Genotoxic Effects of Raxil on Root Tips and Anthers of *Allium cepa* L.

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Abstract — The genotoxic effects of fungicide Raxil, active substance tebuconazole in both mitotic and meiotic cell divisions of *Allium cepa* L. were studied. The bulbs with roots of *Allium cepa* L. were treated with different concentrations of Raxil (1800 ppm, 2400 ppm, 4200 ppm, 6000 ppm) for 3, 6, 12 and 24 hours. For mitotic studies, the root tips of *Allium cepa* L. after having grown to a certain length were stained accoording to aceto orcein squash procedure. To determine the effects of Raxil on meiotic cell division was used M1 generation. All concentrations and treatment periods of Raxil induced a number of chromosomal aberrations in root tip cells and in anthers of *Allium cepa* L. Additionally Raxil decreased the frequency of mitotic index and caused pollen fertility. A linear relationship was observed between increase of chromosomal abnormality with decrease of mitotic index and pollen fertility.

Key words: *Allium cepa* L., chromosomal abnormalities, fungicides, mitotic index, pollen sterility.

INTRODUCTION

Fungicides are most commonly used against diseases of agricultural crops in many countries of the world. Although fungicide application results in quick and high control of the dieases, the widespread use of these chemicals may cause environmental and food contamination (Tort and Turkyilmaz, 2003). Pollution is a major problem of some countries. It is well established that pollution lowers the quality of life in various aspects. Environmental pollutants may be mutagenic or toxic for all living organisms (Grover 1999; Yuzbasioglu *et al*. 2008). Constant use of these chemicals may result in changing the hereditary constitution of the organism (Wuu and Grant 1966; Wuu and Grant 1967). When some chemicals accumulated within food chain to a toxic level, these chemicals affect directly the public health. In context, Dryanowska (1987) and Cantor *et al*. (1992) showed that the frequency of cancer increases among people who have been exposed directly or indirectly to pesticides. Drugs and pesticides should be screened before the use in order to select those which are least toxic (Mann 1977). More than 200 are known bioassays for determining the biological effects of environmental pollutants in literature (Waters *et al*. 1990) but plant assay systems are more useful than most of the other systems for assessing chromosome damage and somatic mutations induced by environmental pollutants (Constantin and Owens 1982). Generally, toxic effects of environmental pollutants cause genetic damage on plant cells. But toxicity is not always correlated with genotoxicity (Kovalchuk *et al*. 1998).

Tebuconazole belongs to triazole fungicides, which are broad-spectrum fungicides intensively used on several crops (Copping *et al*. 1984). Tebuconazole is effective in control of the white rot. The white rot is a disease which can be seen on the garlics and onions (Fullerton *et al*. 1995). The fungicide Raxil is a commercial form of tebuconazole and is used extensively in the agricultural area. But there is no study available on the cytogenetic effects of this chemical in the plant systems. Induction of mitotic abnormalites in root tip cells of plants may cause a decrease of mitotic index (Kovalchuk *et al*. 1998; Bushra *et al*. 2002). The

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abnormalities occurring in meiosis may result in pollen sterility (Pagliarini 2000; Viccini and Carvalho 2002).

The aim of this study was to investigate the chromosomal abnormalities induced by fungicide Raxil in the root tips and anthers of *Allium cepa* L., and also to determine the relation between mitotic chromosome abnormalities with mitotic index and meiotic chromosome abnormalities with pollen sterility.

MATERIALS AND METHODS

The fungicide used in this research was tebuconazole (CAS No:107534-96-3) that trade name is Raxil. Its molecular formula is $C_{16}H_{22}Cl\,N_3O$.

The plant used as test material was *Allium cepa* L. (2n=16). Ten clean and healhty bulbs of *Allium cepa* L. were chosen for each treatment group. Before starting to the experiments, dry scales of bulbs were removed and then the bulbs with root of *Allium cepa* L. were germinated in distilled water at room temperature. When the roots reached 1,5-2 cm lenght, they were treated with different concentrations of fungicide Raxil diluted with distilled water (1800 ppm, 2400 ppm, 4200 ppm, 6000 ppm) for 3, 6, 12 and 24 hours. Controls were also treated with distilled water for the same time periods. The concentrations were chosen according to theirs growth inhibitions at 24 h.

