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Phytotoxic Effects of 4-Chlorophenol and 2,4-Dichlorophenol in the Germination of Seeds of *Phaseolus vulgaris* and *Zea mayz*

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Abstract

Soil contaminated with pesticides may reduce plant development due to their toxicity. The aim of this study was to evaluate the influence on the germination of *Zea mayz* and *Phaseolus vulgaris* of the two main intermediates of the 2,4-D degradation, which are 4-chlorophenol (4-CP) and 2,4-dichlorophenol (2,4-DCP). Maize and bean seeds were treated with distilled water (control treatment) and increased concentrations of 4-CP and 2,4-DCP (0.006, 0.1, 1.0, 1.5 $g \cdot L^{-1}$). It was assessed seed germination and calculated various parameters. The parameter most affected by chlorophenols was the index of germination rate, being the *P. vulgaris* seeds most affected. 2,4-DCP was the compound most toxic for both plants. The germination index was dependent doses for both plant models tested. The results indicate that 4-CP and 2,4-DCP affected the index of germination rate but not influenced in other parameters of germination of *Zea mayz* and *Phaseolus vulgaris.* Maize was most tolerance to both chlorophenols in the assessed concentrations. 2,4-DCP was the most toxic of chlorophenols tested.

Keywords

Chlorophenol, Bean, Maize, Phytotoxicity, Seed Germination

1. Introduction

Chlorophenols are an important class of organic environmental contaminants that are widely used as pesticides and wood preservative products. These compounds are also generated by pesticides degradation and they are precursors of the highly toxic dibenzo-*p*-dioxins and dibenzofurans during incineration processes. 2,4-dichlorophenol (2,4-DCP) is used in the production of the herbicide

2,4-dichlorophenoxiacetic acid (2,4-D) and others herbicides. The Environmental Protection Agency (EPA) has classified 2,4-DCP and 4-chlorophenol (4-CP) as toxic, carcinogenic and persistent chemical compounds and a priority environmental contaminant [1]. Although there is evidence on the toxicity of 2,4-DCP and 4-CP in animals [2], reports on plant toxicity are still few.

Soils treated with the herbicide 2,4-D can be later used to plant vegetables and other crops because this compound is considered to have a rapid degradation, but the disappearance of its possible intermediaries (2,4-DCP or 4-CP) in the soil treated with the herbicide is not controlled. This can cause harm to planted crops or consumers because very little is known about whether the chlorophenols produced accumulate in plant structures through absorption and sequestration into organic molecules and whether these organic molecules can enter the food chain.

The concept of using plants for the remediation of organic pollutants emerged a few decades ago with the recognition that plants were capable of metabolizing toxic pesticides. Since then, phytoremediation has been shown to efficiently reduce chemical risks associated with various classes of organic pollutants, including chlorinated compounds, pesticides, explosives, and polycyclic aromatic hydrocarbons [3].

To implement phytoremediation processes it is necessary to study the effect of pollutants on plant models. Differences between monocotyledonous and dicotyledonous plants have been substantiated with respect to the structure of the seed and vascular and root systems, cell wall proteome as well as the microflora associated with the root system [4] [5]. These differences suggest that there are discrepancies in the metabolism, as well as, in adsorption, transport and degradation of toxins, which would affect the resistance of plants to polluting compounds.

Phytotoxicity is a measure of the delay or inhibition of seed germination, inhibition of plant growth or any adverse effect on plants caused by specific substances. Thus, phytotoxicity bioassays can detect any substance capable of generate temporary or long-term stress on the germination capacity of seeds, roots growth and dry matter evolution. Bioassays must respond not only to known compound but also to complex mixtures of phytotoxins, must be simple, reproducible and fast (to avoid plants adapting to the toxic compounds) [6].

