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PHYTOTOXICITY POTENTIAL OF 2,4-DICHLOROPHENOL ON *ALLIUM CEPA* AND *VIGNARADIATA*- A PRELIMINARY SCREENING

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ABSTRACT

2,4-Dichlorophenol is a commercially produced substituted phenol used entirely in the manufacture of industrial and agricultural products. Purpose of the present investigation was to study the ecotoxic effect of 2,4-Dichlorophenol on two plant systems, *Vignaradiata* and *Allium cepa*. 2,4-Dichlorophenol inhibited germination, root elongation and showed relative higher levels chromosomal aberrations in a dose dependent manner. Germination Index was reduced to 93.31 (3 mg/l treatment) and to 24.42 (10 mg/l treatment) as compared to control (100%), root elongation was reduced to 93.31 (3 mg/l treatment) and to 46.21 (10 mg/l treatment) as compared to control (100%), catalase activity increased with increase in 2,4-Dichlorophenol concentration in both seeds and roots, superoxide dismutase activity decreased from 27.54 (1 mg/l treatment) to 9.00 (10 mg/l treatment) in roots whereas it increased in seed dose dependently and protein content of root and seed decreased in a dose dependent manner (the total protein content of seed was higher than that of root) in *Vignaradiata* whereas in *Allium cepa*, the Mitotic Index (MI) decreased significantly with increase in 2,4-Dichlorophenol concentration and chromosomal aberrations increased with increase in toxicity. Elongated nucleus, bridge, fragment, vagrant, loss and stickiness were observed.

Keywords: 2,4-Dichlorophenol, *Vignaradiata*, *Allium cepa*, Germination, Chromosomal Aberration.

INTRODUCTION

The indiscriminate use of pesticides and herbicides in agriculture, as well as the increase of pollution in ecosystem due to industrial development, justifies the evaluation of the toxicity of these chemicals. They can be transformed into mutagenic or carcinogenic agents by plants which are the first living beings in the food chain, or get bioaccumulated in them as such absorbing the nutrients of polluted environments and acting as the toxic agents' vectors to humans **[1].** 2,4-Dichlorophenol is a commercially produced substituted phenol used entirely in the manufacture of industrial and agricultural products. As an intermediate in the chemical industry, 2,4-DCP is utilized principally as the feedstock for the manufacture of the herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4-D derivatives (germicides, soil sterilants, etc.) and certain methyl compounds used in mothproofing, antiseptics, and seed disinfectants. 2,4-DCP also reacts with benzene sulfonyl chloride to produce miticides or further chlorinate to pentachlorophenol, a wood preservative **[2].**Its identification as a metabolic intermediate and degradation product of various commercial products by plants **[3],** microorganisms **[4]**and sunlight **[5]** has been well established. In the search for relevant short-term test systems, plant materials have proved to be useful in basic research as well as a part of a test battery in environmental monitoring. In a workshop on "Higher plant systems as monitors of environmental mutagens" it was stated: "Plant systems seem especially well suited for research in at least the areas of basic mechanisms, screening, and environmental monitoring" **[6].** Plant systems are commonly used for toxicity tests due to their easy handling and cost effective methodologies. Also, the physiological changes report toxic effects in a very short span of time. Germination studies have been commonly used for checking ecotoxicity of various pollutants **[7].** Mung bean (VignaradiataL.) also known as green gram is one of the most important pulse crops, widely cultivated in a large number of countries. It has tremendous value in agriculture as a good source of plant protein for its high digestibility, good flavor, and high protein content and free from flatulent effects which are common to pulses **[8].**

METHODOLOGY

Preparation of 2,4-Dichlorophenol:

Analytical grade 2,4-Dichlorophenol was purchased from Loba Chemie, India. Stock solution of 2,4-Dichlorophenol with a concentration of 50 mg/l was aseptically prepared in 0.1% methanol and the volume was made up by adding distilled water. Working solutions of 1-10 mg/l concentrations were prepared from stock solution by dilution with distilled water.

Seed Treatment with 2,4-Dichlorophenol:

Seeds of *Vignaradiata* were purchased from the local market. They were soaked in sterilized water overnight followed by treatment with 1% Sodium hypochlorite (NaOCl) solution for 5 minutes for surface sterilization to avoid fungal contamination and thoroughly washed with sterilized distilled water **[13].** 15 healthy and equal sized sterilized seeds were arranged equi-spacially on the periphery of sterilized petri dishes lined with Whatman (grade 41) filter paper, laid on absorbent cotton, moistened with 5ml of 2,4-DCP solution of appropriate concentrations (1-10 mg/l) in a 90 mm petridish respectively **[14].**The petri dishes were sealed with parafilm to avoid water loss and were placed in a plant growth chamber with controlled temperature (20/25°Cmin/max) and light facility (8 hrs light period and16 hrs dark period). For 5 consecutive days, each day 2ml of freshly prepared solution of appropriate concentration was added to the respective petridishes. The experiment was run in triplicates. Seed germination in distilled water was used as control. Germination, marked by the appearance of radical was assessed after the incubation period.

