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An investigation of the genotoxic and cytotoxic effects of myclobutanil fungicide on plants

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Abstract: Myclobutanil is a chemical pesticide commonly used in the production of some vegetables and fruits like greenhouse peppers, grapes, and apples. The aim of this study was to investigate the genotoxic and cytotoxic effects of myclobutanil fungicide on the *Allium cepa* plant, the model organism. Randomly amplified polymorphic DNA and inter simple sequence repeat-PCR techniques were performed on the DNA of *A. cepa* exposed to the different myclobutanil doses and time periods. The nucleus anomalies and abnormal anaphases were investigated using a light microscope. PCR analyses showed that myclobutanil causes some DNA sequence changes on the onion genome depending on the increase in the fungicide dose and exposure time. It was determined that myclobutanil has a serious genotoxic effect, even in low doses like 25–50 ppm.

Keywords: pesticides; toxicity; DNA-markers; *Allium cepa*

Myclobutanil [2-(4-chlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)hexanenitrile] is a steroid demethylation inhibitor and a triazole chemical used as a fungicide (Han et al. 2018) against *Leveillula taurica*, *Venturia inaequalis*, and *Uncinula necator* (Kumar et al. 2019) causing summer patch, scab, powdery mildew, rusts, and dollar spots in the production of greenhouse peppers, grapes, and apples (Fonseca et al. 2019).

Myclobutanil persists in the environment and can accumulate in the ecosystem via the food chain, thereby presenting a great risk to humans and other organisms. Recently studies have reported that myclobutanil residue is present in air, soil, water, and food (Campos-Manas et al. 2019). While the fermentation process significantly reduced pesticide residues like propiconazole (48%), it was reported that this reduction is much less for myclobutanil, around 20% (Navarro et al. 2011).

The interactions between pesticides and DNA molecules may cause various nuclear anomalies in plant cells (Abass et al. 2017). The simplest uses of PCR performed with randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) primers for mutation analysis detect the presence or absence of particular regions of DNA (Stapulionytė et al. 2019). Recently, RAPD-PCR profiles obtained from the genomic DNA of organisms exposed to pesticides have been used to evaluate the genotoxicity of different agents in organisms at the DNA sequence level (Salama et al. 2019).

In this study, the genotoxic and corruptive effects of myclobutanil fungicide on the DNA and cell nucleus of *A. cepa* were investigated using DNA-based PCR techniques and a cell staining technique.

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MATERIAL AND METHODS

Preparation of plant and fungicide material. Commercial *A. cepa* was chosen as the plant material, and the onion bulbs were germinated in 15 cm glass tubes at room temperature. Fungicide treatments were applied when the germinating bulbs reach a root length of about 1 cm. Myclobutanil was provided via commercial purchase. According to the user's manual, the water-diluted form of myclobutanil fungicide at dose of 73.5 ppm was recommended for agricultural use (Agrobest Group Co., Turkey). I prepared dilution series of myclobutanil as 10 ppm, 25 ppm, 50 ppm, 75 ppm, 100 ppm and 150 ppm with sterile water and each concentration was applied to onion roots for 24 h, 48 h, and 72 hours. The negative control was performed with sterile water only. After myclobutanil treatment roots were either fixed in fixative solution (3 : 1 ethanol, glacial acetic acid) for microscopic analysis or stored at –20 °C until DNA extraction.

DNA isolation. Onion roots were ground with liquid nitrogen, and the fine powder was transferred to 2 mL eppendorf tubes. The total plant genomic DNA was isolated from the root samples using a cetyl trimethylammonium bromide isolation procedure (Doyle & Doyle 1987). The quantity and quality of DNA were detected using a Nanodrop UV-Spectrophotometer (Shimadzu, Japan). Plant DNA samples were diluted with sterile deionized water to 2 ng/μL for PCR analysis.