Seed germination is a complex process, starting from water uptake of dry seeds and continuing to elongation of the embryonic axis [7]. A seed germination test, germination index (GI) has been used as a rapid, reliable and reproducible technique to indicate the damaging effect of different industrial waste on plant growth. GI combines effect on germination and root growth for what has proven to be a very sensitive parameter [8].

The vulnerable and complex process of seed germination also depends on decisive and specific changes in tissue and cell properties. By definition, seed germination starts with the uptake of water by the quiescent, dry seed followed by the elongation of the embryonic axis. This usually culminates in the rupture of

the covering·Layers and emergence of the radicle, generally considered as the completion of germination [9].

Taking in account that plat toxicity of chlorophenol compounds is still few known and, by other hand, the existence of substantial physiological and morphological differences between monocotyledonous and dicotyledonous plants, this work was conducted with the aim to evaluate comparatively the phytotoxical effects of 2,4-DCP and 4-CP on one plant model representative of both monocots (maize-*Zea mayz* L.) and dicots (bean-*Phaseolus vulgaris*) by means of germination study.

2. Material and Methods

2.1. Plant

As a dicotyledonous and monocotyledonous plant models were used *Phaseolus vulgaris* variety Cuba C-25-9-R and *Zea mayz* L. P 7928 respectively. Both plants were supplied by Seeds laboratory from Agricultural Delegation at Santiago de Cuba. Maize is among the 10 recommended species by U.S. Environmental Protection Agency [10] for the determination of ecological effects of toxic substances while bean was selected because their seeds are similar in size with maize.

Seed disinfection was done according to Gebreegziabher and Qufa [11] with slight modifications. In brief, bean and maize seeds were disinfected for 10 minutes with 1% sodium hypochlorite and then four times washed with sterile distilled water during 5 minutes each time.

2.2. Reagents

4-chlorophenol (MERCK), 2,4-dichlorophenol (SIGMA-ALDRICH). These were prepared at concentrations of 0.06; 0.1; 1.0; 1.5 $g{\cdot}L^{-1}$ for 4-chlorophenol and 0.06; 0.1; 1.0 $g{\cdot}L^{-1}$ for 2,4-dichlorophenol in distilled water.

2.3. Phytotoxicity Test

Bioassays were carried out in petri dishes with sterile filter paper on the bottom. Seed germination and root length tests were carried out on distilled water (as a control sample) and different concentrations of two chlorophenols. Ten previously washed seeds were placed on sterile Whatman filter paper. From each concentration, 5 mL was pipetted into a sterilized Petri dish prepared with seeds. Dishes were wrapped with Parafilm® to avoid volatilization of the compounds. It was incubated at 20˚C, with a light/dark photoperiod of 12 hours. The operation was replicated five times (five Petri dishes) following the same protocol for each sample. The number of germinated seeds was then counted, and the length of roots was measured during seven days.

After the 7 days of the experiment, the number of germinated seeds in the sample and the number of germinated seeds in the control were counted for each experimental set. The relative seed germination (RSG) was determined ac-

cording to Equation (1) [6]:

$$\mathrm{RSG}(\%) = \frac{\text{number of seeds germinated in compound}}{\text{number of seeds germinated in control}} \times 100 \quad (1)$$

The root length of the sample and the root length of the control were measured for each experimental set. The root length was measured from the tip of the primary root to the base of the hypocotyl. The relative root growth (RRG) was calculated in percentage following Equation (2) [6]:

$$\mathrm{RSG}(\%) = \frac{\text{mean root length in compound}}{\text{mean root length in control}} \times 100 \quad (2)$$

Germination index (GI) was used to assess if a medium contains detrimental substances for seed germination or for growth of the radicle; it was calculated using Equation (3) [6]:

$$\mathrm{GI}(\%) = \frac{\mathrm{RSG} \times \mathrm{RRG}}{100} \quad (3)$$

Other parameters such as coefficient of the rate of germination (CRG) (Equation (4)) [10] and index of germination rate (IGR) (Equation (5)) [12] were also calculated.

