CYTOTOXICITY EFFECTS OF THE FUNGICIDE RAXIL ON *ALLIUM* ROOT TIPS

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Abstract

Raxil belongs to triazole fungicides used in agricultural areas and it needs to understand its impact in plant cells. The purpose of the study was to investigate its cytotoxic effects in the root tips of onion (*Allium cepa).* Equal sized bulbs of *A. cepa* were treated at the molecular biology and animal physiology lab on 2015, with different concentrations of Raxil (1:4; 1:3; 1:2; 1:1 raxil/water ratio); mean values were calculated comparing with controls of distilled water. For mitotic studies, the grown root tips were cut after 4 days, fixed in carnoy and stained according to aceto-carmine procedure. Slides were viewed with a motic microscope plus 2.0ML, B1 series. The results showed that bulbs were significantly inhibited in root length with an amount of 33.7%, 44.8%, 61.6% and 92.2%, respectively. Increasing raxil concentrations decreased mitotic index from 56.1 at control to a minimum of 21.8 at cases ($p<0.05$). It may be due probably to the inhibition of DNA synthesis or the blocking in the G2 phase of the cell cycle. In the *Allium* test, inhibition of root growth, the appearance of stunted roots and suppression of mitotic activity indicate raxil cytotoxicity. Therefore, we need to monitor the quality of treated plants, in order to define its lethal effects on organism.

Keywords: Cytotoxic effects, fungicides, mitotic index, *Allium cepa.*

Përmbledhje

Raksili i përket fungicideve triazol të përdorura në kulturat bujqësore dhe është e rëndësishme të dihet ndikimi i tij në qelizat bimore. Qëllimi i këtij punimi është studimi i efektit citoksik në rrënjët e qepës (*Allium cepa)*. Bulbet me përmasa të njëjta të *A. cepa* u trajtuan në laboratorin e biologjisë molekulare dhe fiziologjisë shtazore, në 2015, me përqendrime të ndryshme të raksilit (1:4; 1:3; 1:2; 1:1, raport raksil/ujë) dhe llogariten vlerat mesatare të rritjes krahasuar me kontrollet në ujë të distiluar. Për studimet mitotike, majat e rrënjëve priten pas 4 ditësh, trajtohen me carnoy dhe ngjyrosen sipas procedurës me aceto-karminë. Slajdet u vrojtuan me mikroskopin motic plus 2.0ML, seria B1. Rezultatet tregojnë pengim me kuptim statistikor në gjatësinë e rrënjëve të bulbeve përkatësisht me 33.7%, 44.8%, 61.6% dhe 92.2%. Rritja e përqendrimeve të raksilit ul treguesin mitotik nga 56.1 në kontroll, në një minimum 21.8 në raste ($p<0.05$). Kjo ka mundësi të shkaktohet nga pengimi i sintezës së ADN ose bllokimi në fazën G2 të ciklit qelizor. Në testin *Allium*, pengimi i rritjes së rrënjëve dhe i aktivitetit mitotik janë tregues i citotoksicitetit të raksilit. Për rrjedhojë, duhet monitoruar cilësia e bimëve të trajtuara për përcaktimin e ndikimit vdekjeprurës në organizëm.

Fjalëkyçe: Ndikimi citotoksik, fungicide, treguesi mitotik, *Allium cepa*.

Introduction

Fungicides are most commonly used against diseases of agricultural crops in many countries; in Albania it makes up about half of the pesticides used (Tafaj, 2012). They are produced in a diverse range of products with novel modes of action. Considerable use of fungicides to defend plants towards fungal action offer for a long time protection of residues in food and in the environment (Barber et al., 2020). Fungicides may also cause variation in plant genetic information owing to their mutagenic and carcinogenic properties. Constant use of these chemicals may result in changing the hereditary constitution of an organism. To detect harmful effects of different pesticides, cytogenetic studies have been carried out on different plant species (Cantor *et al*., 1992, Bushra *et al*., 2002; Sheno, *et al.,* 2014; Bryson & Brix, 2019).

