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Evaluation of genotoxic and antimicrobial effect induced by fungicide Dithane M-45

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SUMMARY. The cyto-genotoxic potential of fungicide Dithane M-45 on the root meristem cells of *Tulipa praestans* Hoog. cv. 'Unicum' and antimicrobial effects on some bacterial strains were investigated for concentrations lower than those currently used in agricultural practice.

The results indicate a stimulating effect of the treatments with this fungicide on mitotic division, associated with a higher frequency of chromosomal aberrations for all concentrations tested, and for all root harvesting periods. The high sensitivity of root meristem cells of *Tulipa praestans* cv. 'Unicum' to the fungicide action suggest the potential of this species to be used as a plant-system for detecting the mutagenicity and genotoxicity, mainly the clastogenic and aneugenic effects, of various chemicals. However, only a slight antibacterial effect against the tested bacteria was observed in the paper disc and agar well diffusion assays.

Keywords: Antimicrobial effect, cytotoxicity, dithane, genotoxicity, tulipa

Introduction

For a long time, chromosomal aberrations were a criteria for assessing the reproductive process in plants and have been correlated with the morphological and taxonomic changes, fertility - sterility relationships, mutations and other characteristics.

Genotoxicity and cytotoxicity testing of chemical compounds on various plant species is a simple, fast and sensitive enough technique (Grant, 1982), validated by the United Nations Environment Program (UNEP), World Health Organzation (WHO) and US Environmental Protection Agency (US EPA) (Türkoğlu, 2009) to be efficient for monitoring of environmental pollutants (Ma, 1999; Yi and Meng, 2003; Bolle *et al*., 2004).

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The pesticides, widely used throughout the world, contain a wide variety of different substances both in terms of composition and in terms of their properties, being used to kill or remove the harmful organisms. The extensive use of pesticides to control pests, and their long persistence in the environment, led to the initiation of research on developing systems for testing and evaluation of their cyto-genotoxic potential. While the use of pesticides has become a necessity, their ingredients induce acute toxic effects in different non-target organisms, as well as in experimental systems (Sudhakar *et al*., 2001). In the case of plants, pesticides can act as a mutagen to induce the occurrence of chromosomal aberrations and cytotoxicity (Chauhan *et al*., 1999; Yuzbasioglu, 2003; Popescu *et al*., 2013; Şuţan *et al*., 2014). The active substances of pesticides can have adverse or killing effects not only on target organisms (plant pathogens and pests), but can be also harmful to non-target organisms such as the soil bacteria. Among all bacteria from soil, some species that are involved in biodegradation process and useful for soil fertility can be sensitive to chemical substances. Some soil bacterial strains are plant pathogens, or humans and animals opportunistic pathogens. When is exposed to pesticides and fungicides, the soil microbiota might be adversely affected (Hussain *et al*., 2009).

Knowing that many pesticides, respectively insecticides, fungicides or herbicides are highly effective in inducing chromosomal aberrations, we initiated a study on the cyto-genotoxic effects of pesticide Dithane, in various concentrations, on the genetic material in somatic cells that are in mitotic division, in the species *Tulipa praestans* Hoog cv. 'Unicum'.

In this paper, we present relevant data to support the reliable use of the above mentioned plant system for detecting genotoxic effects of pesticides, evaluating changes in the mitotic index and identifying different types of chromosomal aberrations occurred as result of the exposure of meristematic root cells to pesticide action. Also, we estimated the effects of pesticide Dithane M-45 on several bacterial strains isolated from cultivated soil.

Materials and methods

Dithane M-45 is a very well-known multisite fungicide, which belong to the ethylene bisdithiocarbamates group. The active ingredient of fungicide tested is 80% mancozeb, as a coordination product of Zinc ion and Manganese ethylene bisdithiocarbamate.

Mancozeb is a broad spectrum contact fungicide with protectant activity against a wide range of plant diseases.

