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EFFECTS OF CARBENDAZIM ON DNA DAMAGE AND RETROTRANSPOSON POLYMORPHISM IN *Zea mays*

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Abstract

Carbendazim (CBZ, methyl 2-benzimidazolecarbamate) is used widely in agriculture against fungal diseases. We aimed to determine the effects of CBZ (0, 0.1, 0.2 and 0.4 mM) on long terminal repeat (LTR) retrotransposon polymorphism and genomic template stability (GTS), DNA damage using ınter-simple sequence repeats (ISSR) and inter-retrotransposon amplified polymorphisms (IRAP) marker techniques. Our results showed that all doses of CBZ compose retrotransposition polymorphism. In additional, our result showed that all CBZ treatment decrease genomic template stability (GTS) and DNA damage increased. These results suggest that carbendazim, widely used in agriculture, can influence unfavorable growth and development of both target and non-target organisms and impend risk to organisms even in trace levels.

Key words: carbendazim, DNA damage, genomic template instability, IRAP, ISSR

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1. Introduction

The use of pesticides in agriculture is rising day to day. The application of pesticides in agriculture is one source of pollution. Fungicides are used commonly against fungal diseases in agriculture. CBZ is a wide spectrum, systemic, widely used fungicide (Yunlong et al., 2009). It is used in agriculture against fungal diseases. Also, it is used as a preventive in paint, papermaking, leather industry and in fruits. The intensive application of CBZ can have effects on non-target plant and animal organism. At the same time, it has been reported that it causes toxic effects in human such as downregulation of immunity, endocrine deterioration, spermatogenic and fertilite failure (Morinaga et al., 2004; Rajeswary et al., 2007; Singhal et al., 2003; Yu et al., 2009). In additional, recently, the toxicity and genotoxicity effects of CBZ were reported in vitro or ex vivo assays (Dere et al., 2016; Li et al., 2015; Verma and Srivastava, 2018). According to these articles, it causes radical oxygen species and therefore it increases, oxidative stress and contributes to lipid peroxidation and DNA damage (Đikić et al., 2012).

CBZ does not cause DNA damage, gene mutations and structural chromosomal aberrations. Some authors have stated that it is aneugen, but not clastogen (Descordier et al., 2009; Sarriff et al., 1994; Verma and Srivastava, 2018). Besides, Tomlin (1994) reported that CBZ inhibited the function of mitosis and cell shape by binding to tubulin proteins.

The aneugenic effects of CBZ are explained by binding to b-tubulin and disrupting the normal formation of microtubulus (Descordier et al., 2009; Sarriff et al., 1994; Verma and Srivastava, 2018). It has been suggested that the inhibition of microtubulus formation and the inhibition of chromosomal migration during mitosis may cause testicular atrophy in rats (Vigreux et al., 1998). In additional, Li et al. (2015) indicated that CBZ has effects on genomic

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DNA methylation and the gene expression, photosynthesis inhibition, amino acid, selenium and nitrogen metabolism, and nutrient accumulation.

Especially, epigenetics influences such as DNA methylation and retrotransposon activities have a significant impact on the medicinal and crop plants. Some authors have reported that DNA methylation changes may cause phenotype variations (Becker and Weigel, 2012; Henderson and Jacobsen, 2007). Howewer, the effects of CBZ on LTR has not been elucidated. Therefore, in this study, we aimed to examine CBZ effects on LTR retrotransposon polymorphism, GTS and DNA damage. These results will be important for CBZ, widely used in agriculture.

2. Material and methods

2.1. Plant material and growth conditions

The Department of Field Crops, Faculty of Agriculture, Ataturk University (Turkey) provided the seeds of Zea mays cultivar RX9292. 0.5% sodium hypochlorite was used for surface sterilization of the seeds for the period of 5 min. Then, the sterilized seeds were sown Murashige and Skoog (MS) containing different concentrations carbendazim (0, 0.1, 0.2 and 0.4mM). Following a week, the seedlings were collected and put in a freezer at the temperature of 80ºC for performing other analyses.

