World Journal of Environmental Biosciences
 eISSN 2277- 8047
Available Online at: **www.environmentaljournals.org**
Volume 4, Issue 1: 1-6

Open Access **Research Article**

Seeds of *Vigna radiata* as a Model to Study the Ecotoxicity Potential of 2,4,6-Trichlorophenol

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Abstract:

2,4,6-Trichlorophenol is a ubiquitous, persistent and toxic pollutant having various industrial applications resulting in its wide presence in industrial wastewaters. Non-ionized form of the compound is highly bioaccumable. Therefore, purpose of present investigation was to study the ecotoxicity of 2,4,6-Trichlorophenol on seeds of *Vigna radiata* (Mung bean). The seeds were allowed to germinate in increasing concentrations of 2,4,6-Trichlorophenol and parameters like percent seed germination, root elongation, germination index and protein content were determined after 5 days treatment. 50% effect concentration was found to be 16.40 mg/L. 2,4,6-Trichlorophenol inhibited germination and root elongation in a dose dependent manner. Germination Index was reduced to 14.81 with 16mg/L treatment and to 2.97 with 20mg/L treatment. Root elongation was reduced to 44.61% with 16mg/L treatment. Protein content in roots increased with increasing 2,4,6-Trichlorophenol concentration and was not significantly affected in shoots. This is probably the first report on ecotoxicity potential of 2,4,6-Trichlorophenol on *Vigna radiata,* suggesting its use as a model for toxicity assessment of chlorophenols. The change in protein content is indicative of some defence mechanism existing within the plant suggesting its phytoremediation potential, which could be explored further.

Keywords: 2,4,6-Trichlorophenol, *Vigna radiata*, Germination, EC50, Ecotoxicity

1. Introduction:

With an era of acceleratory industrialization, more and more industrial waste water containing organic and inorganic pollutants is being discharged into the environment. Xenobiotics produced as a consequence of anthropogenic activities is a serious cause of environmental pollution. Amongst the various halogenated organics released, 2,4,6-Trichlorophenol (2,4,6-TCP) constitutes a fundamental pollutant that is highly toxic, bioaccumable, persistent and ubiquitously present due to its wide industrial application (Barber *et al.,* 1995; Ruzgas *et al.,* 1995). The industrial application of 2,4,6-TCP ranges from its use as a preservative for wood, leather and textile goods (Männistö *et al.,* 1999; Kharoune *et al.,* 2002), to a bactericide & fungicide (Fragiadakis *et al.,* 1981). It is used as a precursor for the synthesis of herbicides (Rappe, 1980). It is also produced by chlorination of lignin and is therefore present in kraft paper mill effluent (Karn and Balda, 2013). Such immense production and faulty waste disposal practices have led to its tremendous distribution across ecosystems. Chlorophenols are considered possible carcinogens to human by the International Agency of Research on Cancer (IARC, 1999) and they have been reported to obstruct photosynthesis in plants (Zha *et al.,* 2006; Tissut *et al.,* 1987). Thus, chlorophenols are significantly toxic giving considerable reasons to broadly study and explore their toxic potential. Disposal of wastewater into field crops is a means of managing wastewater, therefore, it is important to analyse how plants respond to such chemical attacks, as bioaccumulation of chlorophenols lead to their flow in the food chain (Doust *et al.,* 1994). Various studies have been conducted to assess the toxicity of chlorophenols on different plant systems, Sharma *et al.* (1997) reported comparative toxicity and metabolism of different chlorophenols by *Lemna gibba,* Fragiadakis *et al.* (1981) studied the metabolism of ^{14}C-2,4,6-TCP in hydroponic tomato plants, O'Keefe *et al.* (1987) studied the metabolism of phenolic compounds by water hyacynth. But to the best of our knowledge there has been no report on the toxicity assessment of 2,4,6-TCP on *Vigna radiata* till date. Therefore, the present investigation was carried out with an objective to study the toxicity of 2,4,6-TCP on seeds of *Vigna radiata* as it is a common

agricultural crop and a cheap source of protein, constituting an important part of natural ecosystem. It has been extensively used for toxicity assays due to its stress sensitivity, fast and cost effective methods that can be performed in limited space under controlled conditions (Panda *et al.,* 2003; Kumar and Singhal, 2009). These kinds of bioassay are also preferred for toxicity tests because of ease of seed handling, fast metabolism and translocation of nutrients, rapid germination rate and absence of additional nutrients for plant growth (Wang *et al.,* 2002). Growth changes are the first physiological signs that can be monitored after exposure to toxic compounds, therefore seed germination and root growth are important tools to analyse toxicity (Shoaib *et al.,* 2011). Hence, *Vigna radiata* can serve as a model for evaluation of 2,4,6-TCP ecotoxicicity.