For mitotic studies, the root tips of *Allium cepa* L. were fixed in Carnoy, and hydrolyzed in 1N HCl for 15 min. in an oven at 60°C. Squashes were prepared in aceto- orcein stain (Darlington and La Cour 1979). To determine the effects of this chemical on mitotic index, 3000 cells were scored in the control group and in each treated group. The mitotic index was calculated for each treatment as a number of dividing cells/100 cells.

Control and treated plants were sown to obtain M_1 generation. M_1 generation was used to determine the effects of Raxil on meiotic cell division. Young unopened flower buds of M_1 plants were fixed in Carnoy for 24 hour and squashes were prepared according to the method of Darlington and La Cour (1979). The same procedures and stain used for meiotic analysis were applied to opened flower buds to test pollen sterility. While pollen grains, which were round and stained with aceto-orcein, scored as normal, small and shriveled unstained pollen grains were scored as sterile.

In this study, the results were evaluated with the X^2 test. The significance of the results of each treatment group was determined by comparing them with those of the control group. In addition, statistical analysis was carried out with Student's t-test for the analysis of mitotic index, percentage of abnormality and pollen sterility.

RESULTS

Microscopic examination of squashes of *Allium cepa* L. root tip meristem cells showed that Raxil treatments induced a number of mitotic abnormalities when compared with control. The increase of mitotic abnormalities was dependent on the increasing treatment period and concentration. The most common abnormalities were stickiness, c-mitosis and disturbed metaphase. In addition, at anaphase and telophase, fragments, bridges, lagging chromosomes and irregular anaphase were also observed. However, the frequency of micronuclei was not significant. The types and percentage of these abnormalities are given in Table 1 and their photographs are in Fig. 1.

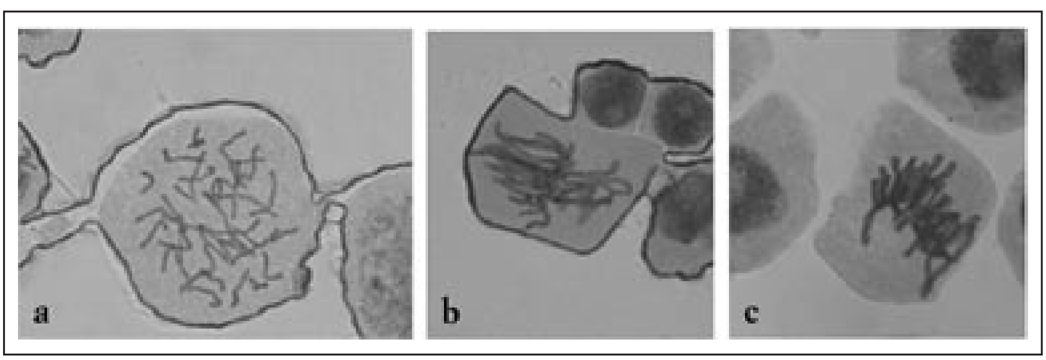

Fig. 1 — Some mitotic division abnormalities observed in the root tips of *Allium cepa* L. with different concentration of Raxil. a) C-mitosis (6h-6000 ppm); b) Stickiness (12h-6000 ppm); c) Distributed metaphase (24h-6000 ppm). (x1000).

Raxil caused a decrease in mitotic index (MI) at all the treatment groups. MI decreased with the increasing of concentration in treated plants with different concentrations and treatment periods (Table 1).

In this study, Raxil also affected meiotic cell division. Abnormal chromosome behaviours were displayed at all the treatment groups. The degree of chromosomal abnormalities was significant when compared with control (Table 2). These abnormalities were formation of univalents and quadrivalents at metaphase I, bridges, laggard chromosome, fragmets at anaphase, telophase I and II, and formation of micronuclei. At meiosis, synchronization disorder was the chromosomal aberration with highest frequency at all the treatments groups. Different types of chromosomal aberration are shown in Fig. 2.

Tebuconazole significantly increased the pollen sterility. The highest percentage of pollen