2.2. Isolation of gDNA

Extraction of genomic DNA (gDNA) from seedlings was performed by employing the method presented by Sigmaz et al. (2015). gDNA samples loading into agarose gel 1% (w/v) in 0.5xTBE (Tris-Borate- EDTA) buffer at 70 V was performed for the period of 150 min. The nanodrop spectrophotometer (Qiagen, Qiaxpert Instrument, Germany)was utilized for checking quality and quantity of the amplified DNA products. Pure of sample DNA were used in ISSR and IRAP marker techniques.

2.3. IRAP amplification

As seen in Table 1, six primers had been used to generate IRAP profiles. The method used by Sigmaz et al. (2015) was practiced in IRAP PCR reactions. PCR amplifications were performed in a Bio-Rad thermocycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). The reaction mixtures (20 µL) were fixed in the way described below: 45 ng of g DNA, 1× buffer (10 mM Tris–HCl, 50 mM KCl, pH=8.3), 1.5 mM of magnesium chloride, 0.2 µM of each dNTP, 0.5 U of Taq polymerase (Cinagen Co., Iran) and 10 pmoL of each primer were added.

Table 1. Details of banding pattern revealed through IRAP and ISSR primers. (R = A, G; Y= C, T)

Markers/Primers	*Primer/primer Combination*	*Length of amplified bands*	*Control*	*0.1 mM*	*0.2 mM*	*0.4 mM*
IRAP	Nikita	250- 1500	11	+96, +168, +529	+96, +168, +529, +882	96, +168, +529, +882, +935+1268
	LTR6149	400- 1800	15	+218	+218, +496	+218, +537, +772
	LTR6150	350- 1300	8	+569	-118, +267, +569, +883, 1023	118, +267, +569, +883, 1023, +1350
	5' LTR1	500- 2300	9	-334, +210	+210, +546, +984	+210, +546, +984
	5' LTR2	200- 1400	17	+196, +429, +885	+196, +423, +885, +1245	+196, +423, +885, +1245
	Sukkula	500- 1700	8	-360	-	+115, +364, +772, +1027
Total bands			68			
% Polymorphism				16.1	26.5	36.8
GTS				83.9	73.5	63.2
ISSR	$(CA)_6R$	500- 1200	15	+ 110, +245	+ 110, +245	+ 110, +245, +368
	$(CA)_6RY$	400- 1500	15	-86, +324,+483	+324,+483, +527, 634	+324,+483, +527, +634, + 699
	$(CA)_6RG$	600- 2000	8	-	+152	+152, +456
	$(GT)_6YR$	750- 2100	13	+193, +258, +534	+193, +534, +964	+193,+ 258, +534, +964
	$(GT)_6AY$	300- 1400	18	- 775	-	-775, +372, +698, +881
	$(AGC)_4GR$	400- 1600	10	+334, +486	+334, +486	+334, +486
	$(AGC)_4GY$	600- 1800	15	-623	-623, +541, +882, 1029	623, +541, +882, 1029
	$(AGC)_4AY$	500- 1900	8	+228,+491	+228,+491, +776, +993	+228,+491, +829, 1150, +1485
Total bands			103	14	20	29
% Polimorfizm				13.6	19.4	28.1
GTS				86.4	80.6	71.9

The PCR program for the IRAP was as follows: an initial denaturation 5 min at the temperature of 94°C, 35 cycles of 45s at the temperature of 95°C, 40 s at the temperature of 55°C to 63°C (Table 1), 2 min at the temperature of 72°C, which was followed by a final extension of 5 min at the temperature of 72°C.

