Anatomic, Physiologic and Cytogenetic Changes in *Allium cepa* L. Induced by Diniconazole

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Received July 10, 2014; accepted October 12, 2014

Summary In this study, the possible cytotoxic effects of diniconazol fungicides on root tip cells of *Allium cepa* L. were investigated. Germination percentage, root length, weight gain, frequency of micronuclei (MN), chromosomal aberrations (CAs) and anatomical changes were used as toxicity markers (indicators). Seeds were divided four groups as one control group treated with tap water, and three treatment groups treated with 25, 50 and 100ppm doses of diniconazole for 72 h. As a result, germination percentage, root length and weight gain decreased, and MN level and chromosomal aberrations increased in the treatment groups compared to the control group. As a result of microscopic examination, stimulation of the chromosomal damages such as C-mitosis, fragment, chromosome bridge, binucleus cell, abnormal polarization, sticky chromosomes or unequal chromatin distribution by diniconazole treatment were observed. Also, in the root tip cells, diniconazole application caused anatomical damages such as cell deformation, nonspecific vascular tissue, flattening cell nucleus and necrosis.

Key words Diniconazole, Chromosomal aberration, Micronucleus, Physiology.

Crop diseases and pests have increased with the development of technology in agricultural production. Thus, the losses in agricultural production due to the diseases and pests have been worked in order to reduce crop losses (PMRA 2010). A pesticide is a chemical compound intended for preventing, destroying, repelling or mitigating any pest (Ecobichon 2001). They usually act by disrupting some component of the pest's life processes to kill or inactivate it.

Although the benefits of pesticides are undeniable, applications in recent years have been focused on their effect on human health and environment. They can harm plants and animals ranging from beneficial soil microorganisms and insects, non-target plants, fish, birds, and other wildlife. Also, pesticides are one of the reasons for water pollution and soil contamination (Sanders 1969). Generally, toxic effects of environmental pollutants cause cytogenetic aberrations on plant cells. Pesticides should be screened before their use in order to select those which are least toxic (Mann 1977), but toxicity is not always correlated with genotoxicity (Kovalchuk *et al.* 1998). Fungicides are a pesticide type against fungus. Although fungicide applications result in effective control of the diseases, the widespread use of these chemicals may cause environmental and food contamination (Tort and Turkyılmaz 2003). Diniconazole, a triazole fungicide which inhibits sterol biosynthesis in fungi, is one of the groups.

Fernex DS is the brand name of a systemic, broad-spectrum fungicide produced by Turkey and contains the active ingredient Diniconazole. Diniconazole was systemic fungicide in closed formulation [(*E*)-(RS)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1*H*-1,2,4-triazol-1-yl)pent-1-en-3-ol] (IUPAC). Diniconazole is used for the prevention of diseases such as leaf curl, riding, rust, and

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DOI: 10.1508/cytologia.80.51

powdery mildew in plants and grapes.

From this point, in this study, the effects of diniconazole on root tip cells of *Allium cepa* L. were investigated. To determine the toxic effects, anatomic, physiologic and cytogenetic parameters such as germination percentage, root length, weight gain, frequency of micronuclei (MN), chromosomal aberrations (CAs) and anatomical changes were investigated.

Materials and methods

Chemicals

FERNEX DS (1% *Diniconazole*) was obtained from Fertil Chemistry, Konya, Turkey.

Preparation of root tips

In this study, 25 healthy and proximate equal-sized *Allium cepa* L. seeds were selected. The seeds were washed in running tap water and placed into clean 50-mL glass beakers. The seeds were divided into four groups ($n=25$):

Group I (control) was treated with only tap water, for 72 consecutive hours.

Group II was treated with 25 ppm dose of diniconazole, for 72 consecutive hours.

Group III was treated with 50 ppm dose of diniconazole, for 72 consecutive hours.

Group IV was treated with 100 ppm dose of diniconazole, for 72 consecutive hours.

Analysis for germination percentage, root length and weight gain

The root lengths were determined by radicle formation bases. At the end of 72 h, the root lengths of the germinated seeds were measured with a millimetric ruler. The weight gain was determined by calculating the differences between the weights of seed before and after diniconazole treatment by using a sensitive balance. The germination percentage of the seeds exposed to diniconazole was calculated using the following Eq. (1):

$$\text{Germination (\%)} = \frac{\text{Germinated seeds}}{\text{total seeds}} \times 100 \quad (1)$$

Micronucleus (MN) assay

The root tips were fixed for 6 h in Clarke's fixator (3: glacial acetic acid/1: distilled water) and washed for 15 min in ethanol (96%), then stored in ethanol (70%) in the fridge at +4°C until making the microscope slides. The root tips were hydrolyzed in 1 N HCl at 60°C for 20 min, treated with 45% CH₃COOH solution for 30 min and stained for 24 h in acetocarmine. After staining with acetocarmine, the root meristems were separated and squashed in 45% CH₃COOH solution (Staykova *et al.* 2005). For MN analysis, 1000 cells were scored on each slide. Micronucleated cells were examined at $\times 500$ magnification using a binocular light microscope (Japan, Olympus BX51). For the MN scoring, the following criteria were adopted (Fenech *et al.* 2003): (i) the diameter of MN should be a tenth of the main nucleus, (ii) MN should be separated from or marginally overlap with main nucleus as long as there is clear identification of the nuclear boundary, and (iii) MN should have similar staining as the main nucleus.