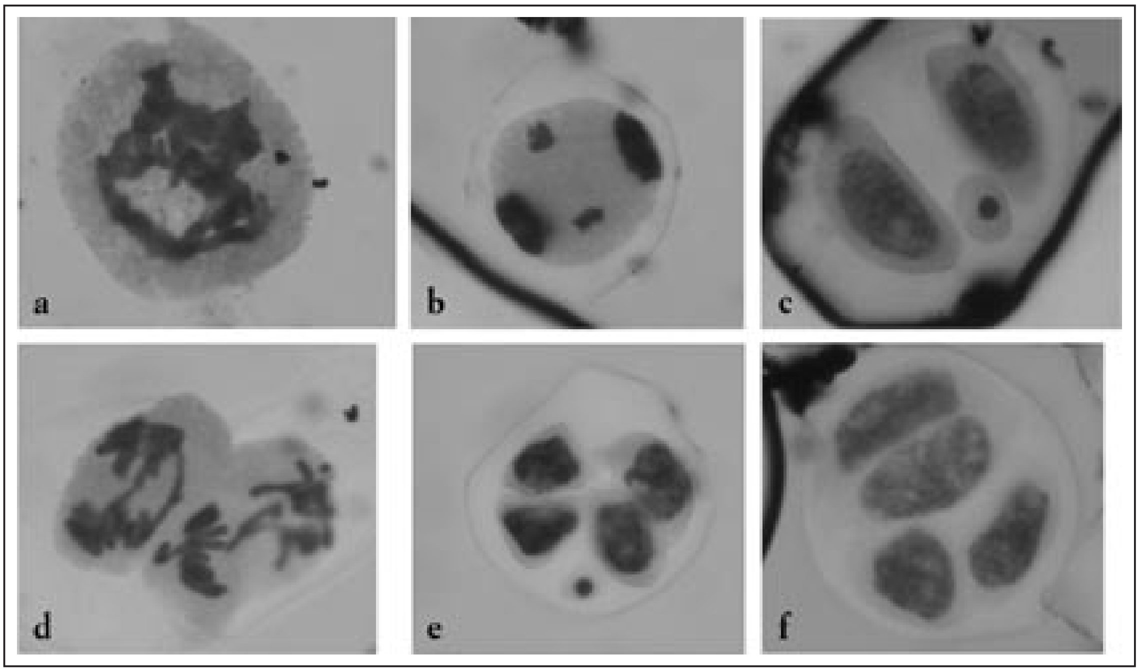

Fig. 2 — Meiotic division abnormalities occurring most frequently with Raxil. a) Formation of quadrivalent at metaphase I (12h-6000 ppm); b) Laggard chromosomes at telophase I (12h-6000 ppm); c) Formation of micronucleus at diad (3h-4200 ppm); d) Bridges at telophase II (24h-1800 ppm); e) Micronucleus at tetrad (24h-6000 ppm); f) Synchronization disorder (6h-6000 ppm). (x1000).

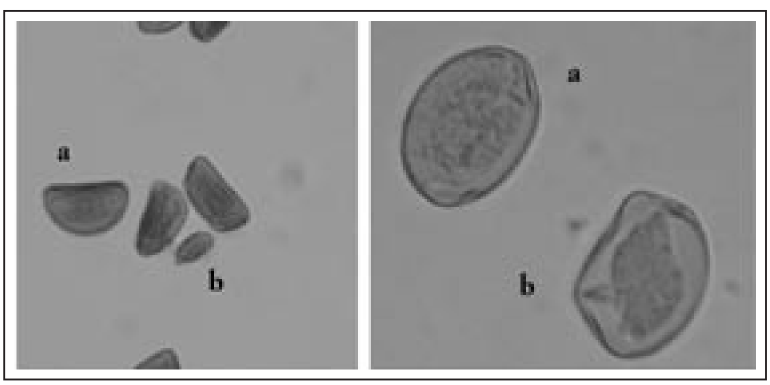

Fig. 3 — Some examples of fertile (a) and sterile (b) pollens observed after treatment with 6000 ppm concentration of Raxil for 12 h. (x1000).

Table 1 — Chromosome abnormalities and mitotic index in the root tips of *Allium cepa* L.exposed to different concentrations of Raxil.