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2. Materials and Methods:

2.1. Preparation of 2,4,6-TCP Solution

Analytical grade 2,4,6-TCP was purchased from Merck, India. Stock solution with a concentration of 50mg/L was aseptically prepared in 1% methanol solution. 2,4,6-TCP solutions of 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 mg/L concentration were prepared from stock solution by dilution with distilled water.

2.2. Seed Treatment with 2,4,6-TCP

Seeds of *Vigna radiata* were purchased from local market. They were soaked in sterilized water for 6 hrs followed by treatment with 0.1% $HgCl_2$ and 70% ethyl alcohol solutions for surface sterilization and thoroughly washed with sterilized distilled water. 15 healthy and equal sized seeds were placed on a Whatman (grade 41) filter paper, laid on absorbent cotton, moistened with 2ml of 2,4,6-TCP solution of appropriate concentration (2,4,6,8,10,12,14,16,18 &20 mg/L) in a 90mm petridish (Mosse *et al.,* 2010; Verma *et al;* 2011). The petridishes were sealed with tape to avoid water loss and were placed in a plant growth chamber with controlled temperature (20/25°C min/max) and light facility (8 hrs light period and 16 hrs dark period). For 3 consecutive days, each day 2ml of freshly prepared 2,4,6-TCP solution of appropriate concentration was added to the respective petridish. 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.

2.3. Phytotoxicity Assessment

After 72hrs incubation, root length was determined. Also, percentage seed germination, percentage root elongation and germination index (GI) were calculated according to Zucconi *et al.* (1981) as follows:

$$\text{Seed germination (\%)} = \frac{\text{No. of seeds germinated in treatment}}{\text{No. of seeds germinated in control}} \times 100$$

$$\text{Root elongation (\%)} = \frac{\text{Mean root length in treatment}}{\text{Mean root length in control}} \times 100$$

$$\text{Germination Index} = \frac{\text{Seed germination (\%)} \times \text{Root elongation (\%)}}{100}$$

2.4. Estimation of Protein Content

Total protein content of root and shoot was estimated by Folin-Lowry method (Lowry *et al.,* 1951).

2.5. Statistical Analysis

EC50 was calculated by GraphPad Prism Version 5, using non-linear regression analysis. The experiment was conducted in triplicates and statistical analysis was performed using SPSS, Version 20.0 by one-way ANOVA followed by post hoc LSD test at significance level of $p<0.05$

3. Results and Discussion:

3.1. Seed Germination

After 5 days treatment with 2,4,6-TCP, a significant dose dependent decrease in percentage seed germination was observed from 10 mg/L concentration (91.66±7.22 %), maximum decrease being noted with 18mg/L (20.83±7.22 %) and 20mg/L (20.83±7.21 %). 100% seed germination was recorded from 2 mg/L to 8 mg/L concentrations, revealing insignificant toxicity at lower concentrations (table 1). Post-hoc test showed significant difference ($p<0.05$) between percentage seed germination with 10 mg/L, 14 mg/L, 16 mg/L and 18 mg/L 2,4,6-TCP concentrations. Previous studies have reported the inhibitory effect of phenols on seed germination (Kuiters, 1989). Since, water uptake is required for shedding the seed coat for radical emergence (Castro *et al.,* 2000), inhibition of seed germination may be due to the hydrophobic nature of 2,4,6-TCP 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 (Williams and Hoagland, 1982).

