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Original Research

The Effects of 2,4,5-Trichlorophenol on Some Antioxidative Parameters and the Activity of Glutathione S-Transferase in Reed Canary Grass Leaves (*Phalaris arudinacea*)

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Abstract

It is known that trichlorophenols may provoke free radical formation, leading to oxidative stress in living organisms, but no effort has been undertaken to evaluate oxidative changes in plants exposed to 2,4,5-trichlorophenol (2,4,5-TCP) that commonly exists in a soil environment.

In this work the effect of 2,4,5-TCP on selected parameters of an antioxidative system, the content of hydrogen peroxide and the activity of glutathione S-transferase in the leaves of reed canary grass (*Phalaris arudinacea*), has been investigated.

An increase in the content of total and reduced forms of glutathione and ascorbate, as well as free phenols concentrations, was shown. Moreover, a rise in the activities of catalase, guaiacol peroxidase, and glutathione S-transferase along with arisen content of hydrogen peroxide was observed. The obtained results showed that oxidative stress was provoked in the examined plants that had been exposed to 2,4,5-trichlorophenol.

Keywords: reed canary grass, 2,4,5-trichlorophenol, hydrogen peroxide, antioxidant enzymes, ascorbate, glutathione

Introduction

For many years people have interfered with the environment by introducing numerous chemicals into the air, water and soil thus disturbing its natural balance.

Chlorophenols are a very important group of compounds present in the environment as they are very toxic, have the capacity to accumulate in living organisms and also exhibit considerable resistance to degradation. The widespread presence of chlorophenols in ecosystems is related to the use of these compounds as components or precursors of chemical reagents, dyes, plastics or medicines. Their appearance also results from the production and use of some pesticides. For example, the degradation of 2,4,5-trichloro-phenoxyacetic acid leads to the formation of 2,4,5-trichlorophenol (2,4,5-TCP) [1]. 2,4,5-TCP is used as a herbicide and it is also employed in the protection of wood as a fungicide [2]. The common use of chlorophenols and their precursors leads to the presence of 2,4,5-trichlorophenol in soil, particularly in plough-lands, areas adjacent to saw-mills and timber and yards and soils polluted with industrial wastes.

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The uptake of chlorinated phenols from a contaminated environment by various plants was proved. Chlorella and rice showed the ability to remove 2,4-dichloropenol from nutrient solutions [3]. Various other halogenated phenols were shown to be taken up by other plants [4].

An early reaction of plants exposed to biotic and abiotic stresses is often related to the overproduction of reactive oxygen species (ROS), like hydrogen peroxide, superoxide radical or hydroxyl radical. The production of ROS is stimulated by extreme climatic conditions – the influence of UV irradiation, ozone and nitrogen oxides - as well as drought and excessive irrigation [5]. It was shown that precursors of chlorophenols, like the herbicide 2,4-dichlorophenoxyacetic acid, are able to produce ROS, like hydrogen peroxide, in leaves [6].

Moreover, in some investigations it has been revealed that chlorinated phenols, like 2,4,5-trichlorophenol and 2,4-dichlorophenol, are able to induce ROS formation in animal cells [7] and it was proved that 2,4,5-trichlorophenol is able to induce oxidative damage to biological structures in lipid and protein peroxidation processes [8].

Catalase (CAT, EC 1.11.1.6.) is an enzyme that utilizes hydrogen peroxide to form water. It has been shown that CAT activity is usually changed as a result of xenobiotic activity. The herbicide atazine increased CAT activity in *Arabidopsis thaliana* [9] and herbicides like dicamba, paraquat and 2,4-dichlorophenoxyacetic acid changed its activity in the investigations on potato tuber cali [10].

Guaiacol peroxidase (POD, EC 1.11.1.7.) belongs to a large family of enzymes that oxidize several substrates, like guaiacol or polyphenols, in the presence of hydrogen peroxide. The activity of this enzyme is usually changed in plants exposed to xenobiotics, e.g.: propanil or an imidazolinone herbicide - mazethapyr [11].