Evaluation of cytogenotoxic activity

The plant used as test material was *Tulipa praestans* cv. 'Unicum' (2n = 16). Three clean and healthy bulbs of *Tulipa praestans* cv. 'Unicum' were chosen for each treatment group. After dry scales of bulbs were removed, onion bulbs were grown in freshly tap water, at room temperature. When the roots reached 1.5-2 cm in length,

they were treated with different concentrations of aqueous solution of Dithane M-45 containing 300, 600 and 900 ppm of active ingredients for 6 hours period. The concentrations were chosen to be lower than those doses used in agricultural field to control different diseases. The control was prepared by exposing the seeds to water only. After 6 hours of treatment went by, roots were transferred into fresh tap water for 24 h, and 48 h respectively.

The roots were collected and fixed in Carnoy 1:3 acetic acid-ethyl alcohol mixture for overnight and then preserved in 70% alcohol at 4ºC for cytological studies. The root tips were hydrolyzed in 1 N HCl at 60^0C for 12 minutes, followed by staining with 2% aceto-orcein at 60^0C for 14 minutes. After proper fixation and staining, appropriate squash preparations were made for each of the treatment and control. Effect of chemical treatment and control on different chromosome plates were observed under light microscope. All observations were made from temporarily prepared slides. To determine the effects of this fungicide on mitotic index, 3000 cells were scored in control group and in each treated group.

Mitotic index (MI) was computed by determining the mitotic cell frequency (prophase, metaphase, anaphase and telophase) by the total number (3000 cells) of cells observed and multiplying the result by 100.

Cytological abnormalities were also observed and scored. Photomicrographs of cells showing chromosomal aberrations as well as showing normal mitosis were taken using an CX31 Olympus microscope.

Percentage of cells showing chromosomal abnormalities such as sticky chromosomes, laggard chromosomes, multipolar anaphases, as well as aberrant interphases (binucleate cells) were recorded at the appropriate mitotic stages.

Statistical analysis

Results are presented as the mean ± standard error of more independent experiments. Statistical significance analysis of the obtained data was performed by the use of analysis of variance (one way ANOVA). Additionally, Duncan's multiple range test was used to compare the means of the treatments. Significant differences were set at $P \leq 0.05$.

Evaluation of antibacterial effect

The disc diffusion method and agar wells method were used for testing the bacterial sensitivity to Dithane M-45 (Valgas *et al*., 2007).

Five bacterial strains isolated from cultivated field soil samples and identified with API test kit were used in our experiment: *Pseudomonas (Chryseomonas) luteola* (95.4%), *Elizabethkingia meningoseptica (Chryseobacterium meningosepticum)* (85%)*, Ewingella americana* (80.4%), *Weeksella virosa* (87.6%), and *Pasteurella pneumotropica* (93.1%).

Pseudomonas (Chryseomonas) luteola is a non-fermenting, Gram negative, strict aerobic rod, motile with polar multitrichous flagella. Colonies are yellow on solid media, either smooth or wrinkled (Hansen and Nielsen, 2011). It is frequently found especially in damp environment (water, soil) and it can cause septicaemia, endocarditis, peritonitis and other infections mainly in patients with immunosuppressive therapy or health disorders. The susceptibility to tetracycline was registered for some clinical strains that were resistant to cephalosporins, but other studies indicate resistant strains to tetracycline (Chihab *et al*., 2004; Doublet *et al*., 2010).

Elizabethkingia meningoseptica (formerly known as *Chryseobacterium* or *Flavobacterium meningosepticum)* is an opportunistic pathogen, that can cause meningitis, sepsis, pneumonia, infection of skin or other organs (Ceyhan and Celik, 2011), mainly in newborns or immunocompromised patients. It is a Gram negative, aerobic, non-fermenting and non-motile, oxidase - positive rod, widely distributed in nature (soil, freshwater and saltwater) and in hospital environments. Because in clinical cases the bacteria are inherently resistant to many antibiotics, *E. meningoseptica* can be considered a potential nosocomial pathogen (Dias *et al.*, 2010).

Ewingella americana is a Gram negative rod of Enterobacteriaceae Family, common in some ecological niches (like some type of food: vegetables, meat, mushroom), but also in human blood, respiratory tract secretions, faeces, urine or wounds (Rozhon *et al*., 2012). It is lactose fermenting, oxidase negative, indole negative and catalase positive, facultative anaerobic rod (Hassan *et al*., 2012). In some clinical cases this bacterium can produce severe infections because the antibiotic resistance.