$$\mathrm{CRG} = \left[\frac{\left(N_1 + N_2 + N_3 + \cdots + N_n\right)}{\left(N_1 \times T_1\right) + \left(N_2 \times T_2\right) + \left(N_3 \times T_3\right) + \cdots + \left(N_n \times T_n\right)} \right] \times 100 \quad (4)$$

where: N_1, N_2,…, N_n is the number of seeds germinated at time T_1, T_2, ..., T_n

$$\mathrm{IGR} = \left(\frac{100}{n}\right) \sum \frac{g_i}{T_i} \quad (5)$$

where: n is the number of seeds used in the experiment and g_i is the number of seeds germinated at time T_i.

2.4. Statistical Analysis

One-way analysis of variance (ANOVA) with Tukey's post-hoc test and significance level of 5% was used to determine the effects of the chlorophenol concentration on the germination parameters, being each plant species independently evaluated. Data was showed in the form of means and standard deviations. All the statistical procedures were performed using Statgraphics Centurion XV (v. 15.2.05; StatPoint, Inc., USA) software.

3. Results and Discussion

Bioassays in plants to determinate the toxicity of organic compounds has been described in literature. Also the study of the germination of the seeds is a requirement to determine if the plants can grow in contaminated soils to apply in phytoremediation [13].

The results in evaluation of germination shown in **Figure 1** revealed that maize and bean exhibited different behaviors toward different concentration of chlorophenols samples with an evident concentration-dependent inhibition.