ISSR-PCR analysis. The primer sequences used in the ISSR-PCR process were provided from the University of British Columbia (Canada). For the selection of suitable primers, a primer screening was performed with 10 ISSR primers (Table 1). The PCR results of five ISSR primers showed clear, reproducible, and unique banding patterns, and these primers were selected for genotoxicity analysis. Amplification of ISSR fragments from plant genomic DNA was performed in a reaction volume of 25 μL containing 8 ng of template DNA, buffer [Tris-HCl, $(NH_4)_2SO_4$, Tween-20], 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.2 mM primer, and 1 IU of Taq polymerase (Solis BioDyne, Estonia). The amplification process was performed using thermal cycler (Thermo Arktik; Thermo Scientific, USA): 4 min at 94 °C and 45 cycles of 45 s each at 94 °C, 50 s at 47–60.5 °C (annealing depended on primer variety) for ISSR amplification, and a 1.5 min at 72 °C, followed by a final extension stage at 72 °C for seven minutes. The amplified DNA fragments were separated on a 1.3% agarose gel containing ethidium bromide (0.5 μg/mL) and photographed digitally using a gel documentation system (Carestream, USA). The nucleotide length of ISSR-PCR products was determined using a 100 bp plus DNA Ladder (Fermentas, USA).

RAPD-PCR analysis. For the selection of suitable primers, a primer screening was performed with 10 RAPD primers (Table 2). The PCR results of four RAPD primers showed clear, reproducible, and unique banding patterns, and these primers were selected for genotoxicity analysis. PCR amplifications were performed in a reaction volume of 25 μL containing 10 ng of template DNA, buffer, and 1 IU of Taq polymerase, 1.5 mM $MgCl_2$, 1 mM dNTPs, and 1 μM of primer. The amplification process was performed using a thermal cycler that was programmed for 85 °C for 15 s, 95 °C for 5 s, and 92 °C for 1 min 55 s, followed by 45 cycles of 95 °C for 5 s, 92 °C for 55 s, 32–36 °C for 1 min, 72 °C for 2 min,

Table 1. Inter simple sequence repeat (ISSR) primers tested for amplification of *Allium cepa* genomic DNA

Primers	Primer sequence (5' to 3')	Temperature (°C)	Polymorphism rate (%)	Appositeness
ISSR-01	AGAGAGAGAGAGAGAGG	52.8	87.5	+
ISSR-04	ACACACACACACACACC	52.8	88.9	+
ISSR-06	GAGAGAGAGAGAGAGAC	52.8	100	+
ISSR-10	GGGTGGGTTGGGGTG	58.8	–	–
ISSR-27	GTGCGTGCGTGCGTGC	59.4	–	–
ISSR-829	TCTCTCTCTCTCTCTCG	52.8	–	–
ISSR-847	CACACACACACACACARC	53.7	86.3	+
ISSR-861	ACCACCACCACCACCACC	60.5	–	–
ISSR-862	AGCAGCAGCAGCAGCAGC	60.5	–	–
ISSR-866	CTCCTCCTCCTCCTCCTC	60.5	88.3	+

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Table 2. Randomly amplified polymorphic DNA primers tested for amplification of *Allium cepa* genomic DNA

Primers	Primer sequence (5' to 3')	Temperature (°C)	Polymorphism rate (%)	Appositeness
P-5	CTGCGACGGT	34	–	–
P-9	GGGAAGAGAG	32	84.6	+
P-11	GGCCGATGAT	32	–	–
P-13	ACCGCCTTGT	32	90.9	+
P-14	CAGCACTGAC	32	–	–
P-16	TGGTGGCCTT	32	–	–
P-17	GTAGCACTCC	32	84.0	+
P-21	ACGGTGCCTG	34	–	–
P-23	CGCCCAAGCC	36	83.8	+
P-24	CGCCCTGGTC	36	–	–

and a final extension at 72 °C for seven minutes. All RAPD-PCR processes for each ISSR and RAPD primers were repeated twice to ensure reproducibility of amplified products.