Phenolic compounds, secondary plant metabolites, are involved in the response of plants to a harmful environment. An increase in phenolics was observed in soybean plants treated with the commonly used herbicide – glyphosate [12].

Ascorbate (ASA) is a major metabolite in plants and, in association with other components of the antioxidative system, protects plants against oxidative damage resulting from aerobic metabolism and a range of biotic and abiotic stresses including the effects of some herbicides like atrazine [13]. The oxidation of ascorbate leads to the formation of dehydroascorbate (oxidized ASA), which can be reduced back to ASA through the so-called ascorbate-glutathione (ASA-GSH) cycle, involving reduced glutathione and glutathione reductase [14].

Glutathione (GSH) is the most abundant low molecular thiol in plants. Due to the chemical reactivity of the thiol group of cysteinyl residue, a broad range of biochemical functions has been attributed to GSH [15]. It plays a crucial role in the protection of plants against various noxious factors. In the investigation on maize, an increase in glutathione content was observed in plants after their treatment with an isoproturon herbicide [13].

Glutathione S-transferase (GST, EC 2.5.1.18) catalyzes the conjugation of a broad variety of electrophilic and hydrophobic compounds with GSH [16]. The resulting GSH conjugates are usually more water soluble and often less toxic than the parent compounds. The results of many investigations clearly show that GST activity is enhanced by the influence of numerous pesticides, including metolachlor, fluorodifen or fenoxaprop in wheat, maize and soybean plants [17, 18].

Up to now, there have been few reports concerning the effect of chlorinated aromatic compounds on plants, and according to our knowledge, no effort has been undertaken to describe the impact of 2,4,5-trichlorophenol on antioxidative parameters, which may prove the free-radical mechanism of the action of this compound on plant cells.

Therefore, the purpose of this work was to determine changes in catalase, guaiacol peroxidase and glutathione S-transferase activities and also the evaluation of glutathione, ascorbate, free phenols and hydrogen peroxide contents in the leaves of reed canary grass (*Phalaris arudinacea*) exposed to a strong environmental toxin – 2,4,5-trichlorophenol. *Phalaris arudinacea* was chosen as a model plant, as it is commonly used in the phytoremediation of organic compounds from soil. Therefore, it would be interesting to find out how this plant would respond to the influence of a chlorinated xenobiotic like 2,4,5-trichlorophenol.

Experimental Procedures

Chemicals

2,4,5-Trichlorophenol (purity 99%), α, α'-bipirydyl, and maleimide ethyl were bought from Fluka, USA. Methanol, diethyl ether and phosphoric acid (HPLC purity) were obtained from Baker J. T., USA. Hydrogen peroxide, Coomassie G-250, trichloroacetic acid, 2-vinylpirydyne, NADPH, dithio-bis(2-nitro)benzoic acid, glutathione reductase, polyvinylpolypirolidone (PVP), sodium versenite (EDTA), 1-chloro-2,4-dinitrobenzene, reduced glutathione and sodium ascorbate were from Sigma-Aldrich, USA. Folin reagent, sodium hydroxide, TRIS, sodium carbonate, ferrum chloride and chloric acid were purchased from POCh, Poland.

Plants

Specimens of reed canary grass (*Phalaris arudinacea*) were taken from a botanical garden (Institute of Biology and Environmental Protection, University of Łódź). Plants were transferred to plastic containers with soil and cultivated at 25°C with a 16-h photoperiod. After cultivation the culms with 5 leaves were cut off and rooted in water. The rooted culms were put into pots containing soil and were grown at 25°C with a 16-h photoperiod. After 3 weeks the soil was treated with 0.5 mg/kg of 2,4,5-trichlorophenol - the compound was dissolved in 1 ml of ethanol and then in deionized water. The above concentration was set as the amount of 2,4,5-TCP, which significantly changed the investigated biochemical parameters.