Chromosomal analysis

The excised root tips were fixed for 24 h in Clarke's solution, and kept in 70% alcohol in the fridge at 4°C. These samples were sectioned routinely and stained using Feulgen. For each group, 10 root tip squashes were prepared and 500 mitotic cells were counted from random fields in each slide (Inceer *et al.* 2003, Wei 2004). Chromosomal analyses were made in anaphase cells in order to identify chromosome alterations such as chromatin bridges, loops and fragments as well as alterations in the centromere and mitotic spindle disturbances, through the appearance of multipolar

anaphases.

Anatomical investigation

To analyze the changes in the anatomical structure of *A. cepa*, the root tips were treated with different doses (25, 50 and 100 ppm) of diniconazole. The root tips were separated and then washed by distilled water. The cross-section of the root tips was taken manually for anatomical studies. These sections were stained by methylene Blue, air-dried and cover-slipped with Entellan (Makbul *et al.* 2008). All the photographs were taken with a binocular light microscope (Japan, Olympus BX51).

Statistical analysis

The statistical analysis of data was carried out using SPSS for Windows version 22.0 statistical software (SPSS Inc, Chicago, USA). Statistically significant differences between the groups were compared using one-way analysis of variance (ANOVA) and Duncan's test. The data are displayed as mean $\pm$ standard deviation (SD), and *p*-values less than 0.05 are considered "statistically significant".

Results and discussion

Physiological Parameters

The effects of diniconazole on *A. cepa* seed germination are shown in Fig. 1 and Table 1. The results from Table 1 clearly demonstrate that diniconazole has a detrimental effect on the germination of *A. cepa* seeds. A negative correlation was observed between diniconazole doses and the germination percentage. The highest germination percentage was observed in the seeds of the control group (in proportion as 100%). The lowest germination rate was observed at 100 ppm dose

Fig. 1. The effects of diniconazole on *A. cepa* seed germination.

Table 1. The effects of diniconazole on *A. cepa* seed germination percentage, root length and weight gain.

Groups	Germination percentage (%)	Root length (cm)	Weight gain (g)	MN frequency (%)
Group I	100	7.00 $\pm$ 0.57 ^d	+1.38 ^a	1.00 $\pm$ 0.82 ^d
Group II	84	5.72 $\pm$ 0.56 ^c	+0.95 ^b	6.90 $\pm$ 3.21 ^c
Group III	70	3.86 $\pm$ 0.44 ^b	+0.59 ^c	22.30 $\pm$ 6.62 ^b
Group IV	44	0.97 $\pm$ 0.25 ^a	+0.27 ^d	29.20 $\pm$ 6.58 ^a

a–d: Statistical significance between means was analyzed using one-way ANOVA followed by Duncan's test as a post-ANOVA test (*p*<0.05). Means with the same letter (vertically) are not significantly different at the *p*<0.05 level.

Fig. 2. Chromosomal aberrations induced by diniconazole in *A. cepa* root tips: a: micronucleus, b: c-mitosis, c: fragment, d: bridge, e: abnormal polarization, f: binucleated cell, g: abnormal distribution of chromatide, h: sticky chromosome, i: spindle abnormalities.

Table 2. CA types and frequency induced by diniconazole.

Groups	FRG	BC	CB	SC	CM	UECD
Group I	00.40±0.52 ^d	00.00±0.00 ^c	00.00±0.00 ^d	00.20±0.42 ^d	00.00±0.00 ^d	00.00±0.00 ^d
Group II	26.70±6.09 ^c	00.20±0.42 ^c	21.30±4.67 ^c	19.10±7.01 ^c	08.90±3.38 ^c	05.40±3.69 ^c
Group III	42.90±6.69 ^b	01.10±0.88 ^b	35.30±6.65 ^b	28.70±6.77 ^b	17.90±3.63 ^b	11.80±5.49 ^b
Group IV	54.40±7.07 ^a	02.50±1.43 ^a	43.90±9.47 ^a	36.50±6.67 ^a	30.20±6.66 ^a	22.50±6.31 ^a

Means with the same letter (vertically) are not significantly different at the $p < 0.05$ level. FRG: Fragment, BC: Binuclear cell, CB: Chromatid bridge, SC: Sticky chromosome, CM: C-mitosis, UECD: Unequal chromosomal distribution

of diniconazole. The 25, 50 and 100ppm doses of diniconazole caused a decrease in seed germination as 16, 30 and 56%, respectively. These results showed that the effects of diniconazole on the germination percentage depend on its dose, and the germination percentage can be considered as a sensitive indicator for diniconazole toxicity.