Weeksella virosa is a Gram negative aerobic rod, oxidase positive, indole positive and catalase positive bacterium. It is unable to grow on MacConkey agar. It can be isolated from urine, blood, cerebrospinal fluid, from the genital tract or ears, eyes, rectal area and can be associated with pneumonia, peritonitis, urinary tract infections and bacteremia (Slenker *et al*., 2012). Some clinical isolates can resist to tetracycline, other strains can be susceptible to this antibiotic (Reina *et al.,* 1990).

Pasteurella pneumotropica is an opportunistic Gram negative pathogen, non-motile, cocobacillary to rod-shaped organism, which can cause rather latent infections in rodents such as upper respiratory infections and pyogenic syndrome (Harlev *et al*., 2009). It can be found in soil, and some studies point out the *Pasteurella pneumotropica* capacity of polychlorinated biphenyls (PCBs) degradation.

After obtaining an overnight culture by incubation at 37°C on nutrient agar, and then a bacterial suspension into 2 mL sterile saline buffer for each bacterial strain, we used all five bacterial suspensions both in filter paper disc diffusion method (PD) and agar wells diffusion method (AW).

In filter paper disc diffusion method dry and sterilised filter paper discs (6 mm diameter) were used (Deliu, 2010; Parihar *et al*., 2010). Each bacterial strain was homogenously mixed and uniformly inoculated with the help of a sterile cotton swab on

dried surface of nutrient agar in Petri dishes (4 mm level depth). We allowed the medium surface to dry (only a few minutes) and the antibiotics to reach room temperature before applying the discs. Then, we discharged the antibiotic disc and the filter paper discs with flamed and cooled forceps and we gently pressed the disc into the solid medium with the help of the same forceps. The distance between discs was about 25 mm. The disc with Tetracycline (30 µg per disc from Bioanalyse) was placed on the inoculated surface as a positive control and sterile distilled water was used as a negative control.

Four filter paper discs were placed on the medium. We impregnated each discs with 5µL of a certain concentration of Dithane M-45 (respectively 300 ppm, 600 ppm and 900 ppm), and the negative control with sterile distilled water by using micropipettes. Then, the inoculated plates were incubated inverted, for 24 h at 37°C, under aerobic conditions.

In the agar wells diffusion method, the bacterial suspensions were homogenously distributed on the nutrient agar in plates by using a sterile cotton swab, after which we cut four 6 mm diameter wells in the solid medium (using a sterile glass capillary tube), spaced at about 25 mm apart from one another (Valgas *et al*., 2007).

In every agar well we put 5 µL of Dithane M-45 (300 ppm, 600 ppm and respectively 900 ppm) and of negative control, by using a micropipette. As in the filter paper disc diffusion method, Tetracycline (Bioanalyse) was used as positive control (30 µg per disc). We allowed the solution to diffuse in the medium and then the plates were incubated inverted for 24 h at 37°C.

The antibacterial effect of Dithane M-45 was evaluated by reading the experimental results. The confluent bacterial growth was observed for each tested bacterial strain. The diameters of inhibition zones were measured in mm from the underside of the plates as a clear zone surrounding the discs, and agar wells, respectively.

Results and discussion

The results obtained in experiments performed in order to highlight the cytogenetic effects of the fungicide Dithane in *Tulipa praestans* cv. 'Unicum' showed a statistically significant increase in the general mitotic index following treatments with the fungicide Dithane in concentrations of 300 ppm, 600 ppm, and 900 ppm, respectively, as compared to the control (Fig. 1).

Transfer of the rooted tulip bulbs to tap water after 6 hours of treatment with Dithane had a stimulating effect, the number of cells undergoing mitotic division being higher and significantly higher after 24 hours and respectively 48 hours of maintenance in tap water, depending on the concentration of the fungicide.

A significantly increase in the general mitotic index values was found in the experimental variant with Dithane in concentration of 900 ppm, both immediately after treatment (5.65 %), and after the transfer of rooted bulbs to tap water for 24 hours (5.86 %) or 48 hours (7.3 %) .