Plants which grew in the same conditions in soil without 2,4,5-TCP were used as controls. The leaves of the plants were harvested after 3, 6 and 12 days of the experiment.

Measurement of Shoot Height and Fresh/Dry Mass of Leaves

Shoot height was measured after 12 days both in control plants and plants exposed to 0.5 mg of 2,4,5-trichlorophenol spiked to 1 kg of soil. Ten shoots were measured both for control and chlorophenol-exposed plants. The fresh and dry weight of leaves (n = 10) was evaluated for both 12-day control plants and plants exposed to 2,4,5-TCP.

Hydrogen Peroxide Content

Hydrogen peroxide was determined as described by Messner and Boll [19]. Leaves (0.5 g) were homogenized in a prechilled mortar with 3 ml of 0.1 M K-phosphate buffer, pH 7.0, containing 10% polyclar. For H_2O_2 determination, 50 µl horseradish peroxidase dissolved in a 0.1 M K-phosphate buffer (60 U/ml) and 50 µl 50 mM ABTS (2,2'-Azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt solution were added to a 1.5 ml extract. Absorbance at 415 nm was measured after 3 min, and compared to a standard curve consisting of a freshly prepared H_2O_2 solution in a 100 mM K-phosphate buffer (pH 7.0). Hydrogen peroxide concentration was expressed in µM H_2O_2 per g^{-1} fresh weight.

Catalase and Guaiacol Peroxidase

Catalase and guaiacol peroxidase were obtained by the homogenization of leaf samples (0.5 g) with 0.1 M TRIS-HCl (pH 7.8) containing 1 mM of EDTA and 0.2 mM of PVP. Then the samples were centrifuged at 12,000 rpm at the temperature of 4ºC for 15 min.

Catalase activity was assayed according to the procedure of Aebi by monitoring the rate of hydrogen peroxide decomposition in a reaction mixture containing 50 mM of potassium phosphate buffer, 0.3 M H_2O_2 and 50 µl of the sample. The consumption of hydrogen peroxide was measured as a decrease in the absorbance at 240 nm [20].

The reaction mixture formed for determination of guaiacol peroxidase contained 0.02 M guaiacol, 50 mM acetate buffer (pH 5.6), 0.06 M H_2O_2 and 10 µl of the sample. The increase in the absorbance at 475 nm was recorded following the formation of the tetra-guaiacol product. The activity of the catalase and guaiacol peroxidase was calculated in mmoles per 1 min. per 1 mg of protein and expressed in units of an individual enzyme activity per 1 mg of protein.

Phenolic Compounds

Free phenols were determined by the homogenization of 1 g of leaves in 6 ml of methanol. The obtained sample was centrifuged at 4,000 rpm for 15 min. The supernatant was separated and the pellet was washed using 3 ml of methanol and centrifuged once again. The supernatants were combined and used for the determination of free phenols. 4 ml of distilled water and 0.5 ml of Folin reagent (dissolved in 3% SDS) were added to 2 ml of ethanol solution. The mixture was incubated at 21ºC for 5 min. Then 1 ml of 5% sodium carbonate was added and samples were incubated at 21ºC for 1 h. Finally, the absorbance of the samples at 660 nm was recorded.

