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Toxicity of phenol solutions treated with rapeseed and tomato hairy roots

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ABSTRACT

Phenolic compounds are frequently found in several industrial effluents. Recently, the use of hairy root cultures has been explored as a new alternative for their treatment. In this study, rapeseed and tomato hairy roots (HR) were used to remove phenol efficiently (100 to 250 mg/L) from aqueous solutions in a short time. The removal efficiencies were 95–80% and 60–70% for rapeseed and tomato HR respectively. Polyethylene-glycol (PEG-3350) addition to the reaction medium, significantly enhanced removal efficiency of rapeseed HR reaching values of 98–88%.

After the removal process the acute toxicity of post-removal solutions was determined by means of AMPHITOX bioassay. Phenol solutions treated with rapeseed HR showed a lower level of toxicity than at the beginning of the treatment. Moreover, PEG addition reduced the toxicity of these solutions. On the contrary, tomato HR could only be applied to reduce the level of toxicity in solutions initially containing low phenol concentrations (100–150 mg/L), without adding PEG. From these results, it is relevant to point out the importance of studying different plant systems and their reaction capacity to find the more suitable ones for removal purposes.

1. Introduction

Phenols are involved in the manufacture of pesticides, tinctures, bakelite and they have also been used for many years as antiseptics [1]. Therefore, they are common pollutants in the effluents of many industries. As a consequence, a large amount of phenolic compounds are effused into natural streams and soil, and pollute the environment [2]. For these reasons, many biological treatments are being explored to remove these pollutants. Hairy roots have been used to test plants' ability to tolerate high levels of phenols [3]. They were also used to remove this pollutant from aqueous solutions with high efficiency [4–6].

Although different plant enzymes are implicated in phenol remediation, it is well known that peroxidase isoenzymes are the main enzymes involved in the removal of phenol and chlorophenols, by hairy roots from different plant sources. For this reason, the removal process is enhanced by exogenously-applied hydrogen peroxide, which is a co-substrate of these enzymes [5,7,8]. However, peroxidases can be inactivated during the reaction and the catalytic lifetime of these enzymes can be significantly extended in the presence of protective additives such as PEG [1]. In this way, it has been demonstrated that PEG would combine with the polymerization products formed during the reaction, because it has a higher partition affinity with polymer products than peroxidases [9].

Depending on the type of phenol treated and the removal process employed, these products can be more toxic than the parent phenol. For this reason it is necessary to evaluate the toxicity of post-removal solutions, since removal of the original compounds is only a partial evaluation of the treatment efficiency.

Among the different organisms which could be selected in order to evaluate toxicity, amphibian's species have shown to be more sensitive bioindicators of aquatic contamination than other vertebrates, because they have a permeable skin that readily absorbs substances dissolved in water [10]. AMPHITOX is a bioassay which utilizes *Rhinella arenarum* embryos to evaluate acute, short-term chronic, chronic and early life stage toxicity [11]. Young tadpoles of *R. arenarum* constitute a very useful tool for toxicity studies, because these organisms are sensitive to toxic samples, available throughout the year, genetically homogeneous and easy to handle in the laboratory [12].

A measure of the whole-effluent toxic impact would be provided by using acute bioassay. In this context, in a previous work, phenol toxicity during the embryonic development of *R. arenarum* in standard conditions was studied, and the induction of both mortality and developmental abnormalities at ecologically relevant concentrations (25 mg/L) was demonstrated [13].

Abbreviations: HR, Hairy roots; PEG, Polyethylene-glycol; RS, Ringer solution; H_2O_2, Hydrogen peroxide; LC_{50}, Lethal concentration 50; LC_{99}, Lethal concentration 99; NOEC, Non-observed effect-concentration; TU, Toxic units; 2,4-DCP, 2,4-Dichlorophenol.

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As it was previously mentioned, tomato (*Solanum lycopersicon* cv Pera) and rapeseed (*Brasicca napus*) hairy root cultures as well as peroxidase isoenzymes derived from them, were used for phenol removal treatment [5,6,14]. However, the toxicity of post-removal solutions, need to be clarified to determine the effectiveness of these treatments. Thus, the aims of the present study were: (1) to evaluate and to compare phenol removal efficiencies obtained with rapeseed and tomato hairy root cultures; (2) to determine the toxicity of post-removal phenol solutions, obtained after treatments performed with hairy roots, using AMPHITOX as a bioassay; (3) to study the effects of PEG addition on the removal efficiency and on *R. arenarum* embryos (acute toxicity test).