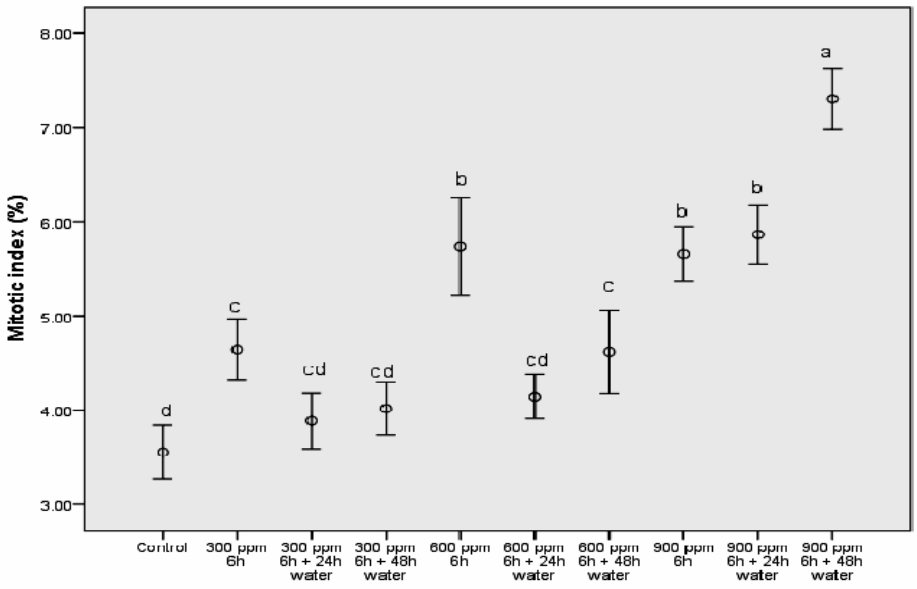

Figure 1. The variation of mitotic index in apical root meristems of *Tulipa praestans* cv. 'Unicum' treated with the fungicide Dithane. The standard errors are ilustrated in the graph as error bars; a, b, c, d: assessment of the significance of differences, by Duncan's Multiple Range Test, $p<0.05$.

Similar results were obtained when assessing the cyto-genotoxic potential of the fungicide Dithane M-45 in Narcissus pseudonarcissus (Şuţan *et al.,* 2014). Using a different formula of the pesticide Dithane (750 g/kg), Asita and Makhalemele (2009) found an inhibitory effect on mitotic division, which could be the consequence of different concentrations tested.

In the case of treatment of the roots with solutions of Dithane in various concentrations (300 ppm, 600 ppm, 900 ppm), the change in the mitotic index was associated with the induction of mitotic and chromosomal aberrations such as vagrants and laggards, metaphases with sticky or depolymerized chromosomes, anaphases with chromosome fragments, C-metaphases, asters in anaphase, bridges in anaphase, multipolar anaphases (Fig. 2). Also, there were observed variations in the number of chromosomes (Fig. 3).

The frequency of chromosome aberrations varied widely depending on the concentration of the fungicide and the time of collection of roots, the highest percentage (0.90 %) being determined for the treatment with 600 ppm Dithane for 6 hours + 48 hours of recovering in tap water. Regardless of the fungicide concentration, the transfer of roots in tap water after the treatment with Dithane was correlated with an increased frequency of chromosome aberrations (Table 1).

Table 1.

The frequency of chromosome aberrations induced in *Tulipa praestans* cv. 'Unicum' following treatment with different concentrations of Dithane.

Treatment duration	**Concentration of Dithane solution**	**Chromosome aberrations (%)**				**Total number of chromosome aberrations** (% ± ES)
		Prophase	**Metaphase**	**Anaphase**	**Telophase**	
Control	0	0	0	0	0	0 ± 0^{c}
6 h	300 ppm	0	0,01	0,02	0,01	$0,01 \pm 0,03^{c}$
6 h + 24 h in water		0	0,02	0,02	0,01	$0,01 \pm 0,05^{c}$
6 h + 48 h in water		0	0,07	0,06	0	$0,03 \pm 0,019^{c}$
6 h	600 ppm	0	0,16	0,26	0,23	$0,16 \pm 0,058^{bc}$
6 h + 24 h in water		0	0,28	0,34	0,21	$0,21 \pm 0,074^{bc}$
6 h + 48 h in water		0	0,36	0,33	0,20	$0,22 \pm 0,081^{bc}$
6 h	900 ppm	0	0,27	0,34	0,32	$0,23 \pm 0,078^{bc}$
6 h + 24 h in water		0	0,66	0,82	0,42	$0,48 \pm 0,178^{b}$
6 h + 48 h in water		0,26	1,60	1,43	0,33	$0,90 \pm 0,354^{a}$
Total number of chromosome aberrations (% ± ES)		$0,03 \pm 0,006^{b}$	$0,34 \pm 0,153^{a}$	$0,36 \pm 0,142^{a}$	$0,17 \pm 0,09^{a}$	$0,226 \pm 0,056$