Total and Oxidized Ascorbate

Ascorbate was determined with α, α'-bipirydyl [21]. Leaves (0.5 g) were homogenized with 0.5 ml of 10% TCA. The samples were frozen for 15 min. and then centrifuged at 10,000 rpm for 5 min. The mixture for determination of ascorbic acid contained 0.3 ml of 0.2 M phosphoric buffer (pH 7.4) and 0.1 ml of the leaf extract. To determine the total ascorbate, 0.1 ml of leaf sample was mixed with 0.2 ml of phosphoric buffer (pH 7.4) and 0.05 ml of 10 mM dithiotretiol. After 15 min. 0.05 ml of 0.5% maleimide ethyl was introduced to the sample to eliminate the excessive amount of dithiotretiol. The colour was developed after the addition of 0.2 ml trichloroacetic acid (10%), 0.2 ml of phosphoric acid (44%), 0.2 ml of 4% bipirydyl (dissolved in 70% ethanol) and 0.1 ml of 3% $FeCl_3$. The samples were incubated at 37°C for 1 h and measured at 535 nm. The calculation of the total ascorbate and ascorbic acid concentrations was based on the standard curve calibrated for sodium ascorbate as a standard.

Total and Oxidized Glutathione

Glutathione was measured using the Griffith method. The reaction was based on the formation of dithio-bis(2-nitro)benzoic acid, which was proportional to the concentration of acid-soluble thiols, mainly glutathione [22]. Plant material – 0.5 g of leaves was homogenized in 2.5 ml of 5% trichloroacetic acid. The obtained extract was centrifuged at 4,000 rpm at 4ºC for 20 min. Then 0.5 ml of supernatant was taken and neutralized by adding 0.1 ml of 0.3 M TRIS dissolved in 2 M KOH. The sample was divided – to one part 5 µl of 2-vinylpiridine was added and then incubated for 60 min. to determine the oxidized glutathione, and the second part was left to determine total glutathione. For the determination of the total and oxidized glutathione, 50 µl of the sample was taken and dissolved in 1 ml of 0.125 M of potassium phosphate buffer (pH 7.5) that contained 0.2 mM NADPH, 0.6 mM dithio-bis(2-nitro)benzoic acid (DTNB) and 6.3 mM of EDTA. The mixture was incubated at 30ºC for 2 min and the next 10 µl of glutathione reductase (50 U/ml) was added. Finally, the samples were measured at the wavelength of 412 nm. The concentration of glutathione in the samples was calculated from extinction of the coefficient for DTNB (13,600 M^{-1} cm^{-1}) and expressed as mmol in a gram of leaf tissue.

Table 1. Fresh weight, dry weight of leaves (n = 10), and shoots length (n = 10) of the specimens of reed canary grass non-exposed (control) and exposed to 2,4,5-trichlorophenol.

Sample	Fresh Weight (g)	Dry Weight (g)	Shoots length (mm)
Control	0.936 ± 0.104	0.062 ± 0.008	344 ± 23
2,4,5-Trichlorophenol	0.876 ± 0.096	0.057 ± 0.007	338 ± 20

Glutathione S-Transferase

Glutathione transferase (GSTs) activity was determined using the Navari-Izzo and Izzo method [23]. Plant material – 0.5 g of leaves was homogenized in 5 ml of 0.1 M of potassium phosphate buffer (pH 7.0) that contained 1 mM of EDTA and 0.2 mM PVP. The extract was centrifuged at 8,000 rpm at 4ºC for 20 min. Then 0.1 ml of sample was added to 3 ml of 0.1 M phosphoric buffer (pH 7.0) that contained 2 mM of 1-chloro-2,4-dinitrobenzene and 2 mM of reduced glutathione. The increase in the absorbance was recorded at 340 nm for 7 min. in a Specord M40 spectrophotometer. A molar extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ for the complex CDNB-GSH was used and expressed in units of enzyme activity per 1 mg of protein.

Protein Determination

Protein concentration was determined according to the Bradford method [24] using bovine serum albumin as the calibration standard.

Statistical Analysis

For statistical analysis a Student's paired t-test was used, which was obligatory for data presented in this work because of interindividual differences. The difference was considered to be significant for $P < 0.05$ and $P < 0.01$. The mean value was calculated from 5 to 6 individuals, whereas for each individual an experimental point was a mean value of three replications.