Raxil is a fungicide with extensive application worldwide, which contains the fungicides tebuconazole and thiram. For practical use, 50 ml of commercial raxil is diluited in 200 ml of water to treat 100 kg of seeds. It disrupts cellular signal transduction pathways, messing up cell division and cellular metabolism by affecting ADN and ARN synthesis and metabolism. Raxil chemicals accumulated within food chain to a toxic level, can affect directly the human health (Fisun & Rasgele, 2009). Fungicides are among the least investigated pesticides for their cytotoxic activities. As all pesticides, raxil effects human health but the direct evaluation is not possible because of ethnic, logistic and practical estimation. Hereupon, onion (*Allium cepa*) is very suitable for cytotoxic studies as a result of some of its advantages: a) The root growth dynamic is very sensitive to the pollutants; b) the mitotic phases are very clear in the onion; c) it has stable chromosome number; d) clear and fast response to the cytotoxic substances; e) spontaneous chromosomal damages occur rarely (Fiskesjö, 1969; 1985).

In the *Allium* test, inhibition of root growth and the appearance of stunted roots indicate cytotoxity and both observations are due to the suppression of mitotic activity (Asita et al., 2013). In this study, we have observed growth inhibition and mitotic division of the root meristem cells of *Allium cepa*, which serve as biomarker regarding raxil's cytotoxicity influence. *Allium* was selected as the test material because of its low chromosome number (2n=16) and larger chromosomal size (Nefic *et al*., 2013).

Material and methods

Small onion bulbs of the same size 16-18 mm weighing about 3-3.5 g, aged maximum 6 months were cut by removing the loose outer scales and scraped, so that the primordial root were immersed into the tested liquids.

The exposure time of bulbs in each experiment was 96 hours at 22°C and protected against direct sunlight. Six onion samples were placed directly in four experimental raxil solutions and in distilled water (1:4; 1:3; 1:2; 1:1 raxil/water ratio) (Figs 1 & 2) during 2015, in the laboratory of Molecular Biology and Animal Physiology, University of Elbasani. Distilled water used as control was divided into three portions, which were successively applied to the onion roots in 24 hour periods, except the first day. Hence each 24 hours, the roots obtained a fresh bath of the controlled solution. After 96 hours, the samples were removed from water bath and raxil solution under investigation. The macroscopic and microscopic tissue morphology examinations followed.

Figure 1. Treatment of some onions bulbs with different raxil concentrations and control water.

For mitotic studies, after the 72 hours exposure, three root tips from two samples per concentration were collected at random and assessed. Root tips, 1-2 cm long, were cut from the treated *Allium* and fixed in alcohol-acetic acid (ethanol: glacial acetic acid in 3:1 ratio) for 24 hours in a refrigerator at 4-6 ºC. The extremity of the roots were cleaned with enough quantity of water for 10 minutes giving the necessary time to dry. A solution of 1N HCl at 60 ºC, (Fisun & Rasgele, 2009) was added to the root tips for 10 minutes. The HCl treatment was repeated.

For each concentration of pesticide, three root tips were transferred individually to a clean microscope slide and cut about 2 mm from the growing tip. The tips were kept while the rest was discarded. The extreme rounded root was put in acetic-carmine stain (Carolina Biological Supply Company, USA) for 10 minutes. A glass cover slip was placed on the root tip and tapped gently to

spread the cells evenly to form a monolayer facilitating the scoring process for normal and abnormal cells in the various stages of the cell stage. The slides were coded and viewed under the Motic light microscope, B1 series. The cells were scored under oil immersion at 1000- x magnification and the most representative of each class of structural aberration were photographed with a Moticam digital camera Images Plus 2.0ML.

Thereabouts 50 cells were scored on each slide, three slides (n = 3) for each concentration, and classified as interphase or one of the division staged (prophase, metaphase, anaphase or telophase). Hereby, roughly 800 cells were accounted referring the case group and the control group (distilled water). The mitotic index (MI) was expressed as the number of cells in the division stages per 100 cells scored. The MI of each treatment group was compared with the negative control group MI using anova, one way analysis of variance at 0.05 level of significance (Agresti, 1992). Mitotic index defines a parameter that permits evaluation of the rate the cell proliferates (Fiskesjö, 1985) and the decrease of mitotic division has been described to refer the potential contained components that are cytotoxic (Cantor *et al*., 1992; Turkoglu, 2007; Blake *et al*, 2018).

