., 1985) like Africa and Asia. These areas are known for their irrigation practices due to limited available water supply and poor drainage system (Marschner, 1995; Nable et al., 1997). Recent reports have stated that the combination of B toxicity and salinity caused less severe adverse effect on plant growth than their additive effects if each was administered separately, implying that in the presence of salinity, B uptake is reduced and vice-versa (Yermiyahu et al., 2008). B tolerance mechanism of plants is found to be quite similar to salt tolerance mechanism, which could mean that plants that are tolerant to salt may resist B toxicity since salt-exclusion mechanisms can reduce the uptake of B (Alpaslan and Gunes 2001). This suggestion may account for the alleviating effect of the combination of B and salinity provided their transport mechanisms are inter-dependent. Sorghum is the world's fifth most important cereal after wheat, rice, maize, and barley in terms of both production and planting areas. Roughly, 90% of the world's sorghum area has been found to lie in the developing countries in Africa and Asia precisely (Léder, 2004). While sorghum constitutes a major source of calories and protein for millions of people in Africa and Asia, it is a staple food for about 300 million people worldwide (Léder, 2004) and an important source of feed, fibre, and biofuel in the developed countries (Doggett, 1976). Although much work have been done on the effects of either or both abiotic stress factors on many plants, less studies have been conducted on epigenetic changes induced by the combination of both stresses and the implications of these changes in sorghum.

The methylation-sensitive amplification polymorphism (MSAP) is a modified version of the standard amplified fragment length polymorphism (AFLP) fingerprinting technique (Vos et al., 1995), by incorporating *Hpa*II and *Msp*I, a pair of isoschizomers that recognize the same restriction site (5' CCGG) but have different sensitivity to methylation of the cytosines. Specifically, *Hpa*II will not cut if either of the cytosines is fully (double-strand) methylated, but will cut if the external cytosine is hemi-methylated (single strand); in contrast, *Msp*I will not cut if the external cytosine is fully- or hemi-methylated (McClelland et al., 1994). Therefore, in a given DNA sample, two major methylation states at the CCGG sites (either full methylation of the internal cytosine or hemi-methylation of the external cytosine) will be readily recognized in the MSAP profiles (Reyna-Lopez et al., 1997; Cervera et al., 2002). Some methylation states at CCGG sites (full methylation of the external or both cytosines) can be differentiated by this method, but only in situations where two or more tissues or developmental stages of the given genotype is being examined. This is because under such situations, any difference(s) in the MSAP profile should reflect differential methylation state at the CCGG sites, and hence, allow the estimation of full methylation of the external cytosine or both cytosines in one versus the other tissue or between developmental stages for the fixed genotype (Zhang et al., 2007).

The aim of this study was to investigate the alterations caused by both stresses on the morphology and cytosine DNA methylation in a sorghum inbred line as detected by methylation-sensitive amplification polymorphism (MSAP). We therefore determined how the morphological res-ponse of this sorghum is related to the observed epigenetic changes and through BLASTN analysis correlated the contributive effects of any responsive gene(s) induced by the stresses.

MATERIALS AND METHODS

The *Sorghum bicolor* L. inbred (pure) line used for the experiments is YN267 (D), which was provided by the Institute of Cereal Crops, Jilin Academy of Agricultural Sciences, Changchun, China. The pure line has been maintained in our laboratory by strict self-pollination for many generations. The sorghum inbred line D seeds were washed with distilled water, surface-sterilized using 8% sodium hypochlorite solution for 5 min and rinsed several times with again with water. The seeds were soaked in moistened cotton mesh in Petri dishes and kept in the incubator at 30°C for 72 h. Seeds were later placed in Petri dishes having sterile cotton mesh, moistened with 20 ml MS (Murashige and Skoog, 1962) containing a constant concentration of NaCl (100 mM) and varying concentrations of B (10 to 400 mM) and a control (CK) of untreated sample. The plates were irrigated twice per day and the excess was allowed to drain into collecting pans.