Cell staining. The fixed onion root tips were stained with 2% orcein dye for 10 min and slightly squashed to accomplish a better spreading of the material over the slide (Fiskesjo 1997). The plant tissue samples were observed with a binocular light microscope using an objective of 40×, and the anomalies were photographed digitally with 1 000 magnification (Olympus BX51, Olympus, Japan) and a digital camera (Kameram 122CU; Micrometrics, Turkey).

Data analysis. Band profiles for each of the ISSR and RAPD primers were compared using the Phoretix1D Pro software version 1.0 for band counting and size determination. The binary data obtained from the software was used for evaluating the genetic distance between samples and for drawing a dendrogram by the unweighted pair group method with arithmetic mean (UPGMA) clustering analysis method. 100 bootstrap replicates for dendrograms were generated. The dendrogram showing genetic divergence between the samples was drawn using MEGA 6 software version 10.1.8. A total of 500 cells of each plant tissue sample for each dose were observed to check the frequency of micronucleus and other anomalies. The results obtained from microscopic analyses were tested using analysis of variance (ANOVA), and the significance was determined at a probability level of $P < 0.05$.

RESULTS AND DISCUSSION

DNA damage induced by pesticides may cause both acute and inheritable diseases (Dhananjayan et al. 2019). Myclobutanil is a forbidden fungicide in some countries like Canada and the USA (Beal 2019), but it is still used freely in other countries. My experimental findings indicate that serious measures should be taken regarding the myclobutanil fungicide.

ISSR-PCR analysis. In this study, different doses of myclobutanil were administered to germinated onion roots for time periods of 24 h, 48 h, and 72 hours. When the ISSR-PCR amplification results were analyzed, apparent changes were detected between the electrophoresis band profiles of the control group and the fungicide-exposed group. It was determined that the five out of 10 ISSR primers (Table 1) produced matchless polymorphic band patterns. Among the 94 bands 88 were polymorphic. Some changes such as the appearance of new DNA bands and the disappearance of available DNA bands were observed between treated and untreated groups (Figure 1). Also, I observed that the variation in band profiles increased in a time and concentration-dependent manner.

In 2017, Sorrentino et al. investigated the genotoxic effect of lead and cadmium on *in vitro* cultures of *Sphagnum palustre*. They obtained reproducible polymorphic bands that indicated a clear genotoxic effect induced by the metals using 10 ISSR primers (Sorrentino et al. 2017). In 2018, Andrade-Vieira et al. analyzed the genotoxic effects of spent potliner (SPL) and SPL derivatives on *A. cepa* and *Lactuca sativa*. They used ISSR markers as an interesting tool to determine the mutagenicity of an environmental pollutant and found that the obtained band differences showed discrepancies between test groups and the control group. They also showed that the negative control had a separate position from the test group samples in the