2. Materials and methods

2.1. Plant material

Hairy root (HR) cultures of *B. napus* and *S. lycopersicon* were obtained by inoculation of sterile leaf explants with *Agrobacterium rhizogenes* strain LBA 9402 as was previously described [15,16].

They were sub-cultured every 30 days in Murashige and Skoog [17] liquid medium enriched with vitamins and kept in an orbital shaker at 100 rpm at 25 ± 2 °C in the darkness. After this period of time, roots were harvested and used for the experiments described below.

2.2. Reagents and instruments

Hydrogen peroxide, phenol, potassium ferricyanide and ammonium hydroxide were purchased from Merck. PEG-3350 and 4-aminoantipyrine were supplied by Sigma and Aldrich, respectively. Human Chorionic Gonadotrophin (Endocorion 5000) was purchased from Elea Laboratory. The other reagents used to prepare buffer solutions were of analytical grade and were purchased from Merck and Sigma. All solutions were prepared using deionized water. Spectrophotometric measurements were made in a Beckman spectrophotometer DU640. Embryos were observed using a Motic digital Microscope DM39.

2.3. Phenol removal assays

Removal reactions were carried out using 100, 150, 200 and 250 mg/L of phenol solution. These solutions were used because concentrations of the same order of magnitude can exist in heavily contaminated sites and in industrial effluents [13].

Phenol solutions were treated with 6 g of HR and 5 mM H_2O_2, in a total volume of 150 mL. The reaction mixture was incubated during 1 h at 25 ± 2 °C in an orbital shaker at 100 rpm [5,6].

After incubation, residual phenol in the reaction mixtures was measured through a colorimetric assay [18] and results were expressed as residual phenol (mg/L).

In the experiments performed to evaluate the effect of PEG-3350 addition to the reaction medium, removal reactions were carried out following the same protocols described above, but in these experiments, 100 mg/L of PEG-3350 was added.

2.4. Phenol determination

Phenol determinations were carried out following the method of Klibanov and Morris [18]. A 5 mL aliquot of tested solution reacted with 0.025 mL of 6.0 M ammonium hydroxide, 0.025 mL of 4-aminoantipyrine (2% aqueous solution) and 0.05 mL of potassium ferricyanide (8% w/v). After five minutes, the coloured compound formed was extracted with 2.5 mL of chloroform. The absorbance of the extract was determined at 510 nm and was proportional to the concentration of phenol in the range of $0–10^{-4}$ M. Removal efficiency was calculated according to Agostini et al. [7].

2.5. Hydrogen peroxide determination

Residual H_2O_2 in post-removal solutions was evaluated using the procedure described by Sergiev et al. [19]. Briefly, samples of 500 µL of post-removal solutions were mixed with 500 µL of 10 mM potassium phosphate buffer, pH 7. Then, 1 mL of 1 M KI was added and the mixture was homogenized. The absorbance was measured at 390 nm, in a Beckman spectrophotometer, and the data were transformed to H_2O_2 concentration using a calibration curve, which was carried out with known concentrations of H_2O_2.

2.6. Test organism and experimental design

R. arenarum toads weighing approximately 200–250 g were obtained in Río Cuarto (Córdoba Province, Argentina). Ovulation was induced by intraperitoneal injection of homogenized homologous pituitary gland [20,21] with 300 IU of Human Chorionic Gonadotrophin (Endocorion 5000, ELEA) [22] in 8 mL of 10% Ringers Solution (RS). Oocytes were fertilized *in vitro* with a testicular suspension. Embryos were maintained in 10% RS until closed both operculum, which is the last stage of embryonic development (s25), according to Del Conte and Sirlin [23].

Bioassays were conducted with *R. arenarum* embryos following the AMPHITOX conditions [11]. Batches of 10 embryos, by triplicate, were placed in 5 cm dia glass petridishes containing 10 mL of tested solutions.