Phytotoxicity Assessment:

After 5 days incubation, root length was recorded. Ten seedlings from each replica were selected for recording the morphological parameters. Percentage seed germination, percentage root elongation and germination index (GI) were calculated according to Zucconi*et al.* (1981) as follows:

Seed germination (%) =

$$\frac{\text{No.of seeds germinated in treatment}}{\text{no.of seeds germinated in control}} \times 100$$

Root elongation (%) =

$$\frac{\textit{Mean roott length in treatment}}{\textit{mean root length in control}} \times 100$$

Germination Index =

$$\frac{\textit{seed germination}\ (\%) \times \textit{root elongation}\ (\%)}{100}$$

Estimation of Protein Content

Total protein content of root and seed was estimated by Folin-Lowry method **[16].**

Estimation of Catalase and Superoxide Dismutase Activity:

Catalase activity was determined by measuring the inhibition rate of hydrogen peroxide (H_2O_2).Superoxide dismutase activity was determined by measuring the inhibition of NitroblueTertazolium (NBT).

Onion Bulb Treatment:

Onion bulbs of almost equal diameter were taken and peeled. The yellow shallows and the root primordia of the bulbs were removed before the onions were placed in the test solutions. Bulbs of *Allium cepa* were placed in boiling test tubes such as their basal ends dipped in distilled water and in growth chamber for 24 hrs. Then these onions were exposed for 5 days to the test solutions (2, 4-DCP solutions of different concentrations), keeping distilled water as control. Test solutions were changed each day with fresh one.

Genotoxicity Assessment:

Cytological experiment was carried out to observe the chromosomal aberrations. The squash technique was applied for the study of the mitotic index (MI) and chromosomal aberrations. Roots were stained in acetocarmine dye by heating for 15 minutes. Afterwards a drop of 45% glacial acetic acid was put on slide after properly cleaning the slide. Root was placed on slide and root tip was cut. Then root tip was squashed by putting a cover slip on it and tapping by finger. Now the slide was observed under bright field microscope. A minimum of around 100 cells were counted from each slide and total no. of cells, total no. of dividing cells and total no. of abnormal cells were counted **[17].**The chromosomal aberration assay was carried out on root cells and Mitotic Index (MI) was calculated using the formula:

Mitotic index (%) = (no. of dividing cells/total no. of cells observed)*100

RESULTS AND DISCUSSION

Seed Germination:

After 5 days treatment with phenol, seed germination reduced to 53.33±6.67 % with 10 ppm 2,4-DCP treatment as compared to control. 100% seed germination was recorded from 1 mg/l to 5 mg/l concentrations, revealing insignificant toxicity at lower concentrations (Table 1). Post-hoc LSD test showed significant difference ($p<0.05$) between percentage seed germination with 7 mg/l, 8 mg/l, 9 mg/l and 10 mg/l 2,4-DCP concentrations as compared to control. The results indicate that lower concentrations of 2,4-DCP does not affect germination significantly however, at higher concentrations inhibition in germination is observed. Previous studies have reported the inhibitory effect of phenols on seed germination **[18].** Since, water uptake is required for shedding the seed coat for radical emergence **[19]** inhibition of seed germination may be due to the hydrophobic nature of phenol which interferes with water activity and absorption inside the seed. Loomis and Battaile (1966) reported enzyme denaturation potential of some phenolic compounds. Mayer and Poljakoff- Mayber (1963) showed that a number of phenol compounds inhibit respiration and early seedling growth in lettuce by affecting mitochondrial metabolism and energy production as they are uncouplers of oxidative phosphorylation. The delay in germination may also be due to difference in seed coat permeability and differential uptake of water and toxin **[20].**

