Determination of Epigenetic Effects of Fipronil on *Vicia faba* by Using CRED-RA Analysis

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***Abstract*—** Nowadays, herbicides are chemicals commonly used gardens or agricultural field. The widespread use of these chemicals has become an environmental problem for all organisms including plants to human and requires urgent precautions. Therefore, the present study was aim to observe epigenetical changes of fipronil herbicides on *Vicia faba* seedlings. The assays five fipronil concentrations (0.5 ppm, 1 ppm, 2 ppm, 3 ppm, 4 ppm) were used. CRED-RA (Coupled Restriction Enzyme Digestion-Random Amplification) was used as a molecular marker to examine epigenetic changes in the experimental groups. The method gave qualitative data about DNA methylation caused by fipronil. The polymorphic percentage of each concentration was calculated. Consequently, it was revealed that five concentrations of fipronil caused DNA methylation changes. In additional, this study further confirmed that the CRED-RA assays are useful in determining potential epigenetics effects on plants.

***Keywords*—** CRED-RA, DNA methylation, Fipronil, *Vicia faba*.

I. Introduction

PESTICIDES can be classified based on their chemical structure. Fipronil is a member of the phenyl pyrazole class of pesticides, which are principally chemicals with a herbicidal effect [1]. Actively marketed in many industrialized and developing countries, its worldwide use is increasing.

Fipronil is highly toxic for crustaceans, insects and zooplankton. Fipronil is recommended for insects control in various crops such as sugarcane, soybeans, corn, eucalyptus and others [2]. However, beyond the target insects, fipronil has demonstrated high toxicity on insects no target [3]. In additional, the genotoxic effect of fipronil on humans has been investigated by using different test systems including micronucleus, comet assay [4], [5]. Many studies have shown that fipronil has genotoxic, cytotoxic and toxic potential [6], [7]. However there is not yet a study examining the epigenetic effect of fipronil on the plants and animals.

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The aim of the present study, was to investigate the epigenetic effect of fipronil in *Vicia faba* seedling by using the CRED-RA technique.

II. Materials And Methods

A. Sample Collection

Fipronil was obtained from Sigma Chemical Company, USA and *Vicia faba* seeds were obtained from the Department of Field Crops, Faculty of Agriculture, Ataturk University (Turkey). *Vicia faba* seeds were surface-sterilized with 0.5 % NaOCl (sodium hypochlorite) solution for 10 min and then washed with sterile water three times.

Seeds were placed on two layers of filter paper moistened with 25 ml distilled water. 25 seeds were used in each petri dish. The dishes were kept at 25 ± 1°C under dark condition until primary roots were grown at 0.5-1 cm length. After, the *Vicia faba* seedlings were exposed to different concentrations of fibronil (0.5 ppm, 1 ppm, 2 ppm, 3 ppm, 4 ppm) for 96 h. Treated seedlings were grown in pots of a peat/soil mix (5 plants/pot) at 25±1°C with a 16 h photoperiod of 60 µmol photons/m^2s provided by white fluorescent lamp, at a relative humidity of 70–75%. Each treatment was replicated three times. Bulk leaves were randomly collected from ten plants for each treatment after emergence of leaf 3 (leaves numbered from base) and were stored at -80 °C.

B. DNA Extraction

Genomic DNA was extracted from powdered plant materials using a method described by Li and Quiros [8]. Approximately 1 g of tissue samples were ground to a fine powder in liquid nitrogen in 2ml eppendorf tubes. Next 1,000 µl of DNA in DNA extraction buffer (100 mM Tris-HCl (pH 8.0) 50 mM EDTA, (pH 8.0) 500 mM NaCl, 20% SDS and 10 mM 2-mercaptoethanol) was added and mixed well. The mixture was incubated at 65^0C in a water bath for 45 min with intermittent shaking at 5-min intervals. The mixture was centrifuged at 12.000 g for 15 min at 4^0C and the supernatant was transferred into a new 1.5ml tube mixed gently with an equal volume of phenol: chloroform: isoamylalcohol (25:24:1). The DNA was precipitated by the addition of 0.6 volume of freezer cold isopropanol left at -20^0C for 10 min. The upper phase was discarded by centrifugation for 10 min at 12000 rpm and at -4^0C. The pellet was washed with 70% ethanol. The dried DNA was allowed to air dry before being dissolved in 100 µl of TE buffer.