2.4. ISSR amplification

Nineteen oligonucleotide primers were tested for ISSR amplifications, among which eight primers were chosen and utilized for future studies (Table 1). The PCR mixture (25µl) was prepared as follows*:* 40 ng of template DNA, 10x buffer, 200 Mm of each of the four dNTPs, 1 U of Taq DNA polymerase, 0. 5 mM of primer and1.5 mM MgCl2. Amplification was carried out in a thermal cycler programmed for an initial denaturation at the temperature of 94°C for the period of 5 min followed with the following 35 cycles of 45 s at the temperature of 94ºC, 1 min at the annealing temperature and 1 min at the temperature of 72°C, ends with a final extension stage of 7 min at the temperature of 72ºC.

2.5. Electrophoresis

The PCR products were blended with 6X gel loading buffer (3ml) and exposed to agarose. Then, electrophoresis was applied to separate them by means of 1. 5% agarose gel (1.5% w/v) in 0.5xTBE buffer (Tris-Borate- EDTA) with was performed 70 V for the period of 150 min. The gel was stained with ethidium bromide solution (2 µl Etbr/100ml 1xTBE buffer) and The Bio Doc Image Analysis System with Uvi-soft analysis package (Cambridge, UK) was utilized for determining the amplified DNA products.

2.6. Analysis

The ISSR and IRAP bands were assessed by means of the TotalLab TL120 computer software program. Genomic template stability (GTS, %), was computed as described below: *GTS*=100-(100 x a/n), where *a* represents the average number of polymorphic bands determined in every specimen treated, and *n* represents the number of total bands in the control specimen.

Polymorphisms in ISSR and IRAP profiles was manifested as the disappearance of a normal band and the appearance of a new band in comparison with the control. The average value was counted up for every experimental group. To compare the sensitivity of each parameter, changes in these values were calculated as a percentage of their control (set to 100%).

In the IRAP and ISSR analysis, the mean values of polymorphism % were computed for every dose. For the purpose of computing the polymorphism value %, the 100 x a/n formula was utilized.

3. Results

DNA concentrations obtained in Nanodrop were in the range of 700-1000 ng/ul. Six IRAP primers presented us specific and stable results in *Zea mays* genome (Table 1). Important changes were seen in IRAP profiles treated by CBZ. As seen in the table, totally 68 bands were appeared in control. 8-17 polymorphic bands (missing or increase) have been identified in CBZ -treated plants out of control for all the primers used in this study. While the lowest polymorphism value (16.1%) was seen the minimum dose (0.1 mM) of CBZ, the highest polymorphism rate (36. 8%) was observed in the maximum dose (0.4 mM) of CBZ.

There was a tendency of GTS values to decrease with an increase in the concentration of CBZ treatments. GTS value (63. 2%) was seen in 0.4mM CBZ, GTS value (83. 9%) was observed in 0.1mM CBZ. Eight ISSR primers gave specific and stable bands in *Zea mays* genome (Table 1). The eight ISSR primers produced a total of 103 ISSR bands. The primer $(GT)_6AY$ gave the highest number of ISSR bands (18), while the $(CA)_6RG$ (Fig. 1), and $(AGC)_4AY$ primers yielded the lowest number of bands (8). While the lowest polymorphism value (13.6%) was seen in 0.1mM CBZ, the highest polymorphism value (28.1%) was observed in 0.4mM CBZ. While the lowest GTS rate (71. 9%) was seen in 0.4mM CBZ, the highest GTS rate (86.4 %) was observed in 0.1mM CBZ.

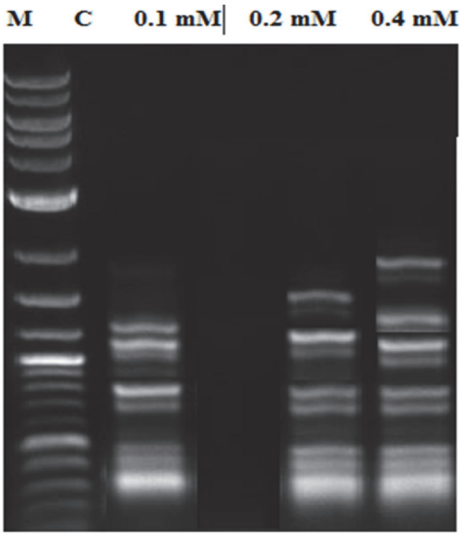


Fig. 1. ISSR profiles of *Zea mays* seedlings subjected and not subjected to CBZ with primers of $(CA)_6RG$