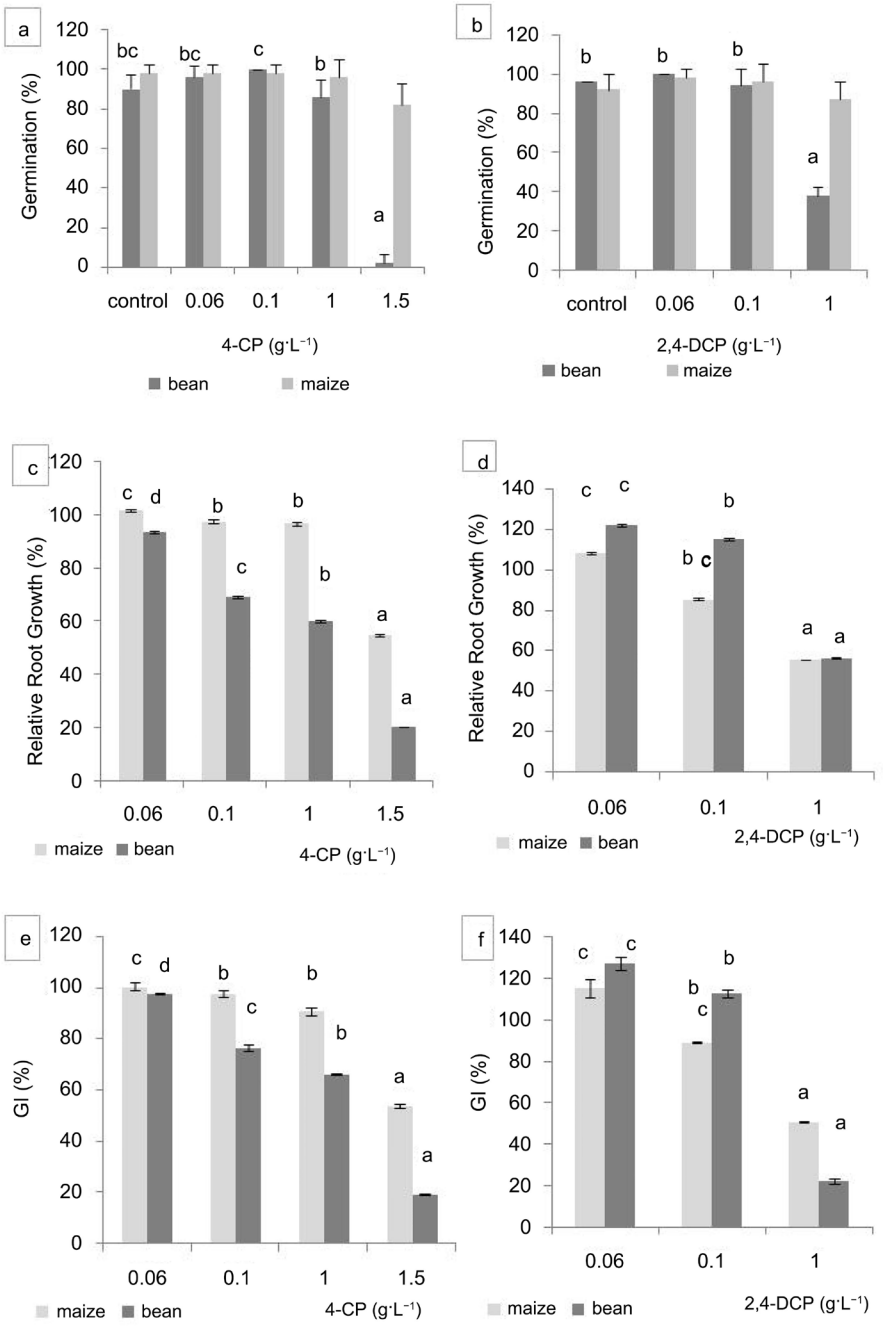

Figure 1. Effects of chlorophenols concentrations at seven days on germination percentage of bean and maize (a), RRG (c), GI (e) in 4-chlorophenol. Effects on germination percentage of bean and maize (b), RRG (d), GI (f) in 2,4-dichlorophenol. Control are seeds without chlorophenol. Different letters reflect significant differences according Tukey ($P < 0.05$, $n = 5$). Capital letter is related with bean while letter is related with maize.

When the germination behavior of bean seeds was analyzed against different concentrations of 4-CP, a delay in the start of germination and a decrease in the total number of germinated seeds were observed compared to a concentration of 1.0 g·L^{-1}, but concentration of 1.5 g·L^{-1} inhibited totally bean germination. In the case of maize, although there was no inhibition of germination, a delay in the

beginning of this was observed, as well as a decrease in the number of germinated seeds when faced with the maximum concentration evaluated. When the seeds were treated with 2,4-DCP, a delay in the initiation of germination was observed for the concentration of 1.0 $g{\cdot}L^{-1}$ in both crops, and it was also observed that there was a greater affectation in the bean crop at the higher concentration, while maize, although it delayed its start of germination, almost reached the control values at seven days.

In both crops the germination at seven days was similar to the control at concentrations up to 0.1 $g{\cdot}L^{-1}$ to both chlorophenols. As from 1.0 $g{\cdot}L^{-1}$ it was observed affectation in the germination percentage. For 4-CP, beans was more affected than maize at 1.0 $g{\cdot}L^{-1}$ and showed a complete inhibition of germination at 1.5 $g{\cdot}L^{-1}$. On the other hand, maize showed greater tolerance to the compound, beginning to affect its germination at the maximum concentration studied (1.5 $g{\cdot}L^{-1}$) (**Figure 1(a)**). In the case of 2,4-DCP (**Figure 1(b)**) germination of bean was different to maize at the same concentration (1.0 $g{\cdot}L^{-1}$) and compared to the control, while maize not present affectation in concentrations assayed.

The comparison of the two plants showed that beans were more affected by both phenolic compounds than maize, being 2,4-DCP the one that most affected germination when equal concentrations were compared.

The germination of the seed is the first step in the life of the plant and is one of the most sensitive in its physiology. Germination can be affected by hormonal interactions, biotic and abiotic factors as the presence of organic contaminants. The seed germination test has been used by multiple researchers to evaluate the toxicity of organic compounds. This test was used to evaluate the degradation of triphenylmethane-based dyes such as malachite green and crystal violet and phytotoxicity of allelopathic compounds [14] [15].