C. CRED-RAs Digestion and PCR

Genomic DNA sample from each treatment were separately digested with HpaII and MspI endonucleases (which cut the

sequence 5´-C/CGG-3´with different sensitivity to cytosine methylation; MspI cuts if the inner C is methylated, whereas HpaII cannot cleave in the presence of methyl groups). After checking digestion on agarose gel, were prepared a PCR reaction cocktail.

CRED-RA-PCR reaction was contained 25 ng digested DNA, 400 µM dNTP, 10 pmol primer, 2,5 mM $MgCl_2$, 1 U *Taq* DNA polymerase and 1X PCR buffer (10X) in a total volume of 20 µL. DNA amplification was carried out in a thermocycler programmed as follows: 1 cycle of 5 min at 95°C, 42 cycles of (1 min at 94°C, 1 min at 36°C and 2 min at 72°C), 1 cycles of 15 min at 72°C.

16 CRED-RA primers were tested with bulked DNA of control treatment (0 ppm fipronil). Only 8 primers amplified polymorphic amplicons and used in CRED-RA PCR reactions. These primers for CRED-RA analyses are; AGAGCCGTCA (OPY-7), CAGGCCCTTC (OPA-1), AATCGGGCTG (OPA-4), CAGCACCCAC (OPA-13), CAGAAGCGGA (OPW-4), CACAGCGACA (OPW-13), TGATGCGTG (OPW-11) and AGGCCCGATG (OPW-6).

D. Electrophoresis

The PCR products (20 µl) were mixed with 6x gel loading buffer (3 µl) and subjected to agarose (1.5% w/v) gel electrophoresis in 0.5x TBE (Tris-Borate- EDTA) buffer at 70 V for 150 min. Amplification products separated by gel electrophoresis were stained in ethidium bromide solution (2 µl Etbr/100ml of 1x TBE buffer) for 40 min. The amplified DNA products were detected using the Bio Doc Image Analysis System and analysed using the Uvi-soft analysis package (Cambridge Electronic Design Ltd, Cambridge, UK).

E. Data analysis

The average number of CRED-RA pattern polymorphisms (%) were calculated for each dose to realize CRED–RA analysis. To calculate the number of polymorphisms (%), the following formula was used 100x *a/n* where *a* is the average number of polymorphic bands detected in each treated sample, and *n is* the number of total bands in the control. Polymorphisms in CRED-RA profiles included disappearance of a normal band and appearance of a new band compared with the control. The average was calculated for each experimental group. To compare the sensitivity of each parameter, changes in these values were calculated as a percentage of their control (set to 100%).

III. Results And Discussion

In total, 15 oligonucleotide primers with % 60-70 GC content were used for CRED-RA analyzing and only eight gave specific and stable results. Compared with the PCR products obtained from the control DNA, fipronil treatments resulted in apparent changes in CRED-RA patterns. These changes are characterized by variation loss of normal bands or appearance of new bands. Increasing concentration of fipronil caused DNA hypo-methylation. The methylation value was 8.5 in control. However, the highest methylation value was 7.4 and the lowest was 5.2 in fipronil applications.

The past decade, studies demonstrated that fipronil has genotoxic effect in animal and plant systems by using the micronucleus assay and comet (single cell gel electrophoresis, SCGE) assays [4], [5]. In additional Schlenk et al. (2001) [9], found that fipronil was very cytotoxic to two crayfish species. This paper is the first report indicating the effects on DNA methylation of treatment with different concentrations of fipronil in plant.

When plants are exposed to environmental stress such as biotic and abiotic, they can be antistress protective action, biochemical, physiology and molecular levels, induced DNA methylation and histon modification. Especially these epigenetically changes may be suggest a mechanism for plants adaptation under stress

The DNA hypo-methylation induced by fipronil could even be responsible for the transgenerational stress resistance. DNA hypo-methylation effect of fipronil may relate to affect the transcription and translation processes of specific genes, to improve the plant resistance under stress conditions.

IV. Conclusions

Herbicides is one of the abiotic stress that cause biochemical, physiological and especially genetic and epigenetic changes. These epigenetic changes have been proved with CRED-RA technique in this study.

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