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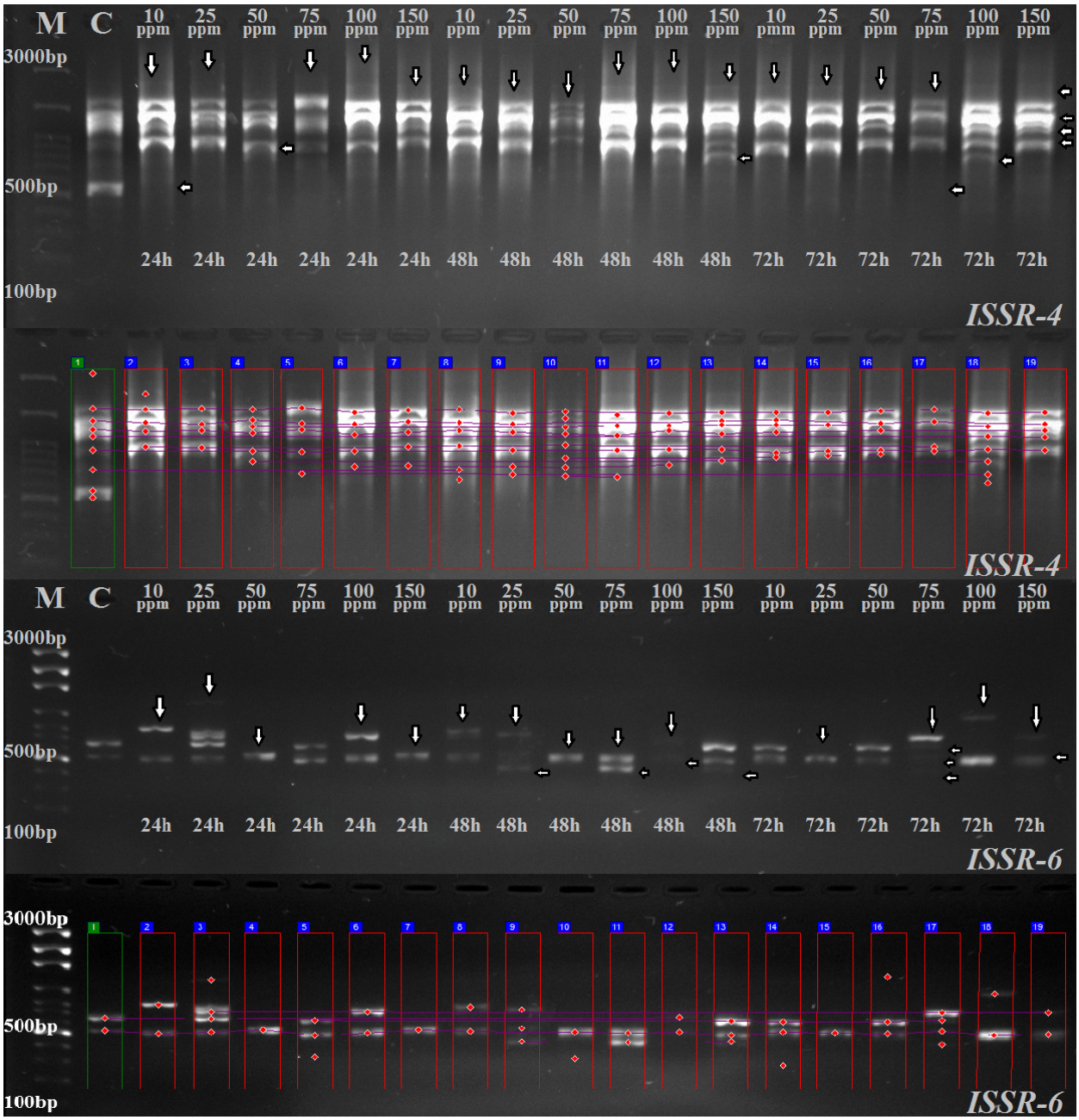

Figure 1. The inter simple sequence repeat (ISSR) banding patterns amplified with ISSR-4 and ISSR-6 primers from *Allium cepa* DNA

M – DNA marker; C – control group; white arrows – the disappearance bands or appearance of new bands; colored frames – band counting and size assignment with Phoretix1D Pro software

dendrogram (Andrade-Vieira et al. 2018). Similarly, I obtained a dendrogram from the ISSR-PCR band data in my study which was drawn using the UPGMA method, and the control group separated significantly from the test group exposed to myclobutanil (Figure 2A).

RAPD-PCR analysis. Among 10 RAPD primers, four (Table 2) of them produced matchless polymorphic band patterns, a total of 76 bands were obtained and 71 of which were polymorphic. RAPD-PCR amplification results showed some significant differences (such as the appearance of new DNA bands and the disappearance of available DNA bands) between the control group and myclobutanil-exposed groups (Figure 3). The P9 primer used was particularly successful in the detection of DNA damage in *A. cepa*