Statistically proved significant variances between the studied samples are confirmed by the statistical account of paired evidence survey; it was by utilizing the Analysis of variance (ANOVA), that allows the *p* value feature between pairs of results (Agresti, 1992). These coupled findings are either distinct (statistically significant) or not identical (statistically insignificant), showing the amount of the risk.

Results and discussion

In Albania, different fungicides are used in agriculture to protect stored food products from damage and thereby to increase productivity reaching about half of the pesticides used (Tafaj, 2012).

As we know, in our country no other study has evaluated if raxil cause cytotoxicity. Introduction of research in environment protection is of great importance, since it enables us to understand the impacts and consequences of cytotoxic substances present in the food. The goal of our research is to give an immediate and important contribution preserving the health of the plants which influence directly in human health. The results show that the use of this fungicide should be under control in agricultural fields. The mean value for root length in the *Allium* bulbs treated in 1:4, 1:3, 1:2 and 1:1 were calculated and compared with the control. The root length of the *Allium* bulbs grown were dependent on concentration of raxil used (Fig. 2), indicating apparent inhibition of root growth compared to the control water (Tab. 1) likewise the appearance of stunted roots.

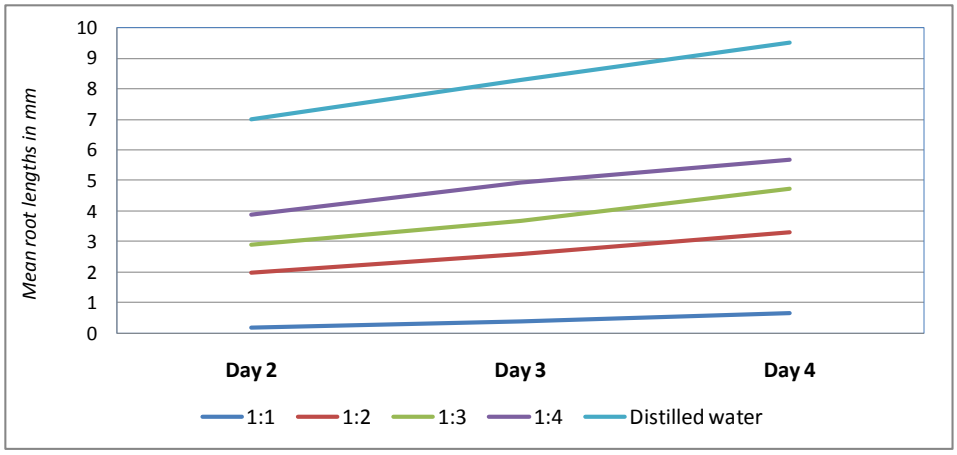

Figure 2. Growth curves (estimated means in mm) of onion roots in four days: treated in distilled and tap water and in different raxil/water concentrations (1:4; 1:3; 1:2; 1:1).

Table 1: Mean root lengths in cm and standard deviation (SD) of onions bulbs in different concentrations of raxil and controls.

Solutions	Day 2 (cm)	SD	Day 3 (cm)	SD	Day 4 (cm)	SD	Growth decrease (%)
1:1	0.2	0.08	0.4	0.09	0.67	0.1	92.2
1:2	2	0.2	2.6	0.52	3.3	0.57	61.6
1:3	2.9	1.13	3.7	1.27	4.75	1.15	44.8
1:4	3.9	0.82	4.95	1.03	5.7	0.96	33.7
Distilled water	7	1.23	8.3	1.19	9.5	1.06	-

Taking into account the cell proliferation, the frequency of mitotic cells progressively decreased with increasing raxil concentration (Fig. 3) reaching a minimum at 1:1 raxil concentration. It suggests that raxil blocks cell division cycle at a stage before mitosis. Reduction of mitotic index over 20% in all cases taken, reaching its highest value over 61.2% at 1:1, shows raxil toxic effect on elongation zone (Tab. 2).

The cytotoxic level of a test chemical can be determined based on the increase or decrease in the mitotic index (MI), which can be used as a parameter of

cytotoxicity in studies of environmental biomonitoring (Smaka-Kincl *et al*., 1996; Sudhakar *et al*., 2001).