The relative root growth showed that for 4-CP, the growth of the maize roots was affected by the concentration of 1.5 $g{\cdot}L^{-1}$, but in beans the affectation started from 0.1 $g{\cdot}L^{-1}$ presenting differences with respect to the concentrations of 0.06 $g{\cdot}L^{-1}$ and control. In general, the roots of bean seedlings were smaller than those of maize, which shows that the effects not only reach the beginning and the number of shoots, but also the quality of what germinates (**Figure 1(c)**). In the case of 2,4-DCP, significant differences were found in the maximum concentration with respect to the minors and the control, causing a greater impairment in the growth of the roots, a result similar to that found in germination (**Figure 1(d)**). A slight increase to 0.06 $g{\cdot}L^{-1}$ was observed as if this concentration stimulated the elongation of the roots but it was not significant.

The germination index (GI) is a factor that relates the relative germination of the seed with the growth of the root. In this case, the GI decreased for both compounds as the concentration increased, the decrease being greater in bean (**Figure 1(e)**, **Figure 1(f)**).

Other authors investigated the seed germination index in various concentrations of other chlorophenol (2-CP) to observe the influence on seedling growth

of tomato and turnip. The extent of germination at lower 2-CP concentrations varied for different species. The two crops only germinate in 2-CP of concentration ranged from 20 to 40 mg·L^{-1}. However further increase in 2-CP concentration (60 - 100 mg·L^{-1}) caused a total failure of seedling growth [16].

The impact of 2,4-DCP on germination of *Vigna radiata* showed similar results confirming that lower concentrations not affect germination significantly however, at higher concentrations inhibition in germination is observed [17]. They comment that the inhibition of seed germination may be due to by the hydrophobic nature of phenol which interferes with water activity and absorption inside the seed. Also, they express the inhibitory effect at higher concentrations in root elongation 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.

Geneve *et al.*, (2018) described that seed coat has a protective role because is impermeable and the embryo absorbs water through the micropylar slit in the coat during germination stage [18]. But, although the tissues that cover the embryo are capable of protecting it against the toxicity, once the breaking of the seed coat begins, growth is affected according to the type and concentration of contaminant [19]. This could be the reason of reduction in root growth without affectation apparent in germination.

When germination rates were analyzed, it was found that CRG was the parameter least affected by the chlorophenols evaluated. When analyzing each chlorophenol more specifically, it was observed that the CRG did not show differences with the control for maize, while beans showed a difference in the highest concentration evaluated for 4-CP (**Figure 2(a)**, **Figure 2(b)**). With respect to the IGR for this compound, it was observed that for maize it showed affectation in 1.5 g·L^{-1}, on the other hand for beans this parameter showed differences in 1 g·L^{-1}, observing no germination at 1.5 g·L^{-1} (**Figure 2(c)**). In the case of the evaluation of 2,4-DCP, differences were observed in the highest concentration tested in both parameters for the two plant models (**Figure 2(d)**). When the two compounds were compared, it was observed that 2,4-DCP was the most toxic for both plants, showing negative effects on germination at a lower concentration than the 4-CP.

In the literature, phytotoxicity effect of phenolic compounds has been related to their lipophilic character (*i.e.* solubility in a lipid medium). A relationship was proposed between hydrophobicity of molecules and their phytotoxicity. Lipophilic substances tend to be more phytotoxic because they pass more easily through the cell membrane enhancing the interaction of the compound with specific plant structures [6] [20].