Table 1: Effect of 2,4-DCP on the germination profile of *Vignaradiata*

Treatment (mg/l)	No. of seeds germinated after 24 hrs	Seed germination %	Average root length (cm)
Control	15.00±0.00^a	100.00±0.00^a	12.47±0.15^a
1	15.00±0.00^a	100.00±0.00^a	12.27±0.25^b
2	15.00±0.00^a	100.00±0.00^a	12.17±0.06^b
3	15.00±0.00^a	100.00±0.00^a	11.63±0.21^c
4	15.00±0.00^a	100.00±0.00^a	11.00±0.10^c
5	15.00±0.00^a	100.00±0.00^a	10.77±0.25^d
6	14.67±0.58^b	97.78±3.85^b	9.97±0.47^a

7	13.67±0.58^{c}	91.11±3.85^{c}	8.00±0.44^{fg}
8	13.00±0.00^{c}	96.67±0.00^{c}	8.00±0.03^{a}
9	9.67±2.08^{d}	64.44±13.88^{d}	7.00±1.08$_{a}$
10	8.00±1.00^{e}	53.33±6.67^{c}	5.77±0.71$_{a}$

Treatment (mg/l)	Root elongation %	Germination Index (GI)
Control	100.00±0.00^{a}	100.00±0.00^{a}
1	98.39±1.39^{a}	98.39±1.39^{a}
2	97.60±0.77^{ab}	97.60±0.77^{a}
3	93.31±0.95^{c}	93.31±0.95^{b}
4	88.24±1.12^{c}	88.24±1.12^{d}
5	86.38±2.64^{c}	86.38±2.64^{d}
6	14.67±0.58^{b}	97.78±3.85^{b}
7	79.94±3.27^{c}	78.23±56.96^{c}
8	64.17±3.34^{c}	58.50±4.65^{c}
9	64.17±2.27^{c}	55.62±1.96^{c}
10	56.15±8.61^{c}	35.46±3.31^{c}
	46.21±5.14^{c}	24.42±0.73^{c}

^{a}P<0.001 (extremely significant), ^{b}P<0.01 (more significant), ^{c}P<0.05 (significant), ^{d}P<0.05 (not significant)

Note: Different letters in each column represents significant difference between treatments at ^{a}P<0.001 to P<0.05.

Germination Index:

Germination Index (GI) is a marker of early plant growth **[21].** GI did not change significantly from control to 2mg/L 2,4-DCP treatment. However, GI decreased dose dependently from 3 mg/l (93.31±0.95) to 10 mg/l (24.42±0.73) treatment (Table 1). Mosse *et al.* (2010) emphasized on the role of GI for determining phytotoxicty of winery wastewater on crop species. Thus, the results are indicative of 2,4-DCP toxicity on the species tested.

Root Elongation:

A significant dose dependent reduction (p<0.05) in percentage root elongation was observed from 3 mg/l (93.31±0.95%) to 10 mg/l (46.21±5.14%) as compared to control (100 %) as showed in Table 1. Earlier reports proposed that some phenols stimulate root growth at lower concentrations (Wang, 1985), but the inhibitory effect at higher concentrations could possibly be due to the impact of 2,4-DCP on cell division in the apical root meristem cells. However, this needs to be verified by further studies.

Catalase and Superoxide Dismutase Activity:

The effect of 2,4-DCP on the activity of SOD and CAT is shown in the table 2. The activity of SOD in root decreased gradually from 27.54±0.22 (µmol/min/gm fresh wt.) at 1mg/l to 9.00±0.00 (µmol/min/gm fresh wt.) at 10mg/l whereas the activity of SOD in seed and CAT in both root and seed increased dose dependently. SOD activity in seed increased to 40.41±0.19 (µmol/min/gm fresh wt.) at 10mg/l while CAT increased to 45.00±0.05 (µmol/min/gm fresh wt.) and 46.75±0.22 (µmol/min/gm fresh wt.) at 10mg/l in root and seed respectively. 2,4-DCP stress is inevitably associated with increased oxidative stress due to enhanced accumulation of ROS in chloroplasts, mitochondria, and peroxisomes. As a result, the induction of antioxidant enzyme activities is a general adaptation strategy which plants use to overcome oxidative stresses **[22].**The ability of plants to overcome oxidative stress partly relies on the induction of SOD activity and subsequently on the upregulation of other downstream antioxidant enzymes **[23].** Abedi and Pakniyat, 2010 reported significant enhancement of SOD activity in seedlings exposed to water stress (Table 2). According to this fact that SOD processing is known to be substrate inducible **[24],** an increase in the SOD activity may be attributed to the increased production of active oxygen species as substrate that lead to increased expression of genes encoding SOD.

Like the present results, SOD in general show simultaneous induction and decline in seeds and roots respectively, which may be a result of its regulation **[25].** The important point here is a decrease in SOD activity in roots, which may reflect the low ROS scavenging capacity and increased damage under this condition.