Morphological analysis and DNA Isolation

The plants were treated for a period of 14 days with the appropriate solution under green house conditions. During this period, morphological parameters such as root length, plant height and colour of leaves were estimated and observed in the fresh samples. The plants were then transferred into plastic pots filled with soil mixed with farm yard manure with no further treatment. Genomic DNA was isolated from the expanded leaves (3rd from the flag leaf) of the plants at the vegetative stage using a modified high salt cetyltrimethylammonium bromide (CTAB) method (Tel-Zur et al., 1999). Further, morphological data was collected at the reproductive level and at the point of physiological maturity, while the plant height, panicle height and panicle weight were measured after harvest.

MSAP analysis

The MSAP protocol used in this study was essentially as reported (Reyna-Lopez et al., 1997; Xiong et al., 1999). The restriction enzymes EcoRI, *Hpa*II and *Msp*I were purchased from the New

England Biolabs Inc. (Beverly Mass). One pair of pre-selective and nine pairs of selective primers was used for amplification (Table 1). A total of over 865 bands were used in the evaluation of the induced polymorphism. The amplification products were separated by running them through 8% denatured polyacrylamide gels for 3 h at 55 W and visualized by silver nitrate staining. Only clear and reproducible bands were scored. An average of four replicates consisting of independent plant individuals and the standard errors (SE) were used to represent tall data. The means were compared at p 0.05. The software origin 7.0 was used for statistical analysis.

Cloning and determination of MSAP Bands

Bands which indicated changes in methylation patterns in a treated plant relative to its control were excised from the silver stained MSAP gels and re-amplified with the appropriate selective primer combinations. PCR products sizes were verified by agarose-gel electrophoresis and then cloned into Pmd18-T vector (Takara Biotechnology Inc., http://www.takara-bio.com) and sequenced with automated vector primers. A BLASTN search of the MSAP bands obtained was carried out using the sorghum genomic sequence (http://www.gramene.org/Multi/blastview) and NCBI (http://www.ncbi.nlm.nih.gov/) for homology analysis.

RESULTS

The morphological changes and cytosine DNA methylation alterations due to B and NaCl toxicity on the sorghum inbred line were evaluated and analyzed using MSAP to determine the DNA cytosine methylation levels and patterns at two toxic concentrations. Alleviation of toxicity was observed at the concentrations of B (300 mM and 400 mM) in combination with NaCl (100 mM) with morphology almost the same as the control. The concentrations of B between 10 to 200 mM were greatly affected (Figure 1).

Morphological analysis after NaCl and Boron stress

At the seedling stage, there was a decrease in plant height by 55.60 and 44.40% at treatments of 300 and 400 mM, respectively when compared to the control. Root length was reduced by 86.10 and 38.90% at both treatments respectively, in comparison to the control plants (Figure 2). Moreover, at maturity, an increase in height of 4.80% was observed for treatment at 300 mM with a decrease of 1.80% at 400 mM when both were compared with the control. Panicle weight decreased by 23.60 and 8.30% at 300 and 400 mM, respectively, in comparison with control but the weight at 400 mM was 69.17 g and at 300 mM, it was 57.00 g implying an increase of 11.57 g (15.3%) at 400 mM (Figure 3). This could be explained by reduced seed filling of glumes at 300 mM (Figure 4).

DNA methylation analysis after NaCl and Boron stress

After analyzing 513 amplified bands which were clear and reproducible, an increase to 19.88% CG methylation level was detected in 300 mM, with a fall to 13.26% in 400 mM. CNG methylation level was also increased at 300 mM having 5.26%, while there was a decrease to 4.68% in 400 mM (Figure 5). CG hyper methylation for 300 mM was 5.47 and 0.78% for 400 mM, while CG hypo methylation was 0.78 and 2.73% at the treatments, respectively. In addition, 0.20% CNG hypermethylation was detected in 300 mM with no significant CNG hyper methylation in 400 mM, while 0.39% CNG hypo methylation was detected in 400 mM with no significant CNG hypomethylation in 300 mM (Figure 6).