Solutions were renewed daily and the test lasted 96 h. Mortality of tadpoles was checked every 24 h and those exhibiting no response behavior towards gentle prodding were considered dead. These dead tadpoles were removed. All experiments were carried out at 20 ± 2 °C. For the experiments of acute toxicity, the number of dead tadpoles was registered. Results were expressed as percentage of mortality.

2.7. Solutions tested

The following solutions were tested for their potential toxicity by means of AMPHITOX: H_2O_2 (0.1 to 5 mM), PEG (50 to 500 mg/L) and post-removal solutions originally containing 100 to 250 mg/L of phenol. Post-removal solutions were obtained after treatments with rapeseed and tomato HR, in two different conditions: with and without the addition of PEG. RS was used as control.

The concentration of H_2O_2 was determined spectrophotometrically using $\varepsilon_{240nm} = 43.6\ M^{-1}\ cm^{-1}$.

2.8. Statistical analysis

All the data were analyzed using ANOVA. In all cases $p \leq 0.05$ was statistically significant. Lethal concentration 50 (LC_{50}), lethal concentration 99 (LC_{99}) and non-observed effect-concentration (NOEC) values were calculated using the EPA Probit Analysis Program [24]. Moreover, toxic units (TU) were calculated as $100/LC_{50}$, for acute toxicity test [12,25].

3. Results and discussion

3.1. Phenol removal assays with and without PEG-3350

Rapeseed and tomato HR constitute an efficient system for phenol removal, as was previously described [5,6]. In the present study, phenol removal (100 to 250 mg/L), supplemented with H_2O_2 (5 mM), was analyzed to establish residual phenol concentration and to evaluate the toxicity of these solutions through an acute toxicity test.

When rapeseed HR were used to remove phenol (without PEG addition to the reaction medium), residual phenol concentration decreased significantly after 1 h of treatment ($p<0.05$) in all solutions tested (100–250 mg/L) (Fig. 1). Since PEG-3350 was added to the reaction mixture, significant low values of residual phenol were obtained, as shown in Fig. 1. These results showed that rapeseed HR were able to remove phenol with high efficiency (95–80%) in a short time, from solutions with initial phenol concentrations ranging from 100 to 250 mg/L, respectively. Moreover, this removal efficiency increased when PEG-3350 was added to the assay medium, reaching values of 98–88%.

When tomato HR were used in the removal process (Fig. 2), residual phenol concentrations detected in the medium, after 1 h of reaction, were higher than those obtained with rapeseed HR. Moreover, the addition of PEG-3350 did not modify significantly phenol removal.

The results presented here, showed that phenol remediation performed by HR depends on the plant species as well as on the concentration of the pollutant used. Singh et al. [4] showed that *Brassica juncea* HR were also capable to remove 50 and 100 mg/L of phenol, but in a longer period of time (3 days). However, when they used high phenol concentrations (200, 500 and 1000 mg/L), removal efficiencies decreased to 97, 47 and 27% respectively in the same period of time.

In previous studies, it was established that peroxidase isoenzymes derived from rapeseed and tomato HR can remove phenol and 2,4-dichlorophenol efficiently and they would be the enzymes more implicated in the removal process. Basic, near neutral and acidic peroxidase isoenzymes could be involved in the metabolism of phenol in rapeseed HR, whereas basic peroxidases would be the main isoenzymes implicated in phenol removal in tomato HR. However, they could be inactivated during the treatment [6,14].

Due to the fact that peroxidases are susceptible to permanent inactivation by various undesirable side reactions during treatment process [26], the addition of some additives, like PEG, which are able to protect the enzyme, have been suggested. Several reports describing the protective effects of PEG on peroxidase activity are available in the literature. Nevertheless, results are sometimes quite different. For instance, Cheng et al. [27] have reported that PEG can improve the efficiency of phenol removal by forming a protection layer in the vicinity of the active centre of horseradish peroxidase (HRP), to restrict the attack of free phenoxy radicals formed in the catalytic cycle. In the same way, Dalal and Grupta [28], indicated that HRP with the addition of PEG (0.1 mg/mL) completely removed p-chlorophenol (1 mM) from the reaction mixture. In contrast, Caza et al. [29], using soybean peroxidase (SBP), did not observe significant changes in phenol removal efficiency by effect of this additive.