Table 2: Effect of 2,4-DCP on antioxidants enzymes of *Vignaradiata*

Treatment (mg/l)	**SOD root (µmol/min/gm fresh wt.)**	**SOD seed (µmol/min/gm fresh wt.)**
Control	0.00±0.00[a]	0.03±0.57[a]
1	27.54±0.22[b]	13.26±0.43[b]
2	24.02±0.30[c]	13.33±0.12[bc]
3	20.54±0.12[d]	14.38±0.36[d]
4	20.12±0.14[c]	16.53±0.11[c]
5	14.42±0.23[c]	17.41±0.08[c]
6	16.58±0.04[c]	18.14±0.15[c]
7	16.45±0.10[c]	21.22±0.19[c]
8	15.30±0.10[c]	24.04±0.05[c]
9	10.00±0.00[c]	34.34±0.30[c]
10	9.00±0.00[c]	40.41±0.19[c]

Treatment (mg/l)	Catalase root (Units/gm root)	Catalase seed (Units/gm seed)
Control	0.00±0.00[a]	0.00±0.00[a]
1	13.04±0.05[b]	19.10±0.09[b]
2	28.35±0.01[c]	19.18±0.11[b]
3	28.20±0.05[c]	19.17±0.15[b]
4	33.33±0.29[c]	20.50±0.39[c]
5	33.60±0.17[c]	23.79±0.26[c]
6	39.54±0.21[c]	28.28±0.27[c]
7	39.25±0.32[c]	32.72±0.15[c]
8	39.00±0.10[c]	37.36±0.40[c]
9	40.00±0.05[c]	40.30±0.20[c]
10	45.00±0.05[c]	46.75±0.22[c]

[a]P<0.001 (extremely significant), [b]P<0.01 (more significant), [c]P<0.05 (significant), [d]P<0.05 (not significant)

Note: Different letters in each column represents significant difference between treatments at [a]P<0.001 to P<0.05.

Protein Content:

Protein content of root and seed decreased in a dose dependent manner and a significant difference was recorded with 1-10 mg/l as compared to control. However the total protein content of seeds was higher than that in roots. The results are shown in Table 3. The increase in protein concentration could be attributed to the induction of stress proteins and/or enzymes in response to 2,4-DCP, exhibited because of the commencement of free radical chain reactions in the membrane, or some other non specific interaction. One possible explanation could be the interference of 2,4-DCP in important metabolic and biosynthetic pathways through the generation of ROS that can associate with a number of biomolecules. 2,4-DCP is adsorbed and absorbed by the roots from solution. Its translocation to shoots is determined by octanol-water partition coefficient (K_{ow}) with the movement decreasing with increasing K_{ow} values suggesting slower movement of the compounds to shoots **[26].** The increase in protein concentration could also be a result of glycosylation and amino acid conjugation of toxic organic compounds that is used as a defensive mechanism by plants **[27].** However, this conjugation can be broken down under acidic conditions **[28]**. Biswas *et al.* (2010) reported that a number of enzymes are induced in response to stress and that class III peroxidise is a common class of stress induced enzymes in plants **[29].**

Therefore, alternations in the amount of antioxidant enzymes and metabolites generated in response to stress could also contribute to the observed pattern suggesting that the plant might have evolved a defence mechanism and thus, could be explored for phytoremediation.

Table 3: Effect of 2,4-DCP on protein profile of *Vignaradiata*

Treatment (mg/l)	Protein root (µg/ml)	Protein seed (µg/ml)
Control	51.30±0.10[a]	81.00±1.00[a]
1	41.00±0.10[a]	75.00±1.00[b]
2	35.00±0.10[c]	72.00±1.00[c]
3	32.70±0.10[d]	66.00±1.00[d]
4	31.40±0.10[c]	61.00±1.00[c]
5	23.76±0.65[c]	61.66±0.57[f]
6	22.43±0.37[a]	57.00±1.00[a]
7	20.33±0.49[a]	52.00±1.00[a]
8	17.53±0.10[a]	51.33±0.57[a]
9	18.60±0.10[a]	40.00±0.00[a]
10	15.40±0.10[a]	37.33±0.57[a]

[a]P<0.001 (extremely significant), [b]P<0.01 (more significant), [c]P<0.05 (significant), [d]P<0.05 (not significant)

Note: Different letters in each column represents significant difference between treatments at [a]P<0.001 to P<0.05.