Results

The differences in shoot heights of control and 2,4,5-TCP-exposed plants were observed, but they were not statistically significant. The growth reduction was also evaluated by the measurement of the fresh and dry weights of the leaves in 12-day-old control plants and the leaves in plants exposed to 2,4,5-trichlorophenol. The obtained results showed statistically insignificant differences in the mass of fresh and dry weight between the leaves of control and 2,4,5-TCP-exposed plants. (Table 1)

An increase in hydrogen peroxide was statistically significant in plants exposed to 2,4,5-TCP. The content of H_2O_2 in the leaves of reed canary grass increased after 3 days and its rise was intensified on the sixth and twelfth days of the experiment (Fig. 1). The increase in hydrogen peroxide levels may prove that the observed changes in antioxidative parameters were linked to an oxidative burst provoked by 2,4,5-TCP in the leaves of the reed canary grass.

Catalase (CAT) is one of the main enzymes that eliminates hydrogen peroxide from cells. The results showed an increase in the activity of catalase on the third and twelfth days of the experiment. But on the sixth day of the test a decrease in enzyme activity was observed (Fig. 2). The decrease in CAT activity on the 6th day was probably compensated for by the rise in the guaiacol peroxidase activity that was the highest at the same time. A statistically significant increase in the activity of guaiacol peroxidase was also observed on the third and twelfth days of the exposure of reed canary grass to 2,4,5-TCP (Fig. 3). Phenolic compounds, due to their antioxidative properties, may neutralize reactive species in plant tissues. The levels of free phenols were considerably elevated and gradually intensified during the time of exposure of the plants to 2,4,5-trichlorophenol (Fig. 4). The increase in the content of total phenols and the rise in the activity of guaiacol peroxidase may prove that phenolic compounds were used by this

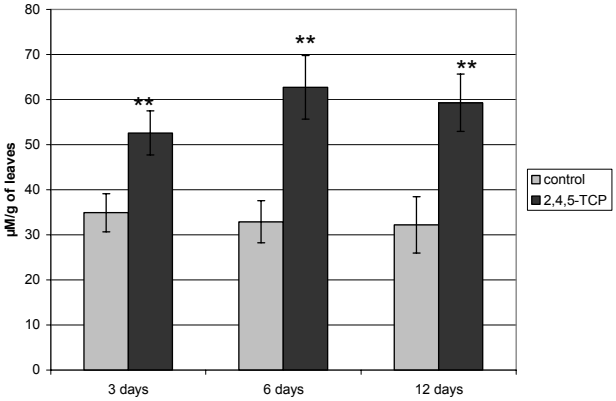

Fig. 1. Changes in hydrogen peroxide content in the leaves of reed canary grass exposed to 2,4,5-TCP.
Significantly different from control (**P < 0.01).

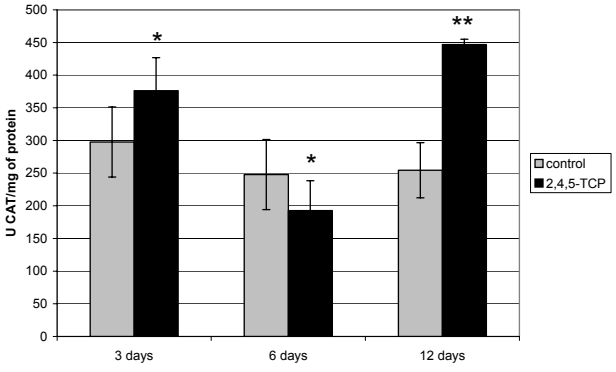

Fig. 2. Changes in catalase activity in the leaves of reed canary grass exposed to 2,4,5-TCP.
Significantly different from control (*P < 0.05; **P < 0.01).