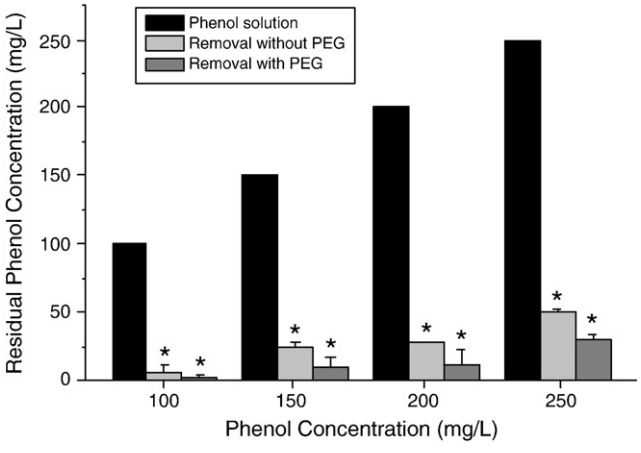

Fig. 1. Residual phenol concentration (mg/L) in solutions treated with rapeseed HR, with and without PEG-3350 addition to the reaction medium.

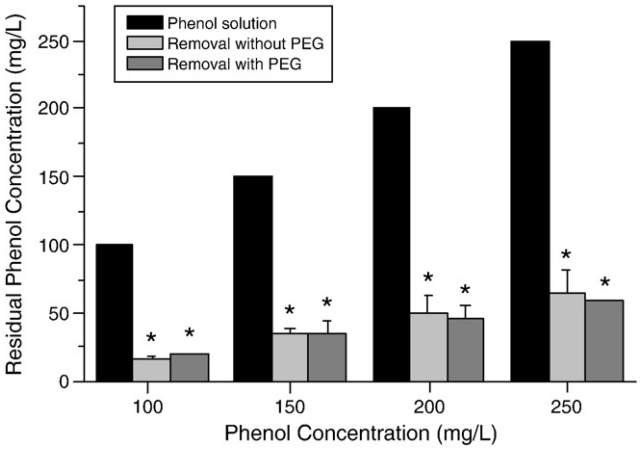

Fig. 2. Residual phenol concentration (mg/L) in solutions treated with tomato HR, with and without PEG-3350 addition to the reaction medium.

In the same way, similar behaviors were observed in this work, because the results were different when PEG-3350 was added to reaction mixtures containing rapeseed or tomato HR and best results were obtained with the former.

This could be probably associated with the variability of peroxidase isoenzymes derived from each plant species [5,6]. Moreover, PEG could interact with reaction products, which might be different considering the source of peroxidase used. In this sense, a variety of reaction intermediaries were formed during the degradation of phenol and chlorophenols, as described Hirvonen et al. [30]. Due to the presence of a complex mixture of these intermediaries, the use of a toxicity test is likely to be needed in order to confirm the detoxifying effect of the removal process. Thus, in the present study the toxicities of post-removal solutions as a whole were measured, to verify detoxification. Studies related to the identification of post-removal products are now being carried out in our laboratory to reinforce the idea of making this HR cultures potentially applicable even at industrial scale, to detoxify large volumes of wastewaters or industrial effluents.

3.2. Toxicity study of H_2O_2, PEG-3350 and post-removal solutions

The toxicity of remanent solutions as well as the toxicity of the different components of the reaction mixture, such as hydrogen peroxide and PEG-3350, after phenol removal process, was analyzed.

Hydrogen peroxide was usually employed in phenol removal process because it is a co-substrate of peroxidases. Then, this compound was added to the reaction medium at a concentration of 5 mM and its toxicity on *R. arenarum* embryos was evaluated.

Fig. 3 shows that survival of larvaes was not significantly affected by H_2O_2 concentrations ranging from 0.1 to 0.6 mM ($p>0.05$). However, a solution containing 1 mM H_2O_2 was toxic and produced a significant mortality (100%) compared to embryos grown in control conditions ($p<0.05$). LC_{50} value was 0.89 mM and NOEC value was 0.6 mM. It should be noticed that mortality changed drastically from 50 to 100% in a concentration range from 0.89 to 1 mM.