Table 2: The effects of different raxil concentrations on cell cycle. MI, Mitotic Index; *, significantly different from control $p<0.05$.

Control & Raxil Concentration ratio	Nr. of cells	No. of mitotic cells	MI	MI decrease, %
Water control	162	91	56.1	0
1:4	163	72	44.1	21.4*
1:3	164	62	38	32.3*
1:2	161	49	30.4	45.9*
1:1	118	26	21.2	61.2*

Allium was selected as the test material in the present study because of its low chromosome number and larger chromosomal size. Significant reduction of MI in root cells, noted in the present study may be due to the inhibition of DNA synthesis or the blocking in the G2 phase of the cell cycle (Lazareva *et al.*, 2003; Sheno *et al.*, 2014).

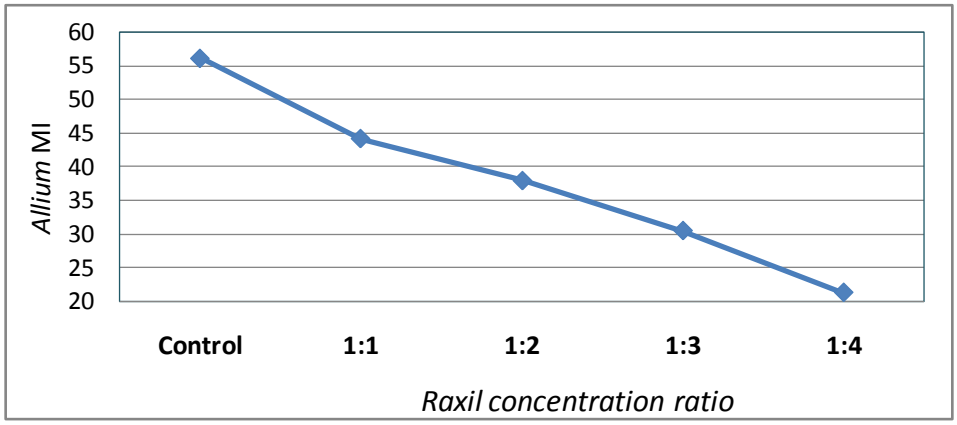

Figure 3. Decrease rate of *Allium* MI at different raxil concentration ratio.

Several other chemicals have been reported to inhibit mitosis (Turkoglu, 2007). Inhibition of mitotic activities is used for tracing cytotoxic substances.