Treatment period	Concentration (ppm)	Total number of cells	Total abnormality	Abnormal Prophase	Abnormal Metaphase			Abnormal Anaphase					MI	Abnormality %
					Distributed metaphase	C-Mitosis	Stickiness	MN	Bridge	Fragment	Lagging Chromosome	Distributed anaphase		
	Control	1050	15	-	5	2	4	-	4	-	-	-	3,7	1,42
3 h	1800	1037	48	1	16*	9	10	-	4	3	3	2	2,49**	4,60***
	2400	1115	53	2	15	9	12	-	3	5	4	3	1,61***	4,75***
	4200	1033	58	-	14	11*	14*	2	6	5	5	1	1,68***	5,61***
	6000	1006	64	2	16*	10*	15*	-	8	7*	4	2	1,36***	6,36***
6 h	1800	1174	58	1	16	7	15*	-	4	5	7*	3	2,37**	4,94***
	2400	1100	70	-	17*	10	20***	1	8	4	8***	2	1,45***	6,36***
	4200	1030	79	2	18*	10*	18***	-	10	5	6*	10***	1,21***	7,66***
	6000	1111	94	2	19*	15***	20***	-	10	6*	7*	15***	0,98***	8,46***
12 h	1800	936	55	-	13	7	13*	-	12*	2	8***	-	2,24***	5,87***
	2400	996	66	2	15*	10*	14*	-	15*	3	7*	-	1,93***	6,62***
	4200	1079	74	1	13	12*	19***	1	17***	4	6*	1	1,61***	6,85***
	6000	1098	86	2	20***	15***	20***	1	17***	4	6*	1	1,19***	7,83***
24 h	1800	1012	62	1	16*	11*	17***	2	6	2	2	5	1,98***	6,12***
	2400	993	66	1	17*	10*	16***	-	12	3	3	4	1,85***	6,64***
	4200	976	72	2	19***	13***	16***	-	11	5	5	1	1,62***	7,37***
	6000	962	78	1	18***	17***	14***	-	13*	4	7*	4	1,22***	8,10***

MN=Micronucleus; MI=Mitotic Index

*P≤0,05; **P≤0,01; ***P≤0,001

Table 2 — Frequencies of different types of meiosis abnormalities after treatment with different concentrations of Raxil.

Treat. period	Concen-tration (ppm)	Total number of cells	Total abnor-mality	Anaphase-Telophase I						Anaphase-Telophase II							
				Univ.	Qadriv.	Brid.	Lag. Chrom.	Frag.	Brid/ Lag.chrom.	Brid.	Lag. Chrom.	Frag.	Brid/ Lag.chrom.	Sticki-ness	MN	OA	Abnor-mality %
	Control	3460	24	12	-	2	8	-	-	1	-	1	-	-	-	-	0,69
3 h	1800	3437	55	25*	-	-	-	5	-	-	-	-	-	6*	4	15***	1,60***
	2400	4555	130	24	11***	9	4	26***	-	-	2	10*	-	3	16***	25***	2,85***
	4200	4170	110	16	7*	9	-	21***	-	1	-	11*	-	7*	18***	20***	2,64***
	6000	3281	98	20	3	10*	7	8*	6*	4	5	8*	-	5	7*	15***	2,99***
6 h	1800	3711	72	12	1	8	5	8*	-	4	-	7	-	8*	7*	12***	1,94***
	2400	3053	83	12	3	2	-	13***	-	3	1	7	-	15***	12***	15***	2,72***
	4200	2980	73	15	2	6	3	5*	3	3	-	6	-	8***	10***	12***	2,45***
	6000	3676	101	17	2	13*	4	10***	2	4	-	3	2	11***	12***	21***	2,75***
12 h	1800	4455	94	11	10*	6	3	5	-	1	-	9	-	16***	9*	24***	2,11***
	2400	2959	70	12	8***	10*	-	11***	-	1	-	3	-	6*	6*	13***	2,37***
	4200	2390	89	14	5***	8*	3	7***	1	1	1	8***	2	13***	11***	15***	3,72***
	6000	2226	93	7	10***	5	5	12***	3	6*	5*	10***	1	10***	9***	10***	4,17***
24 h	1800	3611	82	13	9***	5	2	7*	-	7	-	5	-	9***	10***	15***	2,27***
	2400	2409	66	11	3	7	2	-	-	3	2	5	1	10***	8***	14***	2,74***
	4200	2187	45	4	5*	2	1	3	1	2	1	1	1	10***	5*	9***	2,06***
	6000	3021	92	11	7*	7	4	6*	4	4	3	5	3	10***	14***	14***	3,05***

Treat.period=Treatment peripd; Univ.=Univalent; Quadriv.=Quadrivalent; Brid.=Bridge; Lag.chrom.=Lagging chromosome; Frag.=Fragment; Brid./Lag.chrom.=Bridge/Lagging chromosome; MN=Micronucleus; OA=Other Abnormalities

*$P \leq 0,05$; **$P \leq 0,01$; ***$P \leq 0,001$

Table 3 — Meiotic abnormalities and pollen sterility produced by Raxil during meiotic division.