The results related with the root length and weight gain are given in Table 1. Diniconazole treatment significantly prevented the root length and weight gain of the seeds. A correlation was observed among diniconazole doses with the root length and weight gain. The highest root length and weight gain were determined in seeds of the control group at the end of 72 h. The smallest root length and weight gain were observed in the seeds treated with 100ppm dose of diniconazole. The mean root lengths of the control group seeds were 7.2 times higher than Group IV seeds treated with 100ppm diniconazole, and this difference was statically significant ($p < 0.05$). Maximum weight gain was observed in control group seeds and weight gain of Group IV was 5.1 times lower

Fig. 3. The anatomical appearance of root tips of control group seeds and the anatomical alterations induced by diniconazole in *A. cepa* root tips: a: clear vascular tissue, b: normal cell nucleus, c: cell deformation, d: unclear vascular tissue, e: unusual form of cell nucleus (flat), f: necrotic cell death.

than the control group.

Similar studies examined the effects on the weight gain of other pesticides. Maity (2014) reported that Dithane M-45 fungicide treatment decreased the germination percentage of *Vigna mungo* L. Hepper seeds. Çavuşoğlu *et al.* (2012) studied the toxic effect of Thiamethoxam on *A. cepa* and reported significant alterations in the germination percentage, root length, and weight gain of seeds. Aksoy and Dane (2007) investigated the effect of Fusilade on root and shoot growth of *Lens culinaris* Medic. seeds and determined that lateral root growth was reduced in treatment groups. In another study, Tort *et al.* (2004) investigated the physiological and morphological effects of diniconazole on barley culture form and reported significant decrease in weight gain and root length.

Although the ultimate mechanism of diniconazole toxicity on the physiological parameters is completely unknown, it seems plausible that diniconazole acts as a blocking agent by biochemical pathways in the seed cell. This condition may cause significant alterations in nutrient status and nutrient contents of tissues, and may reduce seed weight and germination percentage (Siddiqui and Ahmed 2000).

Cytotoxicological parameters

In our study, the frequency of MN was recorded and the results are given in Table 1. Microscopic examination of the squashes of *A. cepa* root tip meristem cells showed that there was only 1% MN formation in the control group, but a significant increase in MN formation was observed in all seeds exposed to diniconazole (Fig. 2a). The frequency of MN increased with rising diniconazole doses. There was a certain dose-effect relationship between the MN frequency and diniconazole doses. The highest MN frequency was observed at 100ppm dose of diniconazole. There were statistically significant differences between the MN frequencies of the control and treatment groups ($p < 0.05$). These findings suggest that diniconazole has a toxic activity which induced MN formation in the root tip cells of *A. cepa*. The effect of diniconazole on the frequency of MN in plant cells has not been reported in the literature so far. However, the several studies on other pesticides and chemical agents showed that there was a direct link between MN formation

and exposure to toxic agents. Özen *et al.* (2011) investigated the effect of paraquat on MN formation by the micronucleus test in *A. cepa* root tips and reported that paraquat induced a remarkable increase in the frequency of MN cells in *A. cepa*. In another study, Mustafa and Arikan (2008) investigated the cytogenetic effects of phenoxy herbicide in the root meristem cells of *A. cepa* and reported that MN cells were observed at interphase phase of mitosis division.

In addition, diniconazole treatment caused an increase in the frequency of CAs. CA frequency and types are given in Table 2 and Fig. 2. The CA types such as C-Mitosis (Fig. 2b), fragment (Fig. 2c), bridge (Fig. 2d), and abnormal polarization (Fig. 2e) were observed. The highest CAs observed in this study was chromatid bridge with a frequency of 43.90 ± 9.47 . These findings are also in agreement with the results of the studies carried out by other authors on pesticides. Mustafa and Arikan (2008) investigated the frequency of CAs induced by Quizalofop-P-ethyl herbicide in *A. cepa* and they reported that Quizalofop-P-ethyl herbicide caused CAs such as stickiness, bridges, vagrant chromosomes, c-anaphase, multipolarity and fragments. In a similar study, Singh *et al.* (2007) investigated the cytogenetic effects of profenophos and mancozeb in the root tip cells of *Hordeum vulgare* L. and reported significant inhibition of mitosis and an increase in CAs.

Anatomical observations

In order to identify anatomical changes induced by diniconazole on *A. cepa*, the root tissues were microscopically examined. When seeds were exposed to diniconazole for 72 h, the root cells survived but the root tissues were seriously affected under the presence of diniconazole. The anatomical damages such as cell deformation (Fig. 3c), unclear vascular tissue (Fig. 3d), unusual form of cell nucleus (Fig. 3e), and necrotic cell death (Fig. 3f) were observed when compared to the controls (Fig. 3). Similarly, Pline *et al.* (2002) investigated the effects of glyphosate on the root morphology of cotton seedlings and observed necrotic cells.

Conclusion

The results of the present study indicated that diniconazole caused significant toxic effects in the root cells of *A. cepa*, and this toxic effect induced physiological, anatomical, cytological and genetic alterations in *A. cepa*. The use of pesticides on farmland must further reduce or choose less toxic pesticides for a safe habitat.

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