DISCUSSION

To check for the combined (alleviation) effect of NaCl and B on the growth of sorghum plants, seeds were subjected to different concentrations of B and a constant concentration of NaCl. Although there was overall reduction in morphology when the treated plants were compared with the control, a better recovery was observed for plants treated with higher B concentration (Figures 1 and 2). Past researches have shown that gene expression can be changed via hypo- and hypermethylation of the DNA when stress is applied (Xiong et al., 1999; Chinnusamy and Zhu, 2009). In this study, we observed that in the plants treated with lower B concentration, there was increased methylation predominantly at the CG site (Figure 3a). Methylation patterns at this concentration also showed increased hypermethylation. The genome of organisms has housekeeping mechanism that silences hazardous DNA sequences (Madlung and Comai, 2004). This is associated with methylation changes leading to transcriptional gene inactivtion (Finnegan et al., 2000). The inducement of DNA hypermethylation in this case could possibly have occurred because genes involved in metabolic activities were silenced, which served as a protection during growth and development under stress and subsequently resulted in reduction in morphological parameters. In addition, recent studies in sorghum endosperm have shown that there is more extensive demethylation in the endosperm than in other tissues (Zhang et al., 2011). Plants rapidly employ the regulation of gene expression for physiological survival under stressed conditions either by avoidance or tolerance (Floris et al., 2009).

Another observation is the increased reduced methylation (hypomethylation) in plants treated with higher B concentration (Fig 3b), reflects that more genes involved in stress tolerance were expressed possibly accounting for the improvement observed at this treatment. DNA hypomethyation has often been associated with developmental defects (Finnegan et al., 1996; Bender, 2004). Although DNA methylation inhibits heterochromatic recombination (Bender, 2004), it was

Table 1. Sorghum sequences of adaptors, pre-amplification primers and selective amplification primer combinations used in the MSAP analysis in this study.