4. Discussions

CBZ are organic fungicides, used widespread in agriculture for control of a wide variety of fungi. However it is known that it effects on non-target organisms. This study showed that CBZ caused

genomic template instability, DNA damage and LTR retrotransposon polymorphism in *Zea mays.* Our findings are in accordance with DNA damage results. In additional, this study has been revealed that CBZ changes LTR Retrotransposion polymorfism. LTR retrotransposon polymorphism effects of CBZ have not been elucidated. The transposable elements are in plant genome.

The studies with CBZ effects on DNA damage and GTS using molecular marker are quite limited. Preceding studies have often focused on the genotoxic effects of CBZ on organism, reporting that CBZ cause DNA damage by producing free oxygen radical. Different authors suggest that CBZ have aneugen effect but it doesn't cause gene mutations, structural chromosomal aberrations and clastogen effects (Descordier et al., 2009; Sarriff et al., 1994). The aneugenic effects of CBZ are explained by the disruption of b-tubulin binding and normal microtubule formation. Besides, Tomlin (1994) disclosed that CBZ inhibited mitosis by binding to tubulin proteins. The DNA damage and genomic template instability affected by CBZ can be attributed primarily to its producing free oxygen radical and lipid peroxidation and thus influence DNA integrity. In addition to, via both oxidative and nonoxidative (DNA adducts) mechanisms, CBZ can also cause GTS decreasing and DNA damage increasing.

The authors showed that CBZ caused DNA damage using different techniques such as Choromosal aberration (CA) ,single cell gel electrophoresis assay (SCGE) and the alkaline comet assay (Đikić et al., 2012; Dere et al., 2016; Li et al., 2015; Verma and Srivastava, 2018). Our findings are in accordance with DNA damage results. In additional, this study has been revealed that CBZ changes LTR Retrotransposion polymorfism. LTR retrotransposon polymorphism effects of CBZ have not been elucidated. The transposable elements are in plant genome. Howewer, retrotransposons are inactive plant genomes, they begin to be activated in different biotics and abiotics stress conditions (Alzohairy et al., 2012; Grandbastien, 2004; Sigmaz et al., 2015; Woodrow et al., 2010) These activities are relevant to DNA methylation. At the first time Li et al. (2015) showed that CBZ caused changes in genomic DNA methylation in *A. thaliana.* These findings are in accordance with DNA methylation.

Retrotransposons outnumber the genes in large plant genomes, thereby comprising the bulk of the genome, usually remain silent under most conditions. They are largely quiescent during development, but become more active under various biotic and abiotic stress conditions (Mansour, 2007). Some researchers suggest that retrotransposons can be responsible for the molecular mechanism of plant resistance genes. Some active retrotransposons such as Ttd1 are known to have similar sequence as motifs which are in the transcriptional activation of defence genes in plants (Bichler and Herrmann, 1990; Grandbastien, 2004; Gutterson and Reuber, 2004; Ross and Shen, 2006; Sigmaz et al., 2015). Though it intends to protect the plant, multiple cellular defense mechanisms are available to avoid genomic imbalances caused by retrotransposon activity. The retrotransposons polymorfism will confer new properties by variations in genome size (Grandbastien, 2014; Sigmaz et al., 2015).

5. Conclusions

We reported that CBZ has effects on retrotransposition polimorphism, DNA damage and GTS. Our results suggest that CBZ can cause significant epigenetic changes, leading to silence or reactivation of gene expression.

Our further studies will be focus on the effect mechanisms of CBZ on other epigenetic modifications in plant.

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