Treatment period	Concentration (ppm)	Total number of cells	Total abnormality	Abnormality %	Total pollens examined	Total sterile pollen number	Pollen sterility %
	Control	3460	24	0,69	1860	44	2,36
3 h	1800	3437	55	1,60***	2361	120	5,08***
	2400	4555	130	2,85***	2334	313	13,41***
	4200	4170	110	2,64***	2313	211	9,12***
	6000	3281	98	2,99***	1872	175	9,34***
6 h	1800	3711	72	1,94***	2152	133	6,18***
	2400	3053	83	2,72***	2352	176	7,48***
	4200	2980	73	2,45***	2184	173	7,92***
	6000	3676	101	2,75***	2060	171	8,30***
12 h	1800	4455	94	2,11***	2151	198	9,20***
	2400	2959	70	2,37***	2136	279	13,06***
	4200	2390	89	3,72***	1827	219	11,98***
	6000	2226	95	4,17***	1752	238	13,58***
24 h	1800	3611	82	2,27***	2064	270	13,08***
	2400	2409	66	2,74***	1932	144	7,45***
	4200	2187	45	2,06***	1988	231	11,61***
	6000	3021	92	3,05***	2252	266	11,81***

*P≤0,05; **P≤0,01; ***P≤0,001

sterility was seen after the treatment with 6000 ppm concentration of this chemical for 12 h. This concentration and treatment period also caused the highest percentage of meiotic abnormalities (Table 3). Fully stained pollen grains were considered to be fertile while shrivelled and empty ones were scored as sterile. The examples of fertile and sterile pollens are shown in Fig. 3.

DISCUSSION

Fungal diseases cause extensive crop losses each year. The fungicide tebuconazole is widely used to control fungal diseases in onion and other crops. In study of Epa (1999), it is reported that tebuconazole which is active substance of Raxil in different test systems including an Ames test with Salmonella sp. , a mouse micronucleus assay, a sister chromatid exchange assay with Chinese hamster ovary cells and an unscheduled DNA synthesis assay with rat hepatocytes provided no evidence of mutagenicity. There is no published data available on the cytogenetics effects of tebuconazole in plant systems. Chromosomes of *Allium cepa* L. can be used for testing the potential mutagens in both mitotic and meiotic cells (Grant 1982; Smaka-Kinel *et al.* 1996; Yuzbasioglu 2003; Celik 2006).

Mitotic index is an acceptable measure of cytotoxicity for all living organism (Smaka-Kinel *et al.*, 1996). The cytotoxicity level can be determined by the decreased rate of mitotic index. A decrease below 50% usually has sublethal effects (Panda and Sahu 1985). If mitotic index decreases below 22% of control, that it causes lethal effects on test organism (Antonsie-Wiez 1990). Generally, cytotoxic substances inhibiting mitosis effect the microtubule configuration (Armbruster *et al.* 1991). According to many investigators, abnormalities due to inhibition of spindle formation such as C-mitosis, multipolar anaphases, sticky and vagrant chromosomes, reflect high toxicity of pollutants (Amer and Ali 1974; Haliem 1990; Kovalchuk *et al.* 1998; Lazareva *et al.* 2003).

In the present study, Raxil decreased the mitotic index at all concentrations and at all treatment periods when compared with control. The decrease of mitotic index was dose-dependent. At all treatment periods, the highest concentration of Raxil decreased mitotic activity more than other used concentrations. The percentage of mitotic index decreased with the increase of cells with C-mitosis, disturbed metaphase-anaphase, sticky. Since it decreased the MI in root tip cells of *Allium cepa* L. Raxil can be accepted as a toxic agent in this study.

Raxil significantly increased the percentage of abnormal cells at all concentrations and treatment periods in both mitotic and meiotic cell divisions when compared with control. It has been shown by many investigators that several other fungicides induce chromosomal abnormalities in different plants (MANN 1977; BEHERA *et al.* 1982; BADR 1983; ARMBRUSTER *et al.* 1991; PANDY *et al.* 1994; BADR 1998). In this study, the most common abnormalities were stickiness, C-mitosis and disturbed metaphase in cell division.