Parameter	Primer
Adaptors	
*Eco*RI-adapter I	5'-CTCGTAGACTGCGTACC-3'
*Eco*RI-adapter II	5'-AATTGGTACGCAGTC-3'
*Mse*I-adapter I	5'-GACGATGAGTCCTGAG-3'
*Mse*I-adapter II	5'-TACTCAGGACTCAT-3'
H/M-adapter I	5'-GATCATGAGTCCTGCT-3'
H/M-adapter II	5'-CGAGCAGGACTCATGA-3'
Pre-selective primers	
E-A	5'-GACTGCGTACCAATTCA-3'
M-C	5'-GATGAGTCCTGAGTAAC-3'
H/M-0	5'-ATCATGAGTCCTGCTCGG-3'
Selective primers	
***Mse*I primers**	
1. M-CAA	5'-GATGAGTCCTGAGTAACAA-3'
2.M-CAC	5'-GATGAGTCCTGAGTAACAC-3'
3.M-CAG	5'-GATGAGTCCTGAGTAACAG-3'
4.M-CAT	5'-GATGAGTCCTGAGTAACAT-3'
5.M-CTA	5'-GATGAGTCCTGAGTAACTA-3'
6.M-CTC	5'-GATGAGTCCTGAGTAACTC-3'
7.M-CTG	5'-GATGAGTCCTGAGTAACTG-3'
8.M-CTT	5'-GATGAGTCCTGAGTAACTT-3'
9. M-CCA	5'-GATGAGTCCTGAGTAACCA-3'
H/M primers	
1. H/M-TCT	5'-ATCATGAGTCCTGCTCGGTCT-3'
2. H/M-TCG	5'-ATCATGAGTCCTGCTCGGTCG-3'
3. H/M-TCC	5'-ATCATGAGTCCTGCTCGGTCC-3'
4. H/M-TTC	5'-ATCATGAGTCCTGCTCGGTTC-3'
5. H/M-TTG	5'-ATCATGAGTCCTGCTCGGTTG-3'
6. H/M-TTA	5'-ATCATGAGTCCTGCTCGGTTA-3'
7. H/M-TGA	5'-ATCATGAGTCCTGCTCGGTGA-3'
8. H/M-TGT	5'-ATCATGAGTCCTGCTCGGTGT-3'
9. H/M-TGC	5'-ATCATGAGTCCTGCTCGGTGC-3'
10. H/M-TAC	5'-ATCATGAGTCCTGCTCGGTAC-3'
***Eco*RI primers**	
a. E-AAC	5'-GACTGCGTACCAATTCAAC-3' (combined with 1, 4, 6, 8 and 10 H/M primers)
b. E-AAG	5'-GACTGCGTACCAATTCAAG-3' (combined with 4 and 5 H/M primers)
c. E-ACA	5'-GACTGCGTACCAATTCACA-3' (combined with 3, 4, 5, 8 and 10 H/M primers)
d. E-ACC	5'-GACTGCGTACCAATTCACC-3' (combined with 4 and 6 H/M primers)
e. E-ACG	5'-GACTGCGTACCAATTCACG-3' (combined with 3, 4, 5, 6 and 10 H/M primers)
f. E-AGC	5'-GACTGCGTACCAATTCAGC-3' (combined with 1and 4 H/M primers)
g. E-AGG	5'-GACTGCGTACCAATTCAGG-3' (combined with 1, 3, 4, 5, 6 and 10 H/M primers)
h. E-AGA	5'-GACTGCGTACCAATTCAGA-3' (combined with 4, 5, 6 and 8 H/M primers)
i. E-ATC	5'-GACTGCGTACCAATTCATC-3' (combined with 1, 4 and 5 H/M primers)

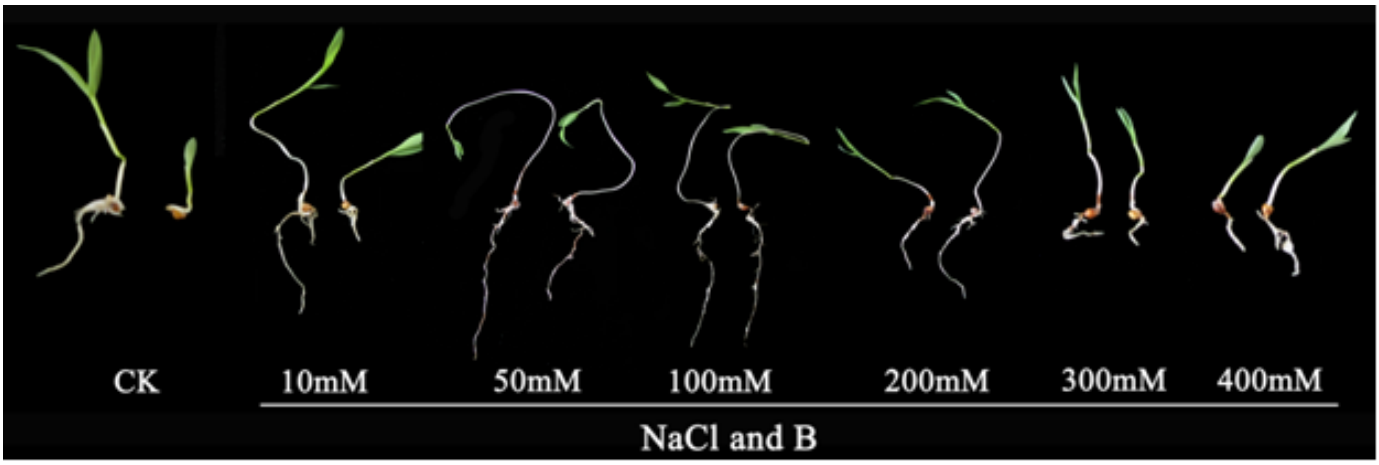

Figure 1. Morphology of the sorghum treated seedlings showing longer, thinner and weaker stems and roots at 10 to 200 mM after NaCl and Boron combination treatment. A recovery of morphology similar to the control was however observed at the 300 and 400 mM treatments.