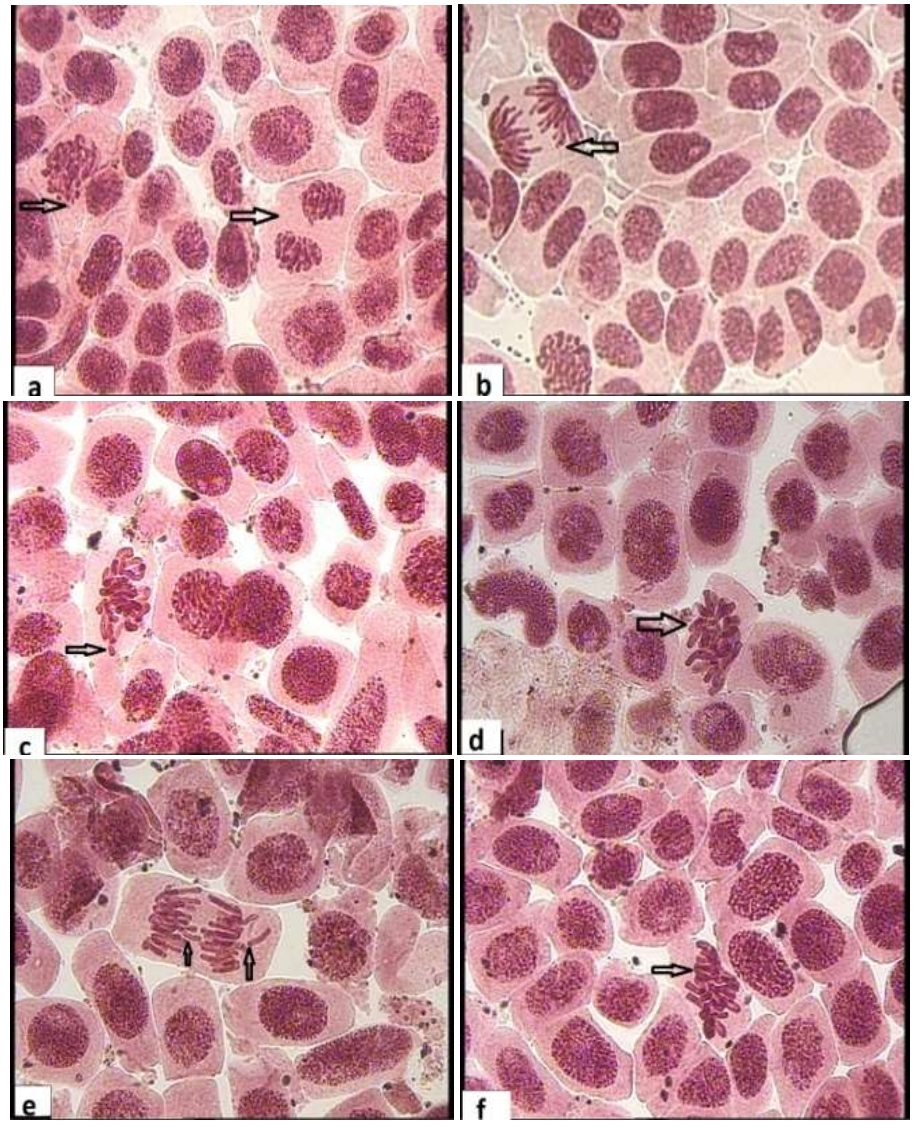

Figure 4. Microscopic parameters for standard use in *Allium* test (labeled with an arrow). All treatments after 4 days. a) Normal prophase, telophase (distilled water); b) normal anaphase; c) laggard chromosome (1:2 raxil); d) c- metaphase (1:3 raxil); e) vagrant chromosome and bridges in anaphase (1:1 raxil); f) sticky metaphase (1:3); magnification 780x.

The cytotoxic degree can be defined by the reduction amount of mitotic index. A mitotic index decrease over 20% of negative control causes lethal effects on test organism while a decrease over 50% has sublethal effects (Sharma & Vig, 2012) and is called cytotoxic limit value. Several investigators have used MI as an endpoint for the evaluation of genotoxicity or antigenotoxicity of different chemical treatments (Sharma & Vig, 2012; Panda & Shahu, 1985). Raxil decreased the mitotic index at all concentrations when compared with control. The same examples are observed furthermore, by (Fisun & Rasgele, 2009) on *A. cepa* by applying fungicide raxil. The reduction of mitotic index was based on the dose used. Increasing concentration of the fungicide is associated with reduced of mitotic index.

The common abnormalities encountered were c- mitosis, stickness, bridges, laggards, fragments (Figs. 4c, d, e, f) usually on metaphase or anaphase at treated samples. Raxil showed genotoxic effects on the chromosome level at the *Allium cepa* test which has perfectly equivalence with mammalian organism (Fiskesjö, 1985). Lack of activity of spindle structure might have conducted to disorders in meta- and anaphases. The error of spindle organization may even lead to split or multipolar spindle. Several publications have outlined the role of certain pesticides in spindle formation (Cantor *et al*., 1992; Sheno *et al.,* 2014; Maity, 2014; etc.). Breaking and fusion induce stickiness and clustering of metaphase and bridge of anaphase owing to forming chromosomes having two centromeres (Wang *et al*., 2007; Pampalona *et al*., 2010). Some cells, when observed in an optical microscope, seemed to be unstructured with a fragile envelope and in some cases rupture of the cellular membrane which are indicative of cell death.

Conclusion

These results indicated that Raxil should be regarded as a cytotoxic agent for plants. Hence, setting "Maximum Permissible Concentrations" (MPC), which would ensure a good and reliable quantity of raxil would not guarantee the quality of treated plants. Hence, the use of this fungicide should be under control in agricultural fields, in order to avoid its lethal effects on organism.

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