Reactive oxygen species (ROS) such as, hydrogen peroxide, $^1O_2$, superoxide and hydroxyl radicals, induce mitochondrial damage, disturbance in Ca^{2+} homeostasis, and apoptosis. Moreover, signal transmission in the central nervous system, as well as ligand binding to membrane receptors, coupling of receptors to proteins and effector enzymes are severely affected by oxidative stress [31]. Thus, the damage produced in *R. arenarum*, could be probably caused by the oxidative stress generated by H_2O_2 excess and this damage could

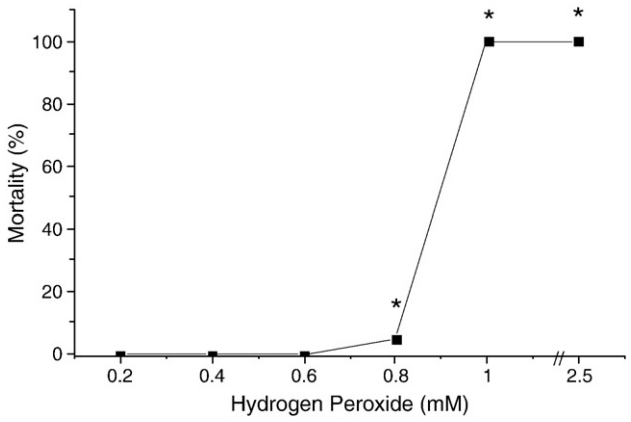

Fig. 3. Mortality (%) produced by hydrogen peroxide (mg/L) on *Rhinella arenarum* embryos at 96 h of exposure (acute test).

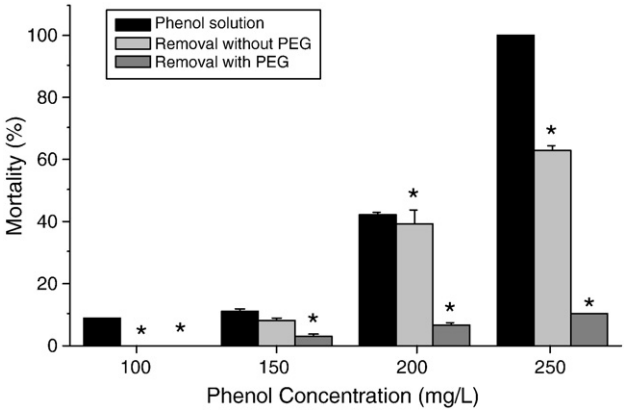

Fig. 4. Effects of the exposure to different concentrations of post-removal solutions obtained by treatment performed with rapeseed HR (with and without PEG addition) on the survival of *Rhinella arenarum* embryos.

explain the lethal effect observed on these embryos, using concentrations higher than 0.8 mM (Fig. 3).

In removal assays performed with rapeseed and tomato HR, 5 mM exogenous H_2O_2 was added to obtain high removal efficiency. Taking into account the results obtained by means of AMPHITOX (Fig. 3), this concentration was toxic. Residual concentration of H_2O_2 in post-removal solutions was determined and it was lower than 0.2 mM, which did not produce toxic effects on the tested organism.

To our knowledge, there are still no studies which investigate the toxicity of H_2O_2 in amphibian's species. However, data obtained from literature demonstrated that H_2O_2 is toxic over a wide range of concentrations (0.1 to 0.88 mM) for several organisms, such as marine algae, *Daphnia magna,* fishes, and bacteria [32,33]. For this reason, it is necessary to determine the residual concentrations of H_2O_2, before releasing it in the environment.

On the other hand, PEG-3350 toxicity was also evaluated by means of AMPHITOX bioassay. Acute toxicity of PEG-3350 was analyzed in embryos exposed to concentrations ranging from 50 to 500 mg/L. PEG-3350 did not produce significant lethality on *R. arenarum* embryos, in all concentrations used. In order to minimize the cost of the process, a concentration of 100 mg/L of this additive was selected for removal experiments. The results obtained are in agreement with those described by other researchers, who have shown that PEG is a chemo protective agent without toxic effects on several organisms. In addition, it has been declared fit for human consumption by the United States Food and Drug Administration [34].