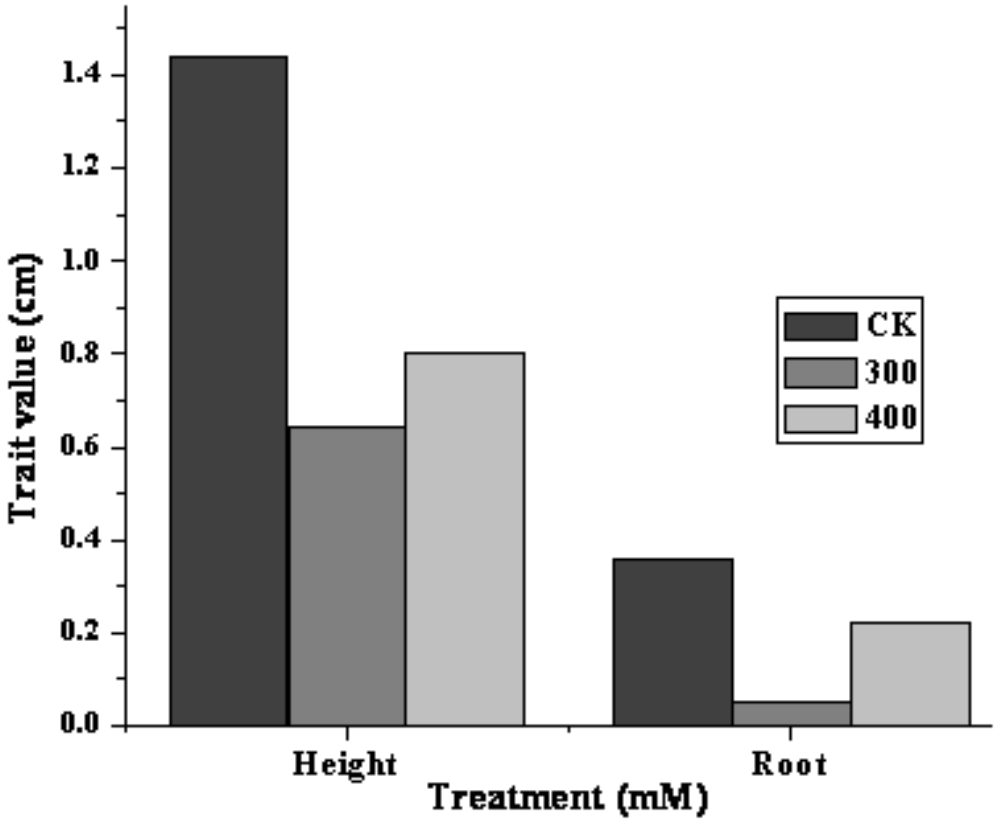

Figure 2. Plant height and root growth rates of the sorghum seedlings at 300 and 400 mM B and NaCl combination and the control (CK).

proposed that pathogen-induced heterochromatin decondensation due to hypomethylation at the pericentromeric sequences may have allowed enhanced recombination of resistance genes present at centromeric regions (Soppe et al., 2002; Pavet et al., 2006). From research by Zhao et al. (2008), increased demethylation in highly heterotic cotton hybrids was observed than in low heterotic hybrids, an implication of the advantageous