Mitotic Index:

Meristematic mitotic cells of *Allium cepa* constitute an adequate material for cytotoxicity and genotoxicity evaluation of 2,4-DCP. Among the higher plants species used to evaluate environmental contamination, the more frequently used are *Allium cepa*, *Viciafaba*, *Zea mays*, and *Nicotianatabacum* **[30].** In particular, *Allium cepa*has been regarded as favorable to assess chromosome damages and disturbances in the mitotic cycle, because *Allium cepa*cells have large chromosomes in a reduced number **[31].** Mitotic index (MI) was determined by scoring approximately 100 cells. MI iscalculated as the percentage ratio of dividing cells and totalnumber of observed cells and the results is expressed aspercentage of the control. The root tip cells of *Allium cepa* after 5 days treatment with different concentrations of 2,4-DCP showed significant chromosomal abnormalities as compared to control. The mitotic index (MI) decreased significantly with increasing 2,4-DCP concentration (Table 4).

Table 4: MI values before and after 2,4-DCP treatment

Treatment (mg/l)	MI
Positive control (EtBr)	19
Negative control	100
3	20
5	13.2
7	10.98
10	4.89

Chromosomal Aberration:

The main types of chromosome aberrations detected were bridges, vagrant chromosomes, stickiness, loss, and fragment (Table 5). Stickiness is considered to be a chromatid-type aberration **[32].** However, stickiness has been shown to be a result of DNA condensation (Österberg*et al.*, 1984) and entanglement of inter-chromosomal chromatin fibers which led to subchromatid connections between chromosomes **[33].**Liu *et al.,* 1992 suggested that sticky chromosomes reflect a highly toxic effect, usually of an irreversible type, and probably lead to cell death. On the other hand, the presence of bridges could be attributed to chromosome breaks, stickiness and breakage and re-union of the broken ends. Sticky bridges might be also the result of incomplete replication of chromosomes by defective or less active replication enzymes **[32].** The spindle irregularities like vagrant chromosomes were also observed after phenol treatment but it was at a lesser extent. The induction of this aberration leads to the separation of unequal number of chromosomes in the daughter cells with unequal-sized or irregularly shaped nuclei at interphase **[33, 34].** All the above mentioned aberrations were induced by different 2,4-DCP concentrations, which demonstrate the high phenol toxicity. For this reason, it is very important to select an effective method for 2,4-DCP removal, which would be able to reduce its concentration.

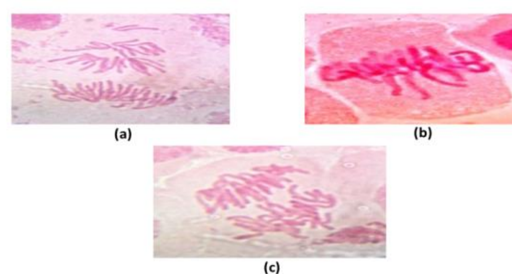

Figure 1: (a) Chromosomal fragment (7 mg/l) (b) Abnormal metaphase (5 mg/l) (c) Bridges (10 mg/l).

Table 5: Chromosomal aberrations in cells exposed to 2,4-DCP

2,4-DCP (mg/l)	Elongated nucleus	Bridge	Fragment
Negative control	4	3	-
3	12	10	11
5	15	15	21
7	10	12	20
10	31	19	13

2,4-DCP (mg/l)	Vagrant	Other aberrations	Total
Negative control	-	7	14
3	4	2	39
5	9	7	67
7	15	13	70
10	4	12	79

CONCLUSION

These studies showed that 2,4-DCP has inhibitory effect on the vegetative growth of *Vignaradiata*. This means that release of such xenobiotics into the environment needs to be considered in order to prevent their hazardous effects. 2,4-DCP did not affect germination to a greater extent but reduced root length considerably. The delay in germination may be due to variation inseed size, seed coat permeability, differential uptake of nutrients, toxins and difference in metabolism. The germination index was also significantly reduced with increasing toxicity. These results suggest that 2,4-DCP imparts negative effect on the growth and development of the species tested. Dose dependent decrerase in Mitotic Index of *Allium cepa* cells treated with 2,4-DCP clearly indicated its cytotoxicity suggesting its role in the inhibition of cell division. The significant chromosomal aberrations observed on treatment with 2,4-DCP reveals the genotoxicity of 2,4-DCP even at lower concentrations. The reduction in total protein content is suggesting its adverse effects on protein synthesis. The enhanced activity of antioxidant enzymes also suggests generation of toxic species. Further studies are required to assess toxicity at cellular and metabolic level but this preliminary study is indicative of chlorophenol toxicity and gives sufficient reasons for its remediation from contaminated sites.

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