Regarding the toxicity of post-removal solutions previously treated with rapeseed and tomato hairy roots (with and without PEG-3350), Figs. 4 and 5 show the results of acute toxicity. Phenol solutions (not treated with HR) were used as controls to compare their acute toxicities with those of the post-removal solutions.

Toxicity decreased significantly in 100 mg/L phenol solutions treated with rapeseed HR (without the addition of PEG-3350), which showed no mortality, compared to phenol solutions without treatment ($p<0.05$) (Fig. 4). Toxicity of 250 mg/L solutions treated with rapeseed HR also decreased; however, this solution produced high mortality (60%) after treatment. In solutions containing 150 and 200 mg/L of phenol, a reduction of the toxicity was observed, but this reduction was not statistically significant. It should be noted that the addition of PEG-3350 to the reaction medium, significantly reduced toxicity in all solutions. Moreover, mortality values were as low as 0, 3.3, 6.66 and 10% for solutions initially containing 100, 150, 200 and 250 mg/L of phenol, respectively. According to these results, it is clear that phenol solutions treated with rapeseed HR, plus PEG, showed a reduction in their toxicities, producing a decrease of 90% in *R. arenarum* embryos mortality, compared to 250 mg/L of phenol solutions.

The toxicity test performed with post-removal solutions obtained after treatment with tomato HR revealed variable toxicity, depending on the concentration tested (Fig. 5). For post-removal solutions containing initially 100 and 150 mg/L of phenol (without PEG-3350), the percentages of mortality decreased significantly ($p<0.05$). When PEG was added the toxicity of all post-removal solutions did not decrease and produce high mortality of the embryos exposed. Then, it was assumed that in the composition of these post-removal solutions, some compounds or reaction products, whose toxicity would be higher than that of the standard phenol solution, could be present. However, no data is available to confirm or discard this possibility. Besides the addition of PEG did not reduce the toxicity of these solutions, in contrast to the results observed when rapeseed HR were used. In the same way, Zhang and Nicell [35] demonstrated that PEG did not reduce the residual toxicity when HRP was used to remove pentachlorophenol (PCP). It may be inferred that the different results found with PEG as an additive may be due to the different nature of reaction products formed during phenol polymerization.

Taking into account these results and those previously obtained [5], it was suggested that tomato HR could only be applied to detoxify waste waters with phenol concentrations until 150 mg/L, and without the addition of PEG-3350.

The 96 h LC_{50}, LC_{99}, and NOEC values obtained by Probit analysis, and calculated TU values for different treatments are given in Table 1. The concentrations needed to reduce 50% of survival (LC_{50}) of the larvae exposed to solutions treated with rapeseed HR, doubled those obtained for tomato HR. In the same way, when rapeseed HR were

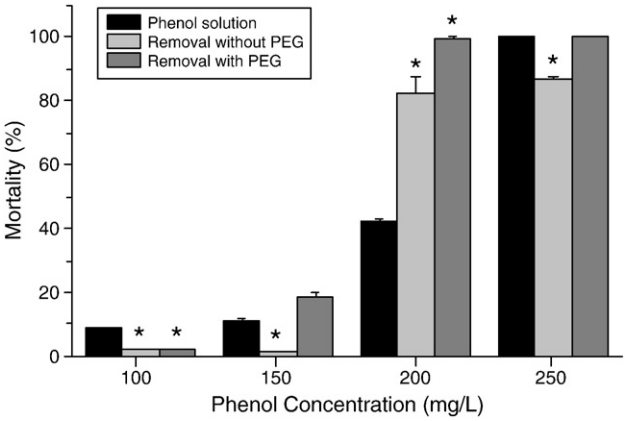

Fig. 5. Effects of the exposure to different concentrations of post-removal solutions obtained by treatment performed with tomato HR (with and without PEG addition) on the survival of *Rhinella arenarum* embryos.

Table 1
Toxicity values obtained on *Rhinella arenarum* embryos expressed as non-observed effect-concentration (NOEC), lethal concentration 50 (LC_{50}), lethal concentration 99 (LC_{99}) and toxic units (TU) for different post-removal solutions and conditions.