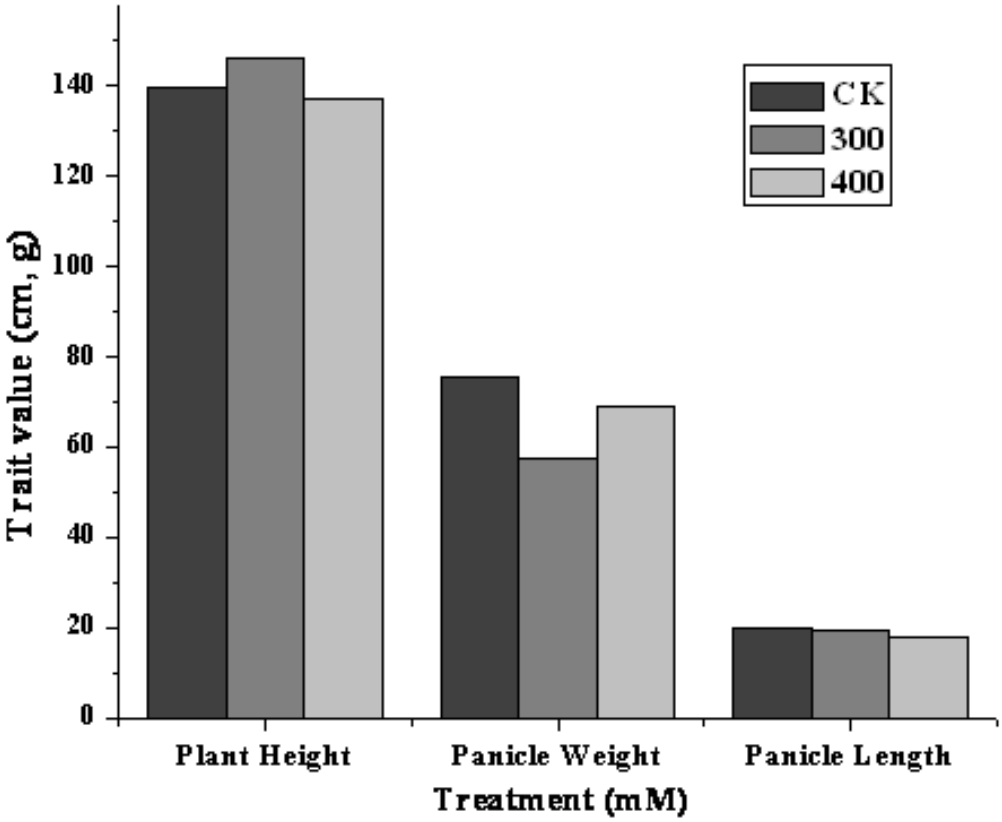

Figure 3. A comparison of the height, panicle weight and panicle length of B and NaCl treated sorghum inbred line D plants at the physiological maturity stage.

Figure 4. Panicles of sorghum plants for control (CK) and those of the B/NaCl treated plants at 300 and 400 mM concentrations. The reduction observed in the pattern of seed filling especially at 300 mM (red arrow) and a higher seed filling corresponded with a higher seed yield at 400 mM.

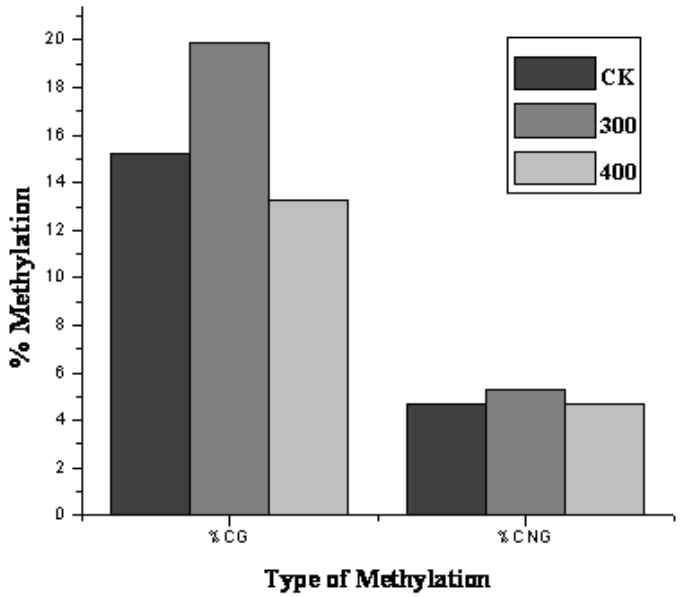

Figure 5. The percentage methylation levels of the B and NaCl treated sorghum inbred line D with the control plants (CK), showing the 300 B/NaCl concentration (mM) treated plants with the highest methylation levels.

