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Effect of Fungicide MAJOR-75 on Growth and Mitosis in Onion (*Allium Cepa*) Root Apical Meristem

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Abstract

Toxic effects of fungicide, MAJOR-75 were evaluated by analyzing root growth and root morphology. The higher concentration (500 ppm) of fungicide an inhibition of root growth and there was a statistically significant difference between control groups. In addition, cyto- and genotoxicity were estimated by observing cytological parameters such as the mitotic index and number of chromosome abnormalities, including chromosome breaks, stickiness, and polar deviations. The mitotic index (MI) of *A. cepa* meristematic cells treated with the 500 ppm of fungicide was significantly decreased in comparison to the negative control. The obtained results indicate that MAJOR-75 had the ability to cause a reduction in the seed germination percentage in the number of different phases of mitosis. The fungicide treatment in root meristem cells of *A.cepa* with different concentrations resulted in the reduction in morphological and mitotic frequency of root tip cells of Onion. As we know, mitotic frequency reflects cell division frequency and is used to determine the root growth ratio as a significant parameter. Regardless of which process is affected, both processes result in a reduced supply of new cells in the root meristem leading to an eventual inhibition of growth.

Keywords: MAJOR-75, Allium cepa, toxicity and rodenticide

Introduction

In agriculture, plant diseases are controlled primarily by chemicals (pesticides, bactericides, nematicides, etc). As many as 400 chemicals are being used as pesticides (Grover and Tyagi, 1980). In modern agriculture, pesticides have become an essential component that increases the yield by reducing the disease rate. Despite the undoubted effectiveness of these pesticides in controlling plant diseases and improving crops yield, many studies have underlined their toxic effects on crop plants. The world agrochemicals market is computed to constitute 35% insecticides, 35% herbicides, 10% fungicides, and the balance 18% by the non-agricultural use, including public health. The corresponding figures for India were 70% insecticides, 22% fungicides, 3% herbicides, and 5% rodenticides and fumigants and they are increasing

day by day. Fungicides, a class of pesticides have long been used to control, prevent and remediate microbial growth. The use of a fungicide is one of the most important aspects in agriculture for the protection of seeds during storage as well as in the field by preventing the growth of fungi that may produce toxins. Fungicides are metabolic inhibitors and their modes of action can be classified into different groups; inhibitors of the electron transport chain, inhibitors of enzymes, inhibitors of nucleic acid metabolism, protein synthesis, and sterol synthesis (WHO, 1994). When they are used to control fungal diseases by killing the fungus that causes the disease. They are most commonly used against diseases of crops in many countries of the world.

Fungicide can be attributed to the various undesirable behaviour of plants, mainly due to the excess and unscientific application of it. This involves reduced photosynthesis, resulting in a decrease in both growth and yield of crop plants, abnormal fruit setting, abnormal flower setting, seed abortion, etc. These problems mandate the need for regulating the application of fungicides so that such undesirable effects on plants can be averted and thereby the chances of pollution can be checked. Fungicides enter our environment in both natural and anthropogenic ways. Once they enter our biological process, it's really difficult to eliminate them from the environment and disturb various biochemical processes, leading to fatal results. Numerous potentially mutagenic chemicals have been studied because they can cause mutagenic, damaging, and inheritable changes in the genetic material. Many thousands of fungicides are present in the environment and new chemicals are being introduced every year. No doubt, the rapid progress of the chemical industry has provided economic and social benefits but at the same time, it has accentuated the environmental and social problems.

Constant use of these chemicals may result in changing the hereditary constitution of an organism (Wuu and Grant, 1966). When some chemicals accumulated within the food chain to a toxic level, these chemicals affect directly public health. Currently, public concern about the impact of fungicides on humans, birds, fishes, and beneficial microorganisms that are exposed directly through various ways and indirectly through diet has increased. This is because fungicides tend to be applied repeatedly over a specific period of the year, so arguably pose a greater environmental risk than other types of pesticides, such as insecticides, which tend to be applied more intermittently to eradicate pest outbreaks when detected (Yoon et al., 2013). The few investigated fungicides were found to exert C-mitotic activity and induce chromosomal abnormalities in several crop plants (Fiskesjo, 1969). Some fungicides were also found to induce chromosomal stickiness, bridges, and lagging (Bielecki, 1974). The interest in the impact of fungicides is mainly related to their toxicity. Like all pesticides, fungicides also affect human health and the environment, hence the need for assessing their effects.