3.2. Root Elongation

An insignificant reduction ($p>0.05$) in percentage root elongation was observed with 2mg/L (99.14±0.74 %) and 4 mg/L (97.42±2.24 %) as compared to control (100 %) and with 4 mg/L (97.42±2.24 %), 6mg/L (96.11±1.27 %) and 8mg/L (96.12±2.22 %) as compared to 2mg/L (99.14±0.74 %) 2,4,6-TCP treatment. However, a significant dose dependent inhibition was observed from 10mg/L concentration (90.93±2.16 %), which reduced to 14.74±2.18 % at 20mg/L 2,4,6-TCP treatment (Table 1). Earlier reports proposed that some phenols stimulate root growth at lower concentrations (Wang, 1985 b), but the inhibitory effect at higher concentrations could possibly be due to the impact of 2,4,6-TCP on cell division in the apical root meristem cells. However, this needs to be verified by further studies. Toxicity was also revealed morphologically as the roots became curved, thin, fragile and sticky. Previous studies have reported that phenolic compounds have a greater effect on seedling growth than germination (Rasmussen and Einhellig, 1977). Compounds distribute themselves between aqueous and lipid phases according to their octanol-water partition coefficient. Glass (1973) therefore, proposed that being lipophillic, uncoupling phenols restrict both, transport and respiration by affecting membrane permeability and biochemical gradient. Numerous studies have proposed that phenolic compounds affect fundamental plant processes such as, respiration and protein synthesis consequently inhibiting plant growth (Demos *et al.,* 1975).

3.3 Germination Index

Germination Index (GI) is a marker of early plant growth (Mosse *et al.,* 2010). GI did not change significantly from control to 8 mg/L 2,4,6-TCP treatment. However, GI decreased dose dependently from 10 mg/L (83.30±5.82) to 18 mg/L (6.47±2.51) 2,4,6-TCP treatment (table 1). Mosse *et al.* (2010) emphasised on the role of GI for determining phytotoxicty of winery wastewater on crop species. Thus, the results are indicative of 2,4,6-TCP toxicity on the species tested.

3.4. EC50

Toxicity of 2,4,6-TCP is represented by 50% effect concentration (EC50). The EC50 value for *Vigna radiata* was reported to be 16.40 mg/L (Fig. a). The result is in agreement with the EC50 value obtained by Wang (1985 a) who reported 16 mg/L as the EC50 value of 2,4,6-TCP on both millet and velvetleaf. In another experiment Wang (1985 b) reported 10mg/L as the EC50 value of 2,4,6-TCP on millet. Sharma *et al.* (1997) reported 2.1µM as the EC50 value of 2,4,5-TCP on *Lemna gibba*.

Table 1: Effect of 2,4,6-TCP on germination of *Vigna radiata* seeds

Treatment (2,4,6-TCP)	Seed Germination (%)	Average Root Length (Cm)	Root Elongation (%)	Germination Index (GI)
Control	100±0.00^{a}	7.68±0.28^{a}	100±0.00^{a}	100±0.00^{a}
2 mg/L	100±0.00^{a}	7.27±0.15^{ab}	99.14±0.74^{ab}	99.14±0.74^{a}
4 mg/L	100±0.00^{a}	7.33±0.06^{ab}	97.42±2.24^{ab}	97.42±2.24^{a}
6 mg/L	100±0.00^{a}	6.87±0.35^{bc}	96.11±1.27^{b}	96.11±1.27^{a}
8 mg/L	100±0.00^{a}	6.43±0.25^{c}	96.12±2.22^{b}	96.12±2.22^{a}
10 mg/L	91.66±7.22^{b}	6.30±0.30^{c}	90.93±2.16^{c}	83.30±5.82^{b}
12 mg/L	87.50±0.00^{b}	5.10±0.50^{d}	67.99±2.30^{d}	59.49±2.01^{c}
14 mg/L	66.67±7.22^{c}	4.83±0.90^{d}	57.99±1.26^{e}	38.71±4.82^{d}
16 mg/L	33.33±7.22^{d}	3.43±0.20^{e}	44.61±3.12^{f}	14.81±3.05^{e}
18 mg/L	20.83±7.22^{e}	2.53±0.50^{f}	30.72±2.30^{g}	6.47±2.51^{f}
20 mg/L	20.83±7.21^{e}	1.43±0.51^{g}	14.74±2.18^{h}	2.97±0.74^{f}

Different letters in each column represents significant difference between treatments at $p<0.05$.