enzyme as the substrates in oxidation reactions. The content of the total ascorbate was elevated between the third and twelfth days of the experiment and was mainly expressed by the rise of its reduced form. Thus, the increase in the ASA/DHA ratio was favourable for the plants exposed to 2,4,5-TCP activity (Fig. 5). The total content of glutathione was elevated during the whole time of the experiment, with its highest increase on the sixth day of the exposure of the plants to 2,4,5-trichlorophenol. An increase in the oxidized, and in particular the reduced form of glutathione, was noted on the third and sixth days of the experiment, so that the advantageous GSH/GSSG ratio was maintained for the investigated plant (Fig. 6). Glutathione S-transferase (GST) plays an important role in the conjugation of xenobiotics with glutathione and/or the elimination of free radicals including peroxides. The activity of glutathione S-transferase increased considerably after three and six days of the exposure of reed canary grass to 2,4,5-TCP activity, which may suggest the conjugation of this xenobiotic with glutathione (Fig. 7).

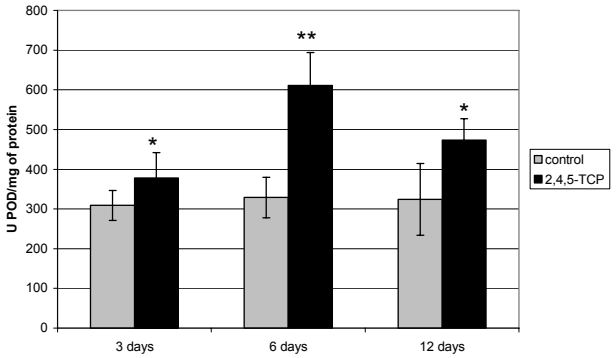

Fig. 3. Changes in guaiacol peroxidase activity in the leaves of reed canary grass exposed to 2,4,5-TCP.
Significantly different from control (*P < 0.05; **P < 0.01).

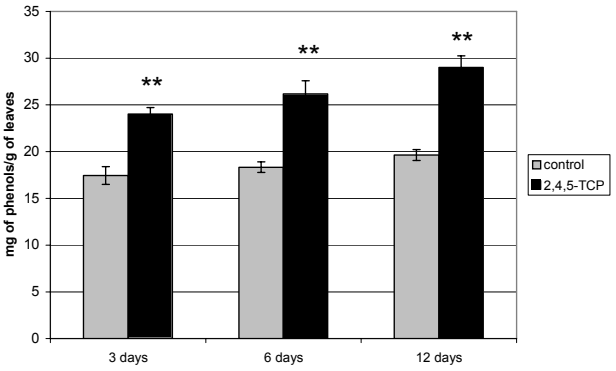

Fig. 4. Changes in free phenols content in the leaves of reed canary grass exposed to 2,4,5-TCP.
Significantly different from control (**P < 0.01).

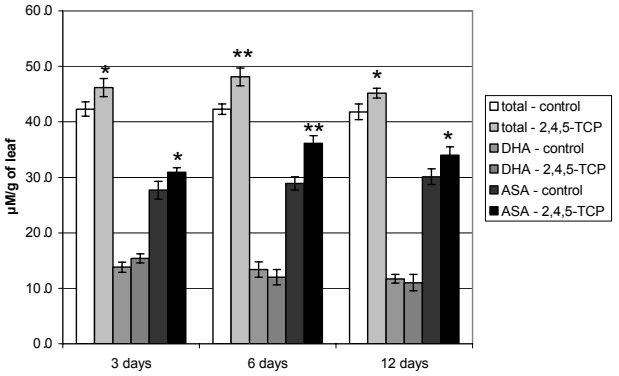

Fig. 5. Changes in ascorbate content in the leaves of reed canary grass exposed to 2,4,5-TCP.
Significantly different from control (*P < 0.05; **P < 0.01).

Discussion of Results

Hydrogen peroxide is an important reactive oxygen species due to its high reactivity, long half-life and high stability in the cell environment [25]. An increase of hydrogen peroxide content is usually related to oxidative stress occurring in plants as a result of the influence of environmental factors, including organic xenobiotics [26]. Thus a statistically significant increase of H_2O_2 concentration in plants exposed to 2,4,5-TCP observed in our work (Fig. 1) may be related to an oxidative burst provoked by this toxin in the leaves of reed canary grass.