Chromosome stickiness is characterized by chromosome clustering during any phase of the cell cycle. Stickiness may be caused by genetic and environmental factors. Several agents have been reported to cause chromosome stickiness (BADR and IBRAHIM 1987; CAETANO-PEREIRA *et al.* 1998). GAULDEN (1987) postulated that sticky chromosomes result from the defective functioning of one or two types of specific nonhistone proteins involving chromosome organization which are needed for chromatid seperation and segregation. The altered functioning of these proteins is caused by mutation in the structural genes coding for them or by the direct action of mutagens (TURKOGLU 2007). Although many studies have been reported on the occurrence of chromosome stickiness, the primary cause and biochemical basis of the phenomenon are still unknown (PAGLIARINI 2000). C-mitosis is one of the consequences of inactivation of spindle apparatus connected with delay in the division of centromere (MANN 1977). Disturbed metaphases and anaphases may be due to disturbance of spindle apparatus which allows that the chromosomes to spread irregularly over the cell (AMER and ALI 1974).

In this study, occurrence of C-mitosis, stickiness and disturbuted metaphase in root cells of *Allium cepa* L. clearly shows the accumulated effect of Raxil on the spindle formation. In addition to the mitotic abnormalities; bridges, lagging chromosomes, fragments were also observed at all the treatment groups.

Meiosis is a critical process in the life cycle of sexual plants. The normal and harmonious course of meiosis ensures gamete viability. The cytologic events of gametogenesis are controlled by a large number of genes. Mutations in these genes cause anormalities that may impair fertility (PAGLIARINI 2000).

In the present study, Raxil significantly induced meiotic abnormalities such as bridges, stickiness, laggard, univalent, micronuclei, fragment, synchronization disorder, formation of quadrivalent.

While stickiness was significantly shown in both mitotic and meiotic cell divisions, abnormalities such as formation of micronuclei and fragments occured during meiotic cell division. Micronuclei are the manifestation of the chromosome breakages and the failure of normal spindle function (DASH *et al.* 1988; GROVER and KAUR 1999). According to SPARROW and SINGLETON (1953), micronuclei are a fair index of fragment production. Bridges and fragments are clastogenic effects, both resulting from chromosomal and chromatid breaks (KOVALCHUK *et al.* 1998). In this study, micronuclei formation, bridge and fragments observed with Raxil may be results of clastogenic effects. Higher frequencies of these abnormalities in meiotic cells shows that reproductive cells are more sensitive than somatic cells to the clastogenic effects.

Synchronization disorder is due to formation of abnormal spindle and failure of cytokinesis. Because of unbalanced and steril gamet production, abnormal spindle is an important meiotic irregularity (DEFANI-SCOARIZE *et al.* 1995; CAETANO-PEREIRA *et al.*, 1998). Chromosomal stickiness, is generally regarded as a physiological effect on chromosomes during division. Pollen fertility may be partial or totally affected depending on the intensity of chromosome stickiness (PAGLIARINI 2000). SING (1992) mentioned that univalent and laggard formation may be due to the failure of pairing and lagging to the failure of moving apart.

These meiotic abnormalities observed with Raxil decreased pollen fertility. The percentage of pollen sterility increased with the increase of the percentage of chromosomal abnormalities. It has been observed that several other fungicides caused meiotic abnormalities and pollen sterility in different plants (MANN 1977; BEHERA *et al.* 1982; PANDY *et al.* 1994; BADR 1998). It is known that significant causes of pollen sterility are inversions and quadrivalents. But in present study, inversions were not obtained.

Quadrivalents are formed because of translocations. Increase in pollen sterility in plants with more number of quadrivalents, could be profitably used to isolate large number of plants showing quadrivalents, which in turn useful for preparation of translocation tester test (Reddy and Annadurai 1992). The consequence of meiotic aberrations can be evaluated by recording pollen and plant sterility. There is a direct relationship between chiasma and pollen fertility, multivalents reducing the latter (Sing 1992). Among the chromosomal causes of plant sterility, the inviable unbalanced gametes may be mentioned resulting from meiotic irregularities, misdisjunction of multivalents (Darlington 1937).

In this study, increase of the chromosomal aberrations was accompanied by decrease in pollen fertility. High pollen sterility was observed at the treatment group with increased formation of translocation and quadrivalents. Present results suggest that quadrivalents are more affective than other chromosome abnormalities on pollen sterility.

As a result, the present study shows that Raxil, commercial formule of tebuconazole, reduced mitotic index of cells because of its cytotoxic activity. Raxil also induced chromosomal abnormalities in both mitotic and meiotic cell divisions. Because of its effect on hereditary material, Raxil is considered as genotoxic. A linear relationship was observed between the percentage of mitotic abnormalities and mitotic index. Pollen sterility was the direct result of meiotic abnormalities which increased due to treatment. These results indicated that Raxil should be regarded as an mutagenic agent for plants. Hence, the use of this fungicide should be under control in agricultural fields.

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