Dryanowska (1987) and Cantor *et al.* (1992) showed that the frequency of cancer increases among people who have been exposed directly or indirectly to fungicides. So those should be screened before use to select which are least toxic (Mann, 1977). Generally, toxic effects of environmental pollutants cause genetic damage to plant cells. Health risks that result from exposure to fungicides have sparked awareness among researchers, triggering the idea of developing nanoencapsulation pesticides to enhance cytoprotection as well as renoprotection of the fungicides. In addition, nanocapsules of fungicides have the slow-release

capability, high bioavailability, and site-specific delivery, which has attracted great interest from researchers. In the present study, the fungicide tested is MAJOR-75, a manganese ethylene bis-dithiocarbamate polymer coordinated with zinc ion, used widely to protect various field crops, fruits, vegetables, and ornamental plants against fungal diseases.

It is registered for use on a variety of vegetable, fruit, nuts, and grain crops and is marketed by different trade names. It is also used for seed treatment of cotton, potatoes, corn, safflower, sorghum, peanuts, tomatoes, flax, and cereal grains. It is reported to cause structural and functional changes in the thyroid of rats and also affect the level of glycogen, proteins, and lipids in testis, liver, and kidney (Axelstad et al., 2011). MAJOR -75 induces genotoxicity and apoptosis in cultured human lymphocytes (Srivastava et al., 2012). Mancozeb reacts with, and inactivates, the sulfhydryl groups of amino acids and enzymes within fungal cells and resulting in disruption of lipid metabolism, respiration, and production of ATP. Since it reacts with sulfur-containing amino acids, it is of direct necessity to check its effect on plants, how plants are being affected at their various concentration. Studies have been carried out to detect harmful effects of fungicides as well as their undesirable residues in water, food, and the environment as they may cause some serious health problems. Chromosomal abnormalities induced by some of these compounds were found to be linked with their capacity to induce mutations and can therefore be regarded as consistent evidence for the evaluation of genotoxicity (Grant, 1982). Plant genotoxicity assays are relatively inexpensive, fast, and give reliable results, and chemicals that cause chromosomal alterations in plant cells also produce chromosomal abnormalities in cultured animal cells that are frequently identical. The plants, being direct recipients of agro toxics, become an important material for a genetic test and environmental monitoring of cases affected by such products. Environmental biologists are presently concerned to safeguard human beings from exposure to chemicals. Unfortunately, the direct assessment in humans is not feasible because of ethnic, logistic, and practical considerations.

Several plant test systems are already in use and are found to be as sensitive and reliable as other short-term tests. *Allium cepa* (onion) has been considered as the best-established test system to indicate the presence of mutagenic chemicals due to sensitive dynamics of root growth, clear mitotic phase, stable chromosome number with kinetic characteristics of proliferation thus suitable for cytogenotoxic study (Barberio et al., 2011). In this study, the effects of MAJOR-75 fungicide were investigated in the mitotic cell division in onion (Allium cepa) root tip cells during germination. This is an excellent approach to establish the adverse effect of major-75 on cellular activities, especially, cell division.

Different parameters of *A.cepa* such as root growth rate, mitotic index, chromosomal abnormalities, etc. can be used to estimate the cytotoxicity and mutagenicity of environmental pollutants viz., fungicide in agricultural fields. When applied to soil, these chemicals retain their effectiveness for a considerable period and potentially pose a risk to the long-term fertility of the soil. The objective of this study was to monitor the morphotoxic and cytogenetic potency of commercial formulation of MAJOR-75 by the *Allium cepa* test system. It was done by recording and calculating the average number and length of root tips (morphotoxic effect) and by calculating mitotic index and frequencies of abnormalities in root tip cells of *A. cepa* L. with a view to detecting their mutagenic potential (cytogenotoxic effect).

Materials and methods

Test chemicals

MAJOR-75 was purchased from a local agricultural store. The other chemicals used in the present study were of analytical grade.