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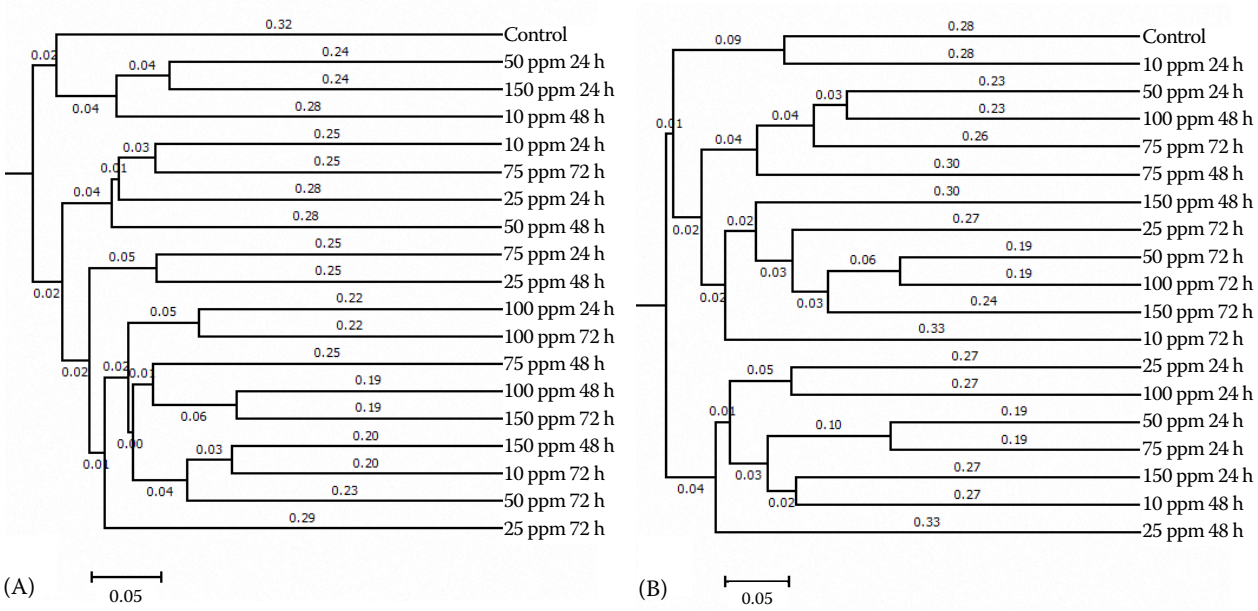

Figure 2. The dendrograms that showed genetic similarity constructed by unweighted pair group method with arithmetic mean analysis method (bootstrap value: 100)
(A) Inter simple sequence repeat-PCR dendrogram, (B) randomly amplified polymorphic DNA-PCR dendrogram

genome. This primer had the most monomorphic bands and clearly showed the changes based on myclobutanil damage in the band profile of *A. cepa* genome (Figure 3).

There are many studies using RAPD-PCR method in genotoxic studies today. For example, the effect of zinc oxide nanoparticles on bean DNA was investigated using RAPD markers in 2019 (Salama et al. 2019). Abass et al. (2017) investigated the genotoxic effects of different plant growth regulators on date palm callus, using RAPD-PCR analysis. They obtained the band polymorphisms showing symptoms of the genotoxic effect with the RAPD markers (Abass et al. 2017).

Stapulionytė et al. (2019) drew a dendrogram based on genetic polymorphism values of RAPD-PCR data using the UPGMA clustering method in a genotoxicity study of soil pollution in *A. cepa*. They determined that branches in the dendrogram showed significant separation of the control group from the test group samples (Stapulionytė et al. 2019). Similarly, in the dendrogram produced from RAPD-PCR data in my study, the control group prominently separated from the test group exposed to myclobutanil (Figure 2B). The difference between DNA bands of control and test group samples was also seen in the RAPD-PCR agarose gel samples (Figure 3).

Both dendrograms of ISSR and RAPD-PCR data showed the same tendency. The control group in both dendrograms separated similarly from the test groups (Figure 2). While the control group in the RAPD dendrogram branched together with the lowest dose and time (10 ppm-24 h), the control group in ISSR dendrogram branched like an out-group.

The comparative data obtained from both ISSR and RAPD-PCR analyses showed that myclobutanil caused DNA damage in a time and concentration-dependent manner in the genome of *A. cepa*.