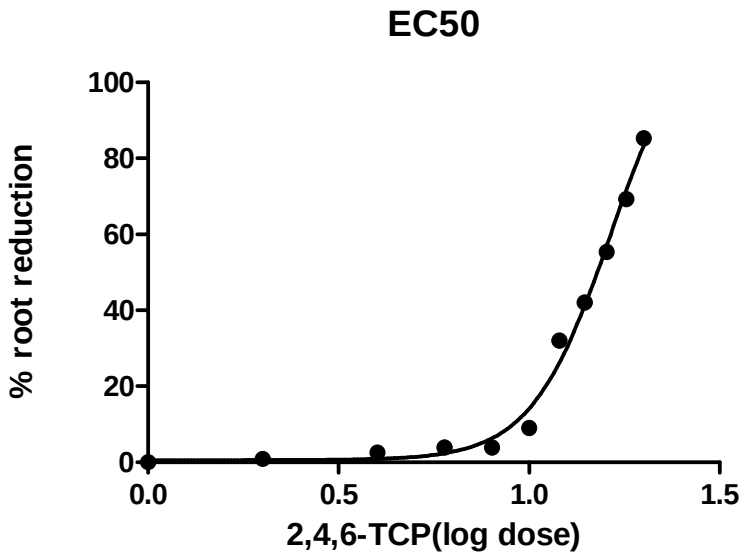

Figure a: Toxicity of 2,4,6-TCP on *Vigna radiata* represented by EC50.

3.5. Protein Content

Protein content of roots increased in a dose dependent manner and a significant difference was recorded with 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 mg/L 2,4,6-TCP treatment as compared to control, values being 1.32±0.08, 1.29±0.02, 1.37±0.12, 1.66±0.05, 1.40±0.10, 1.40±0.10, 1.52±0.68, 2.75±0.22, 4.50±0.10, 5.20 and 0.57±0.03±0.10 µg/µl, respectively. Effect of the treatment on protein content of shoots showed a similar trend, increasing from 3.82±0.02 µg/µl in control to 4.13±0.02 µg/µl with 20 mg/L treatment. However, the increment in protein content of roots was found to be greater than that in shoots (table 2).

The increase in root protein concentration could be attributed to the induction of stress proteins and/or enzymes in response to 2,4,6-TCP, 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 2,4,6-TCP in important metabolic and biosynthetic pathways through the generation of ROS that can associate with a number of biomolecules. 2,4,6-TCP 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 (Briggs *et al.,* 1982).

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 (Davidonis *et al.,* 1982; Edwards *et al.,* 1982; Casterline *et al.,* 1985). However, this conjugation can be broken down under acidic conditions (Sharma *et al.,* 1997). 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 (Passardi *et al.,* 2005). 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. Reduced toxicity in shoots might be because of the compound concentration in roots which minimised its translocation. However, screening for the presence of these antioxidant enzymes is required for further explanation.

Table 2: Effect of 2,4,6-TCP on protein profile of *Vigna radiata*

Treatment (2,4,6-TCP)	Protein(µg/µl)	
	Root	Shoot
Control	0.57±0.03^{a}	3.82±0.02^{a}
2 mg/L	1.32±0.08^{b}	3.81±0.01^{a}
4 mg/L	1.29±0.02^{b}	3.80±0.02^{a}
6 mg/L	1.37±0.12^{bc}	3.82±0.03^{b}
8 mg/L	1.66±0.05^{d}	3.86±0.03^{c}
10 mg/L	1.40±0.10^{bc}	3.88±0.03^{d}
12 mg/L	1.40±0.10^{bc}	3.90±0.04^{d}
14 mg/L	1.52±0.68^{cd}	3.92±0.06^{e}
16 mg/L	2.75±0.22^{e}	4.00±0.10^{f}
18 mg/L	4.50±0.10^{f}	4.09±0.81^{g}
20 mg/L	5.20±0.10^{g}	4.13±0.02^{h}

Different letters in each column represents significant difference between treatments at $p<0.05$

4. Conclusion:

The study shows that 2,4,6-TCP exhibits significant toxicity to the legume, *Vigna radiata* with an EC50 value of 16.4 mg/L affecting germination, root growth and protein content in a dose dependent manner. In general the toxicity of chlorophenols can be attributed to the uncoupling character which interferes with electron/proton movement involved in important cellular metabolic processes and their binding with a variety of biomolecules altering biological functions. Substituted chlorine molecules increase hydrophobicity and reactivity of phenols and octanol-water partition coefficients dictate their translocation in the plant. Damage occurs when defence mechanisms are lower than the generation of reactive oxygen species. Thus, the study provides a conclusive evidence that *Vigna radiata* could be used as a potential model for ecotoxicity study of chlorophenols and could also be explored for its phytoremediation potential.

5. Acknowledgments:

We thank Department of Bioengineering, Integral University, Lucknow, India for providing material and laboratory support to carry out this work.

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