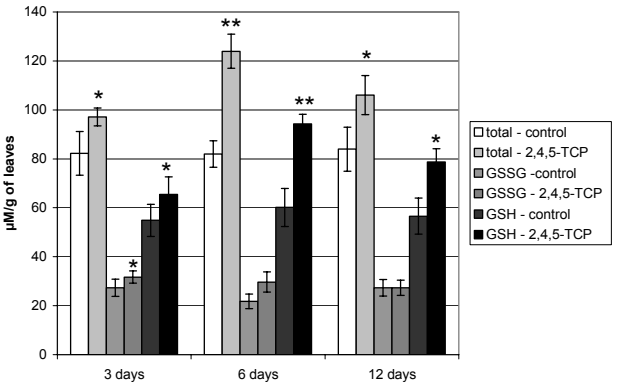

Fig. 6. Changes in glutathione content in the leaves of reed canary grass exposed to 2,4,5-TCP.
Significantly different from control (*P<0.05; **P<0.01)

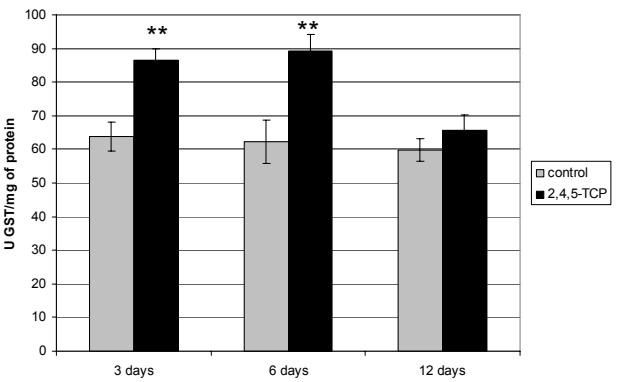

Fig. 7. Changes in glutathione S-transferase activity in the leaves of reed canary grass exposed to 2,4,5-TCP.
Significantly different from control (**P < 0.01).

Catalase is an enzyme that converts hydrogen peroxide to water. This enzyme rapidly destroys a vast majority of H_2O_2 produced in peroxisomes during photorespiration and formed as a result of mitochondrial electron transport, but it allows low steady-state levels to persist presumably to maintain redox signalling pathways [27]. In this work an increase in the activity of CAT in the leaves of reed canary grass exposed to 2,4,5-TCP after 3 and 12 days of the experiment was observed and was probably related to the overproduction of hydrogen peroxide in cells. Similarly, an increase in CAT activity was observed in plants exposed to paraquat (herbicide) which formed ROS, including hydrogen peroxide in cells [28] and plants exposed to a high concentration of natruim chloride, which generates considerable amounts of reactive oxygen species, including H_2O_2. A decrease in catalase on the sixth day (Fig. 2) of the experiment seems to be more difficult to explain; however, it has been revealed that some aromatic compounds may decrease the activity of this enzyme [29].