Nuclear anomalies. Most agricultural drugs have a tendency to be very reactive chemical compounds that can form strong bonds with the various nucleophilic centers of cellular biomolecules like DNA, RNA, and proteins. They may cause various nuclear anomalies in plant cells. Fernandes et al. (2009) observed a pesticide concentration-related increase in the rate of micronuclei and nucleus alterations. The irregular nuclei were in different formations like horn, kidney, and horseshoe (lobed nuclei) shapes (Fernandes et al. 2009). I detected that myclobutanil fungicide caused micronuclei formations and some alterations or aberrations *A. cepa* DNA (Figure 4). It was determined that a significant augmentation

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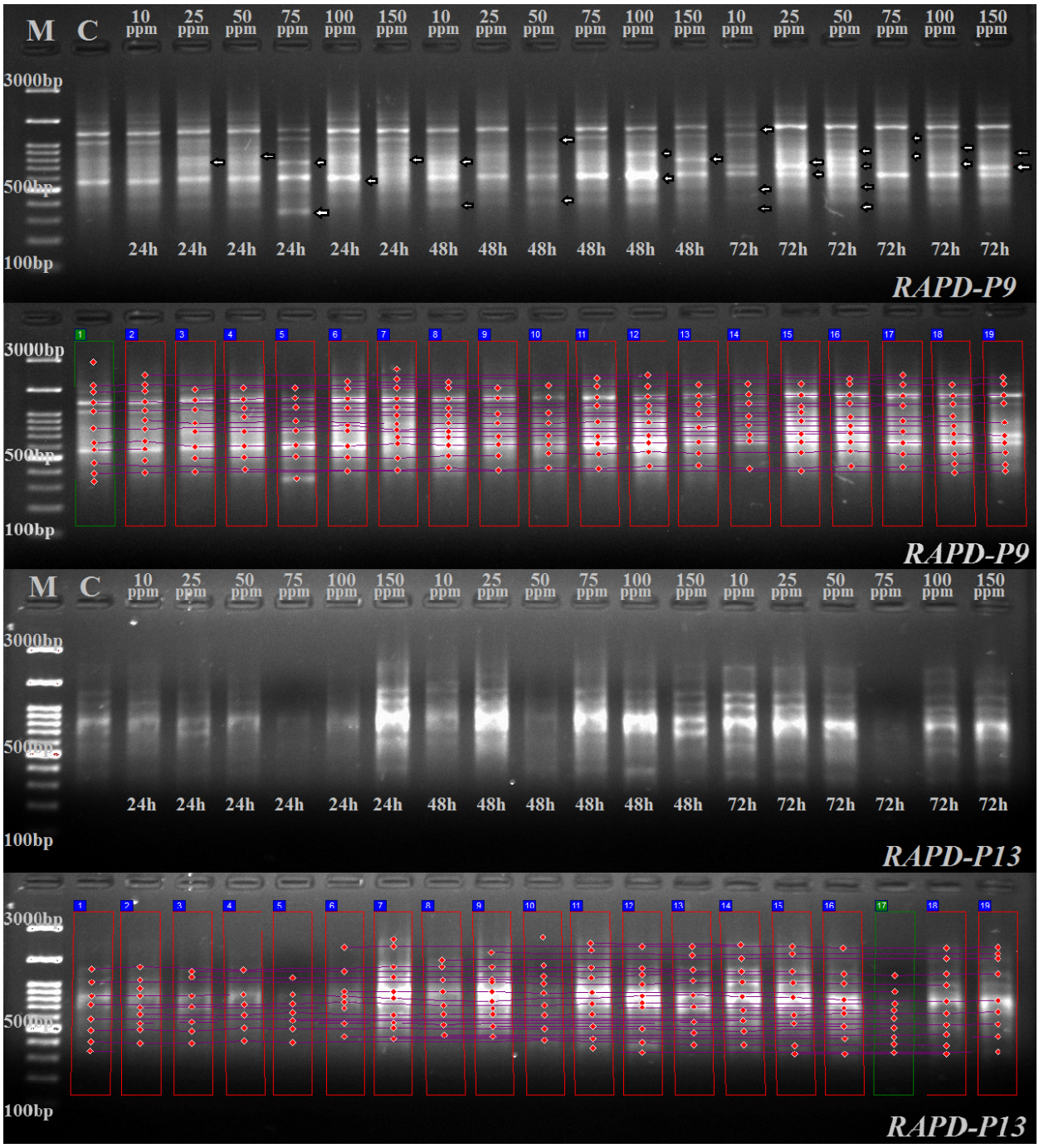