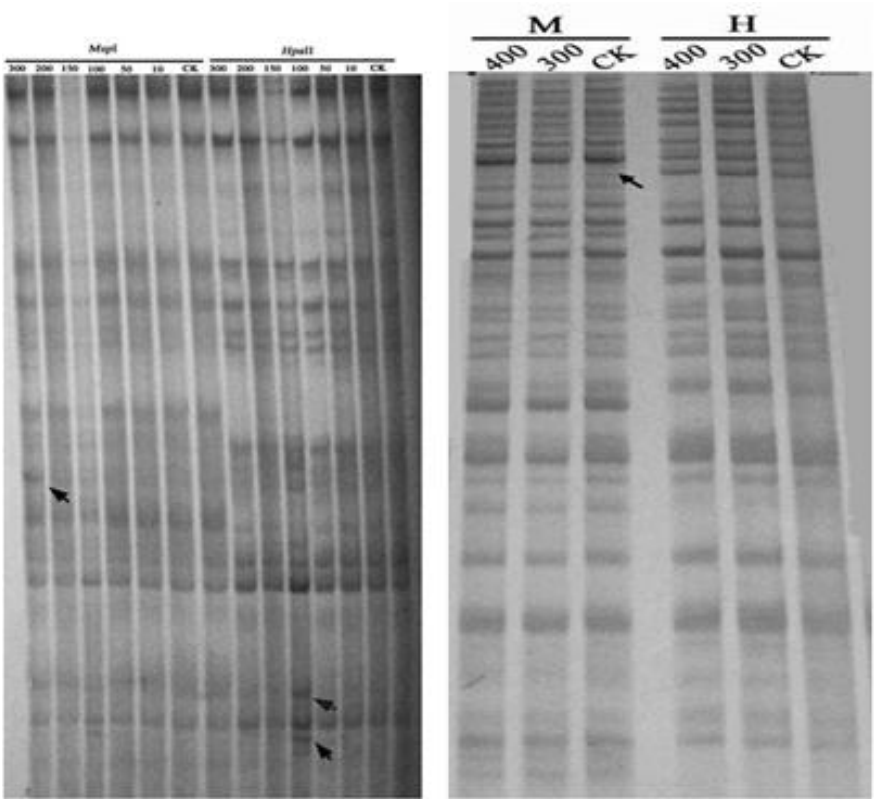

Figure 6. Examples of the MSAP profiles showing different band patterns through the primer combinations *Eco*RI + AAG/ *Hpa*II (*Msp*I) + TTC. Arrows indicate band variants due to the B and NaCl stresses.

effect of hypomethylation in connection with increased heterosis in these plants. Likewise the genomic plasticity and polymorphisms which are adaptive mechanism for plants and their progenies might be increased when disorder in DNA rearrangement is intensified (Lucht et al., 2002; Kovalchuk et al., 2003). The minimal recovery observed from the morphology could likely be due to the increased CG and CNG hypomethylation at this concentration. The obtained subsets of bands representing methylated alterations in response to the combined NaCl and B treatment have sequences associated with proteins like transposon protein and an amino acid family transporter protein.

Conclusion

The relationship between cytosine DNA methylation and the alleviating effect of the NaCl and B in sorghum plants is still not clear. From the results of this study, sorghum plant growth recovery was observed at an increased concentration of B with NaCl. An overall hypomethylation was also observed at this concentration.

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