It is considered that the formation of sticky chromosomes involves chromatin protein matrix rather than nucleotide sequences. Generally, a consequence of the induction of chromosome stickiness is the formation of ana-telophase chromosome bridges. The aggregate state of the genetic material is irreversible, often resulting in significant decrease of the mitotic index, as well in cell death (Fernandes *et al*., 2009). In our study, the number of cells with sticky chromosomes was extremely low, which can be correlated with the higher values of the mitotic index in treatments with Dithane.

Chromosome breakage can be associated with either an increased affinity to mutagens of some regions of DNA, the presence of highly fragile sites, or the composition of some specific DNA sequences, probably related to the nuclear matrix (Kihlman, 1966). The results of numerous studies showed that chromosome breakage induced by the alkylating agents is produced typically in the heterochromatic regions. In our study with Tulipa praestans, the chromosome breakage occurred both in the terminal and middle regions. The largest chromosomal fragments were observed

following treatment with Dithane in concentration of 900 ppm. The presence of the C-metaphases may be a consequence of partial or complete inhibition of the spindle formation (Fiskesjö, 1993; Fernandes *et al*., 2009), which also explain the formation of cells with a variable number of chromosomes (from 2n = 24 to 2n = 37). In a single cell, the observed chromosome number was lower (2n = 7) than the basic chromosome number in the genus Tulipa (x = 12) (Fig. 3). In this context, it should be noted that, in the case of some C-metaphases, chromosomes with achromatic lesions were observed (Fig. 2).

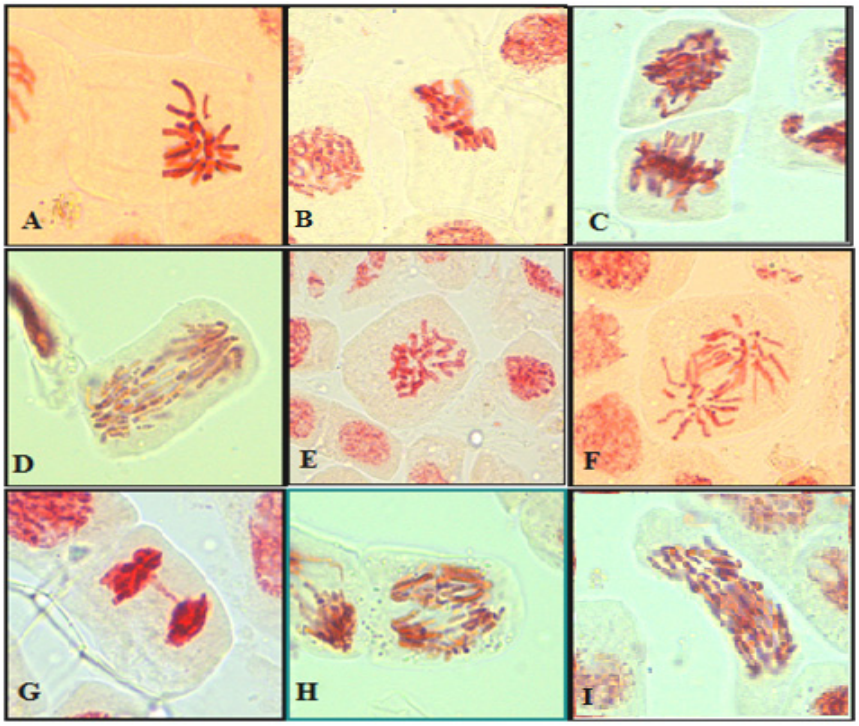

Figure 2. Chromosome aberrations induced by the fungicide Dithane M-45 in root meristem cells of *Tulipa praestans* cv. 'Unicum'. (A) vagrant; (B) sticky chromosomes; (C) chromosome fragments in metaphase; (D) chromosome fragments in anaphase; (E) C-metaphase; (F) aster-like arrays of chromosomes in anaphase; (G) chromosome bridges; (H) multipolar anaphase; (I) abnormal anaphase.