Figure 3. The randomly amplified polymorphic DNA (RAPD) banding patterns amplified with RAPD-P9 and RAPD-P13 primers from *Allium cepa* DNA

M – DNA marker; C – control group; colored frames – band counting and size assignment

($P < 0.05$) in the rate of nuclear alterations occurred according to an increase in concentration and time (Figure 5). Anaphase anomaly formations were observed from a 50-ppm dose. Micronucleus formations were seen in 75 ppm and in higher doses. This evidence showed that the exposure time to fungicide is also an important criterion that caused an increase in DNA damage. Incorrect use of myclobutanil herbicide by farmers may increase the risk even more. The increase of nucleus anomalies and micronucleus numbers seen in the highest myclobutanil dose (150 ppm)

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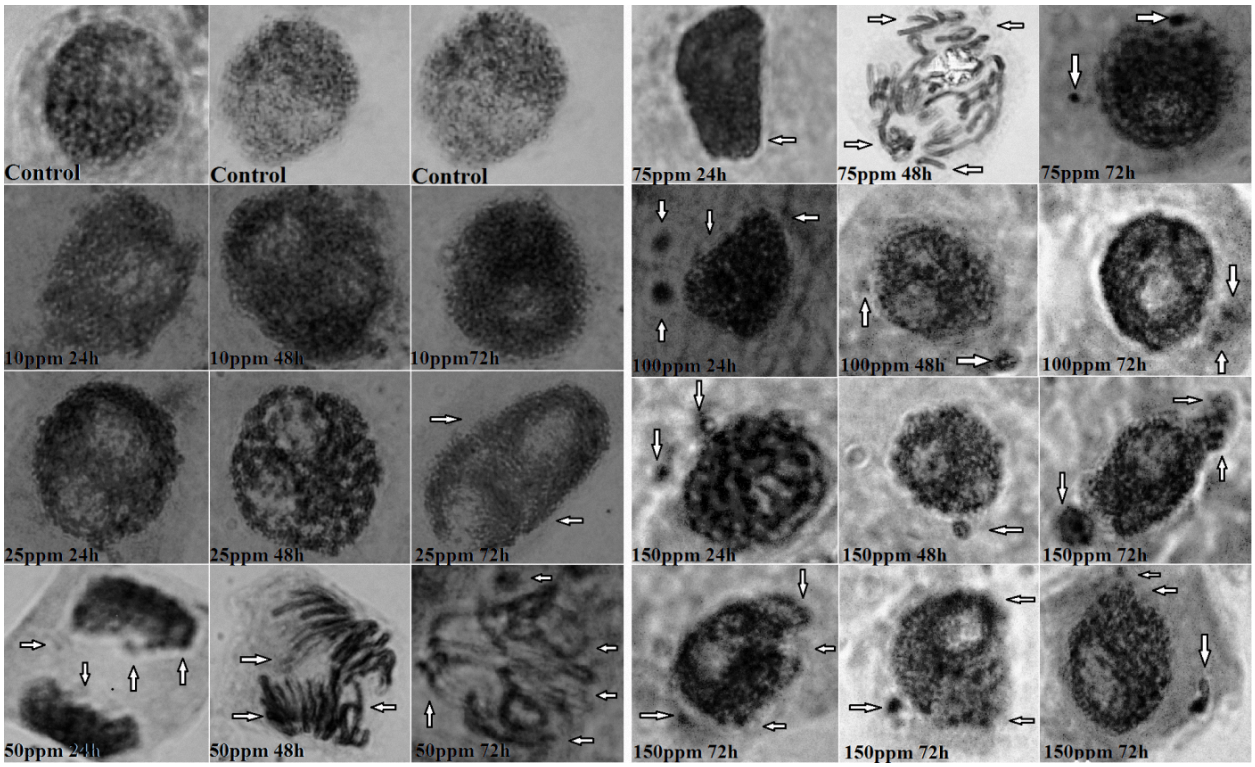