Test organism and fungicide application

Healthy and equal sized bulbs of common onion (*Allium cepa* L.), procured from local market of Kottayam. For experiment, the loose outer scales of bulbs and old roots were removed with the help of sharp and pointed forceps so as to expose the root primordia. Different concentrations of viz., 25, 50, 100 and 500 ppm were prepared by dissolving the calculated amount in distilled water, and control of distilled water was maintained. A set of four bulbs were grown in separate petridishes for each concentration of MAJOR-75 for 48 h. A set of 4 bulbs was also run-in distilled water used as control. The root lengths from the control and experimental sets were measured on the 5^{th} day as described earlier.

Morphological and cytological investigation

After treatment, the bulbs were washed thoroughly under running tap water. On third day, number and length of root tips were recorded for morphological analysis. The root tips from each bulb were plucked and fixed in fixative, Carnoy's fluid (1:3 glacial acetic acid: ethanol) for 24 h and then preserved in 70% ethanol for cytological analysis. Hydrolysis, squashing, staining of cells and preparation of slides was done according to the method outlined by Sharma and Sharma (1980). The slides were mounted and observed under the light microscope. The experiments were repeated 3 times and the statistical analysis was performed according to the results. Slides were coded and scored for mitotic index (at least 500 cells/slide) and mitotic aberrations. Photographs of different mitotic stages and chromosome abnormalities observed were taken using digital camera. Counts of different mitotic stages were also recorded and to calculate percentage mitotic index (MI).

Statistical analysis

One way ANOVA was carried out for statistical analysis of data with the help of SPSS software programme. Difference between control and exposure treatment groups was considered statistically significant at $p<0.05$. The data was expressed as mean value with ± standard deviation (SD) for each concentration.

Results and Discussion

In this study, the toxic effects of MAJOR-75 were investigated with the help of *A. cepa* which is preferred as non-target organism. *A. cepa* is an indicator material for determining the effects of fungicide on non-target organisms. The effects of MAJOR-75 application on selected physiological parameters are shown in

Table 1.

Table 1: The effects of fungicide on the growth of the onion root tips

Concentration	No. of roots	Length of roots (cm)
Control	14	3.1±1.01
25 ppm	13	1.7±2.65
50 ppm	11	1.4±0.41
100ppm	9	0.6±1.79
500ppm	4	0.15±1.15

Data was expressed as mean ± SD
Level of significance compared with control

It was determined that the number of roots and root length decreased with the fungicide concentration increased. In addition, these decreases in root length and number of roots were found to be statistically significant ($p < 0.05$). As a result of experimental studies, it was determined that fungicide application decreased all the selected parameters such as number of roots and root length. Bicakci et al. (2017) reported that diazinone administration decreases the weight increase, root length and germination percentage in proportion to the dose increase in *A. cepa*. Tort et al. (2004) found that diniconazole fungicides reduced the wet-dry weight, chlorophyll a and total chlorophyll content in barley due to increased concentration, and also prevented physiological events such as photosynthetic activity and chlorophyll synthesis. Soykan and Koca (2014) reported that increasing doses of dichlorvos (DDVP) insecticide decreased root length in *A. cepa*. The reason for this is that some of the various structural and numerical changes that occur in chromosomes cause the death of the cells that provide root elongation. In addition, it was emphasized that DDVP inhibited stem prolongation by preventing cell division.

Microscopic examination of *A. cepa* root tip cells showed that fungicide application caused anatomic damage such as non-prominent transmission tissue, cortex cell deformation, cell deformation of epidermis, accumulation of substance in cortex cells, cortex cell wall thickening and flattened cell nucleus. The data obtained for Mitotic Index (MI) are shown in **Table 2.** MI is another parameter used to determine cytotoxicity and is an indicator of cell proliferation. The active MI determined by overall number of dividing cells is commonly used to assess the genotoxicity of toxic chemicals.

Table 2: Mitotic index in *Allium cepa* root tip cells exposed to increasing concentration of MAJOR-75

Concentration	Cells counted	Number of cells in division	% mitotic index (± SD)
Control	450	320.80	71.11 ±1.64
25 ppm	450	314.40	69.86± 4.82
50 ppm	450	309.80	68.84 ±5.35
100ppm	450	289.60	64.35 ±3.20
500ppm	450	252.60	56.13± 4.27