Anaphase chromosome bridges, seen in both anaphase and telophase, might be the consequence of the existence of cohesive chromosome ends, chromosome rearrangements, or chromosome fusions, in which case the chromosome bridges can be multiple and may persist until telophase (Giacomelli, 1999; Marcano *et al*., 2004).

Kuriyama (1982) noted that the polyploidization agents induce the increase of microtubule organizing centers (MTOCs). The MTOCs replicates throughout the cell cycle, simultaneously with the DNA replication (Ghadimi *et al*., 2000). These structures are responsible for cell polarity and chromosome segregation in anaphase, which maintain the numerical chromosome stability and genomic integrity organisms (Sumara *et al*., 2004). The presence of supernumerary organizing centers of microtubules, accompanied by the inhibition of cytokinesis and deregulation of the nuclear spindle formation (Fig. 2), causes unequal chromosome segregation and formation of multipolar anaphases (Ochi *et al*., 2003). In the studies carried out with *Tulipa praestans* cv. 'Unicum', we have seen the occurrence of multipolar anaphases (Fig. 2).

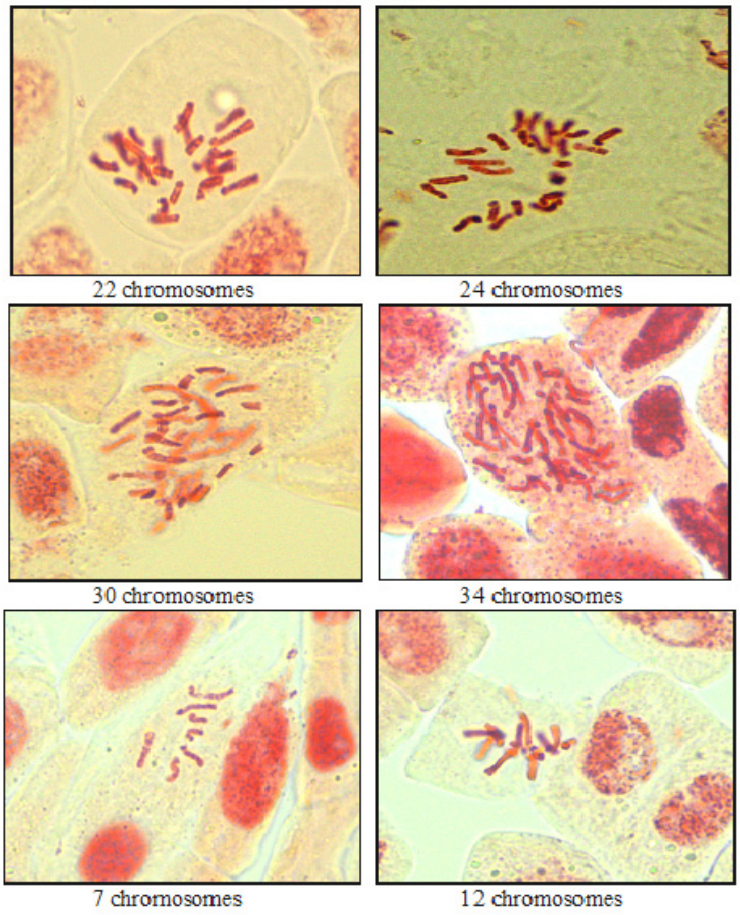

Figure 3. Variation of the chromosome number in root meristem cells of *Tulipa praestans* cv. 'Unicum'.

The antimicrobial activity of different concentrations of Dithane M-45 is illustrated as the diameter of inhibition zone measured in mm, in Table 2.

All three concentrations of Dithane M-45 had only a poor effect against the tested bacterial strains. The largest inhibition zone was found for Dithane M-45 900 ppm on *Weeksella virosa* and *Elizabethkingia meningoseptica*. The diameters of inhibition zones were higher with increasing pesticide concentration (Fig. 4, 5).