Peroxidase (POD) is one of the most abundant enzymes with a broad action spectrum. It is involved in substrate oxidation, cell-wall lignifications, photosynthesis, respiration and growth regulation [30]. The enzyme activity is usually changed in response to environmental stress. For example, temperature and toxic metals affect peroxidase activities. In this work an increase in POD activity was observed between the third and twelfth days of the experiment (Fig. 3). The highest activity of POD was observed on the sixth day of exposure of plants to 2,4,5-TCP and might have compensated for the decrease in CAT activity at this time. Herman et al. (1998) observed an increase in guaiacol peroxidase activity under the influence of the aromatic herbicide 1,10-phenantroline in the leaves of some species of plants [31]. Moreover, a heightened activity of this enzyme has been found in aquatic macrophytes and was related to water pollution by pulp and paper-mill effluents, which usually contain chlorinated phenols and their derivatives [32]. An increase in the phenolic compounds content in the plant response to climatic stresses and pollution has been demonstrated [33], but in the case of toxic aromatic contaminants, research is scarce. Phenolic compounds possess an antioxidant activity mainly due to their redox properties, which can play an important role in adsorbing and neutralizing organic radicals and reactive oxygen species [34]. In our work a considerable increase in the concentrations of free phenols (progressing with time of exposure of plants) under the influence of 2,4,5-TCP was noted (Fig. 4), which might have resulted from the formation of excessive amounts of free radicals in the leaves of reed canary grass.

Ascorbate (ASA) is the most abundant antioxidant in plants and serves as a major contributor to cell redox state (ASA/DHA ratio). ASA is thought to represent the first line of defence against potentially damaging external oxidants like ROS, and may play an important role in mediating response to a stress-generating enhanced oxidative burden [35]. Our results show that an increase in the total content of ascorbate occurred between the third and the twelfth days of the experiment (Fig. 5), which may show that prooxidative changes had been provoked by the 2,4,5-trichlorophenol in leaves of reed canary grass. Plants can sense shifts not only in the total amount but also in the ASA/DHA ratio, and this may be important in the perception of potentially stressful situations as well as in the modulation of compensatory defence responses. An increase in the ASA/DHA ratio in plants usually results in a decreased sensitivity to oxidative agents and thus a decreased oxidative burden caused by a variety of abiotic and biotic stresses [27]. The considerable increase in the reduced form of ascorbate noted in the leaves of reed canary grass was thus favourable for plants, as it protected them from oxidative action exerted by 2,4,5-TCP.

A reduced form of glutathione (GSH) is the most abundant non-protein thiol in cells. Due to the chemical reactivity of the thiol group of cysteinyl residue, a broad range a biochemical functions has been attributed to GSH [36]. It plays a crucial role in protecting plants against various environmental stresses as an important component of antioxidant defence reactions and it is a substrate to glutathione S-transferases for conjugation with various xenobiotics [37]. In our investigations an increase in the content of total glutathione in the whole time of the experiment (Fig. 6) was observed, which may be linked to the overproduction of ROS and/or organic radicals in the cells of the leaves of reed canary grass. The ratio between reduced (GSH) and oxidized glutathione (GSSG) is important in the adaptation of plants to the polluted environment. A high GSH/GSSG ratio is necessary to sustain the role of glutathione as an oxidant and its decrease is unfavourable for adaptation to environmental stress [15]. Our results showed that the changes were more related to the reduced than to the oxidized form of glutathione and, in consequence, the GSH/GSSG ratio was not lowered and the high redox potential was maintained.

The raised GST activity is suggested to be a marker for herbicide resistance [38]. It is possible that chlorophenols may induce GST directly or indirectly by toxic organic radicals, ROS or through lipid peroxidation. GST may be involved in the neutralization of xenobiotics, as well as oxidation products (organic radicals) by their conjugation [39]. An increase in GST activity was observed in reed canary grass treated with 2,4,5-TCP. Relatively high changes in GST activity after 3 and 6 days (Fig. 7) and the changes in redox state of glutathione might suggest that the defence of plants was related both to a direct elimination of ROS and probably also to the conjugation of phenolic xenobiotic by this enzyme.

Conclusions

In conclusion, an increase in the activity of catalase, guaiacol peroxidase and glutathione S-transferase as well as the contents of free phenols, ascorbate and glutathione was observed in the leaves of reed canary grass exposed to 2,4,5-trichlorophenol.

The induction of the antioxidative system and also elevation of the content of hydrogen peroxide, showed that the observed changes were provoked by free radicals formed as a result of 2,4,5-TCP activity on reed canary grass.

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