Figure 4. Nucleus anomalies caused by myclobutanil fungicide
White arrows – micronucleus formations, anaphase anomalies and different formations as horn, kidney, lobed nuclei, etc.

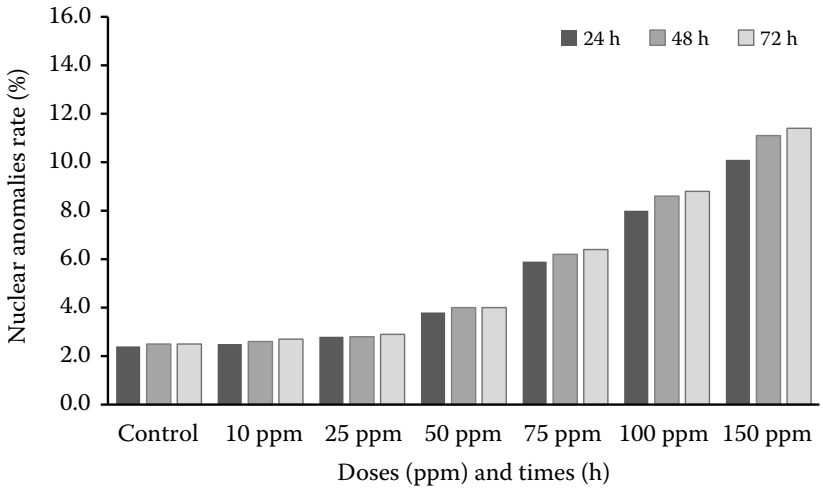

Figure 5. Nuclear anomalies and rates of incidence as time (h) and concentration (ppm)-dependent ($P < 0.05$)

used in assays has verified this risk. My microscopic analyses revealed the presence of many nuclear anomalies such as micronucleus, lobed nuclei, and kidney-shaped nuclei in myclobutanil-exposed *A. cepa* cells (Figure 4).

I also observed anaphase anomalies in myclobutanil-exposed *A. cepa* cells (Figure 4). My results may indicate that other plants and animals in the myclobutanil-treated fields might encounter risks like DNA damage.

CONCLUSION

The recommended dose and time for myclobutanil is 73.5 ppm and 14 days worldwide, but higher doses are used by some farmers to increase effectivity. I tested different myclobutanil doses for three time periods on *A. cepa* and observed acute genotoxicity. My results revealed that myclobutanil has a high genotoxic effect even at low doses like 25 ppm and 50 ppm at 72 hours. DNA damage caused by my-

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clobutanil fungicide can trigger vital or developmental problems in plants. The harmful effects of DNA damage that occur in exposed organisms may continue even after the exposure has ended. The hazardous effects of this fungicide on genetic material may also threaten other animals that feed on myclobutanil exposed plants. The results of this study revealed that myclobutanil should not be used even in low concentrations. The use of myclobutanil must be monitored to prevent any possible dose overflow during fungicide treatment. Acute genotoxicity resulting from the recommended doses in the user manual may increase the accumulation and permanence of DNA mutations in plants. Mutations may cause metabolic problems and a decrease in life quality in myclobutanil-exposed plants. These plants may be affected by negative selection under environmental conditions. Ecosystems exposed to myclobutanil in the years ahead may deteriorate in terms of species number and variety. Prospective studies should focus on the investigation of the metabolic function losses caused by myclobutanil in plants.

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