All five bacterial strains tested in this study, which are inhabitant in soil, can be potential pathogens for humans or animals. Because of their origin, they exhibit sensitivity to tetracycline (according CLSI standards a diameter larger than 15 mm shows a sensitive strain), while the clinical isolates could be resistant to this antibiotic.

Table 2.

Antimicrobial activity of Dithane M-45 in paper disc diffusion method (PD) and agar wells diffusion method (AW).

Variants/ Control	**Tested microorganisms (mm, diameter of inhibition zone)**									
	Pseudomonas luteola		*Elizabethkingia meningoseptica*		*Ewingella americana*		*Weeksella virosa*		*Pasteurella pneumotropica*	
	PD	AW	PD	AW	PD	AW	PD	AW	PD	AW
Dithane M-45 300 ppm	7	7	7	8	-	-	7	9	-	-
Dithane M-45 600 ppm	7	7	7	8.5	7	-	7	9	-	-
Dithane M-45 900 ppm	7.5	8	7.5	9	7.5	-	8	9.5	7	7
Tetracycline 30 µg per disc (positive control)	28		28		24		26		26	

In other studies on the effects of Dithane M-45 on soil bacterial strains (Deliu, 2010), in which higher concentrations of the fungicide were used, the largest inhibition zone was found for Dithane M-45 0.16% and 0.32% (up to 30 mm), while Dithane M-45 0.08% led to inhibition zones which mostly ranged between 7 - 10 mm.

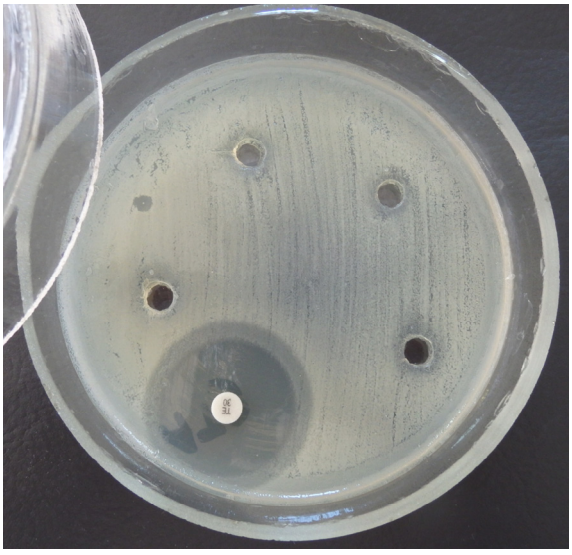

Figure 4. Antimicrobial activity of Dithane M-45 on *Weeksella virosa* - agar wells method.

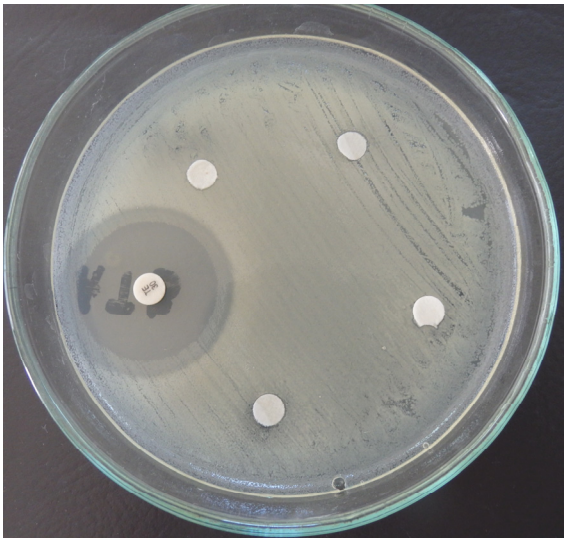

Figure 5. Antimicrobial activity of Dithane M-45 on *Elizabethkingia meningoseptica* - filter paper discs method.

Conclusions

The meristematic root cells of *Tulipa praestans* cv. 'Unicum' proved to be sensitive to the mitogenic effect of the fungicide Dithane, which resulted in a significant increase of the percentage of cells undergoing the cell division. Completion of at least two cell cycles subsequently to the fungicide treatment had also a stimulatory effect, the frequency of cells in one of the phases of mitotic division being in these treatments approximately double as compared to the control.

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