Removal conditions	NOEC (mg/L)	LC_{50} (mg/L)	LC_{99} (mg/L)	TU
Rapeseed HR without PEG	100	323.18	1559	0.309
Rapeseed HR with PEG	46.39	351.14	1797.91	0.284
Tomato HR without PEG	98.30	180.24	260.92	0.554
Tomato HR with PEG	100	172.27	218.7	0.580

used, the LC_{99} were five to six times higher than those obtained with tomato HR, indicating the lower toxicity of the former (Table 1).

However, the use of additives like PEG-3350 produced different results in both systems. In post-removal solutions derived from treatments with rapeseed HR, a positive effect was observed in all solutions tested (LC_{50} 351.14 mg/L). Meanwhile, in solutions treated with tomato HR, LC_{50} was lower with PEG-3350 than the same solution without PEG-3350.

The higher LC_{99} values determinated by Probit analysis corresponded to post-removal solutions derived from rapeseed HR with the addition of PEG-3350 to the reaction medium, indicating the low toxicity of these solutions.

In order to become independent of the chemical composition of the sample and to determine the magnitude of hazard due to chemical stress, the obtained data was expressed as TU. The maximum value recommended by USEPA for TU is 0.3, regarding quality control of industrial effluents [36].

Post-removal solutions obtained from rapeseed HR without PEG-3350 showed a TU value around 0.3, which represents the maximum value recommended for US EPA. With the addition of PEG-3350, this value decreased to 0.28, representing the lowest toxicity of all treatments studied. On the other hand, post-removal solutions obtained from treatments with tomato HR with and without PEG-3350, showed higher TU values than the reference value (Table 1). These results clearly showed that post-removal solutions obtained by treatment with rapeseed HR are less toxic than those derived from tomato HR.

Rapeseed HR showed high capability to remove phenol and this efficiency was increased when PEG-3350 was added to the reaction medium compared to tomato HR. Although residual phenol was higher in post-removal solutions treated with tomato HR than in solutions treated with rapeseed HR, this pollutant is not responsible for tadpoles mortality. This affirmation is consistent with the fact that phenol concentrations lower than 50 mg/L did not produce mortality on *R. arenarum* embryos [13].

Since some reaction products of enzymatic catalysis may still remain in the aqueous phase after treatment and may also be toxic, a complete transformation of substrate may not result in complete reduction of toxicity, as was shown by Zhang and Nicell (2001) [35], using HRP. Moreover, the compounds responsible for the toxicity would probably be present in trace quantities or the toxicity would be due to synergistic effects of several species [1].

Furthermore, a color change (from uncolored to red) was observed in the reaction mixtures during oxidation performed with tomato HR. This change would indicate the formation of new chromophores, most probably quinones and their derivatives. This color change was not registered in solutions treated with rapeseed HR, which gives more evidence that different reaction products with different toxicities exist in post-removal solutions, depending on the hairy roots specie used.

4. Conclusion

Rapeseed and tomato HR have the ability to transform and remove phenol solutions with high efficiency. The removal efficiencies were 95–80% and 60–70% for rapeseed and tomato HR respectively. PEG-3350 addition to the reaction medium, significantly enhanced removal efficiency of rapeseed HR reaching values of 98–88%, however it was not capable to enhance the removal efficiency of tomato HR.

When the toxicity was evaluated by means of AMPHITOX test, variable results were observed. Post-removal solutions, obtained with rapeseed HR, produced less toxicity on *R. arenarum* embryos than solutions treated with tomato HR. The addition of PEG-3350 was efficient to reduce toxicity of post-removal solutions treated with rapeseed HR. However, PEG-3350 was not able to reduce toxicity from post-removal solutions treated with tomato HR.

Since removal efficiencies are variable depending not only on the concentrations and toxicity of a certain xenobiotic, but also on the removal abilities of different plant species or *in vitro* cultures derived from them, it is relevant to point out the importance of studying different plant systems in order to find the more suitable for remediation purposes.

According to these results both HR cultures could be used for the treatment of effluents contaminated with phenol, however, rapeseed HR with the addition of PEG-3350 could be a more efficient removal system, considering the low toxicity of post-removal solutions on *R. arenarum* embryos.

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