Data was expressed as mean ± SD
Level of significance compared with control

After the administration of fungicide, the MI was decreased and the lowest MI was obtained in 500 ppm compared to the control group. As a result of cytogenetic analysis, it was found that MAJOR-75 application caused a decrease in Mitotic index. All these cytogenetic damages can be explained by MAJOR-75 toxicity. It is known that, attacks the macromolecules such as DNA, causing mitotic abnormalities, DNA fragmentation and mutation. In addition, all these damages are thought to be triggered by inducing intracellular oxidation and free radical formation (Dias et al. 2014). There are some studies conducted in the literature using different pesticides in a manner that confirms our results on cytogenetic effects. For example, Kuchy et al. (2016) reported that endosulfan, dichlorvos and carbendazim pesticides cause damage in the form of bridges, fragments, sticky chromosomes, c-mitosis, multiple polarity and ring chromosomes in root tip cells of *A. cepa*.

In order to tolerate the toxic effects of chemicals, the plants have developed mechanisms such as activation of the detoxification system, reduction of transport to other tissues, thickening of the cortex cells, increasing the epidermis cells and deposition of suberin in the cell wall (Baker, 1981). As a result of these mechanisms, it is inevitable that some anatomical changes occur in the plant and thus the effects of chemicals can be reduced. For example, Liu et al. (2004) reported that toxic substance exposure caused a thickened cell wall in *Vicia faba* L. and that this anatomical change acted as a barrier to restrict the transport of toxic substances to other cells. Demirtas et al. (2015) reported that 25, 50 and 100 ppm doses of Dinicanazole fungicides promote anatomic damage such as cell deformation, nonspecific transmission tissue, flattened cell nucleus and necrosis in *A. cepa* root tip cells. In the present work, at concentration of 25,50,100 and 500 ppm the increase in abnormality was found highly significant in comparison to control.

A progressive concentration- and duration-dependent inhibition of the mitotic activity was observed in meristematic cells. The MI was minimum at the highest concentration of the fungicide tested. The genotoxicity was estimated on the basis of chromosomal aberration frequency and the highest percentage of abnormal cells were observed at the highest concentration of fungicide used. Occurrence of sticky chromosomes, dislocation of spindle stickiness, extended cells, disturbed spindle, Micronucleus in prophase stickiness, Cells with nuclear bud, micronucleus formation, stickiness, Vacualization, c-mitosis,

vagrant chromosome, cytoplasm destruction, abnormal chromosome coiling and inactivation of the spindles. This indicated the aneugenic potential of the fungicide.

The observable characters to be recorded range from macromorphological effects if the plants are allowed to grow enough to produce a stem, to the mitotic index, including the presence of micronuclei, and the effects on chromosomes during mitosis. The MI is defined as the number of cells undergoing mitosis divided by the total number of cells. This character can be measured with a variable number of repeats, each with a microscope observation of a given size measured on the slide at a given magnification. All stages of mitosis should be included in the count. Both higher and lower MI with respect to the control can be related to an alteration of mitosis mechanisms as a result of cytotoxic effects (Leme and Marin-Morales 2009). Generally, the concentration of fungicide tested induced a dose-dependent inhibition of MI, which could be due to intracellular stress, including DNA damage, preventing the cells from entering mitosis. Mito depressive action may be due to a negative interference of the active substances contained by the fungicide tested, with specific proteins and enzymes that influence DNA polymerase (Hidalgo et al. 1989), DNA synthesis, microtubule formation, impaired nucleoprotein synthesis and reduced level of ATP provide energy for spindle elongation, microtubule dynamics and chromosomal movement (Majewska et al. 2003).

Conclusions

The results of the present study indicated that MAJOR-75 caused significant toxic effects in the root cells of *A. cepa*, and this toxic effect induced morphological, cytological and genetic alterations in *A. cepa*. MAJOR-75 showed highly significant effect on mitotic process and the frequencies of all the mitotic anomalies showed a good correlation with the concentration of the insecticide. This study showed that MAJOR-75 used in agriculture can potentially induce cyto- and genotoxic effect on crops and ultimately damage biota and human health. The results may be considered as providing a warning or an indicator that the anthropogenic pesticides enrichment may be a potential risk to the environment. Such studies have a special value, because they help to solve in short-term the problem of using the pesticides at such doses, on which they keep their proper function, but have insignificant cyto and genotoxic effects. The use of pesticides on farmland must further reduce or choose non-toxic pesticides for a safe habitat. In a long run, the use of this pesticide may have a negative impact on eukaryotic genome including plants and animals.

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