Also, the toxicity of chlorophenols can be attributed to the uncoupling nature by interfering with the activity of some elements of the respiratory chain, being able to separate it from the coupled proton pump to generate ATP, so that the latter is not generated, an action that is increased with the number of chlorines

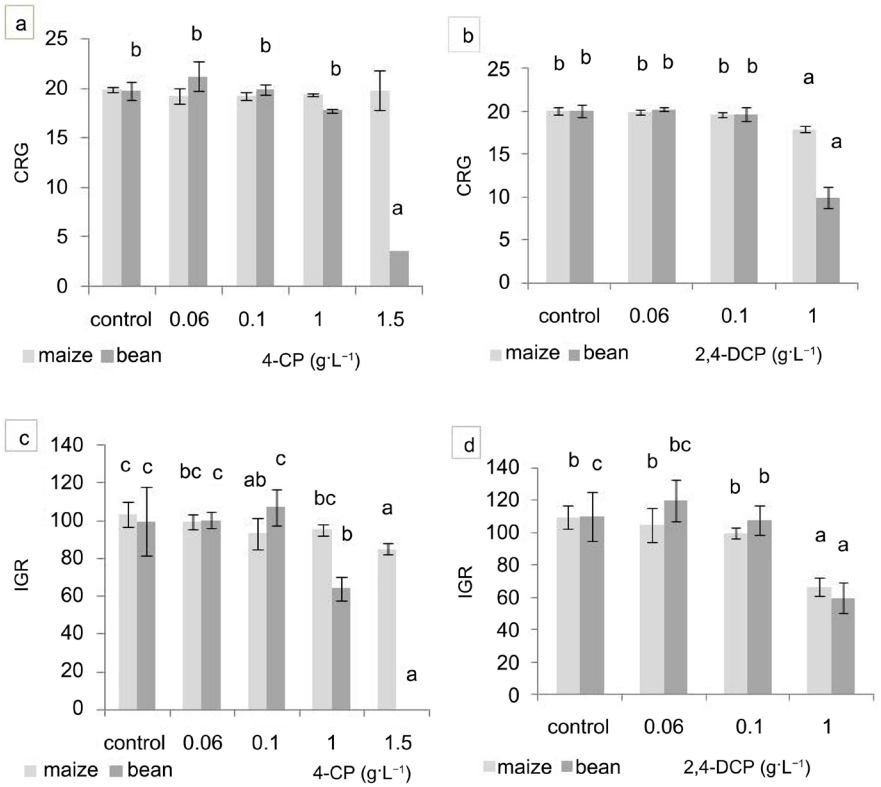

Figure 2. Effects of chlorophenols in germination rate for selected plant species: CRG (a) and IGR (c) for 4-CP. CRG (b) and IGR (d) for 2,4-DCP. Different letters reflect significant differences according Tukey ($P < 0.05$, $n = 5$). Capital letter is related with bean.

attached to the phenolic ring [2] [20]. Phenols can also bind to a variety of biomolecules by altering their biological functions [21]. The most detailed studies of these effects have been carried out in bacteria, but few studies have explored the causes of the affectation in higher organisms, specifically in plants.

The studies carried out showed that 4-CP and 2,4-DCP at concentrations similar to or greater than 1 $g \cdot L^{-1}$ could affect the germination and development of the roots of the plants that are in contact with them, an aspect that should considerate when plants are to be grown on land contaminated with 2,4-D and its intermediates. It is also important to consider when planning phytoremediation trials with these plants or the like.

4. Conclusion

Based on the results obtained when evaluating the phytotoxicity of 4-chlorophenol and 2,4-dichlorophenol, it was concluded that beans (*Phaseolus vulgaris*) were more sensitive to the chlorophenols studied than maize (*Zea mays*). 2,4-DCP was the chlorophenol that most influenced plant physiology by delaying germination and root growth at a concentration of 1.0 $g \cdot L^{-1}$. Beans showed an inability to germinate against 1.5 $g \cdot L^{-1}$ of 4-chlorophenol, however, maize could grow at this concentration with only a 20% decrease in germination. This knowledge is important not only to cultivate in soils contaminated with 2,4-D herbicide or

their intermediates but also to develop a phytoremediation strategy to deal to contamination with this compounds.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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