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Set of Tests for Chlorpyrifos Toxicity Screening

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Abstract. A set of test systems/endpoints for chlorpyrifos screening has been proposed in the present research. The set includes *Myzus persicae*, rats, *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae*, and several endpoints: aphids' mortality; mitotic index; chromosomal aberrations and micronuclei on rats; spot-test, clonal assay, induction of superoxide anions, Zimmermann's test, test of "visible" mutant colonies, CFGE on microalgae and yeast. The aim of the present study was to evaluate the reliability of the proposed by us set of test systems/endpoints for chlorpyrifos toxicity screening. Chlorpyrifos (CPF) treatment was for 5 sec with concentrations in the range: 5 – 10000ppm on aphids; 25.6ppm on rats; for 30 min with 6.5 to 100ppm on *Chlamydomonas reinhardtii* and 100 – 10000ppm on *Saccharomyces cerevisiae*. Dose-dependent mortality of aphids was found. Further, a 2-fold reduction in the mitotic index, about 7-fold increase in chromosomal aberrations, and about 4-fold increase in the total number of micronucleated polychromatic erythrocytes were measured in rats. The LD_{50} values for aphids, *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae* were calculated - 31.5, 36.56 and 66.05 ppm, respectively. The mutagenic potential was expressed mainly of low size and pigment mutations in *Chlamydomonas reinhardtii*, and reverse point mutations in *Saccharomyces cerevisiae*. A correlation between the recombinogenic and pro-oxidant activity of CPF in yeast was found. The cytotoxic, DNA damaging and mutagenic activity did not follow the dose response model in yeast. Based on our data, CPF possesses clastogenic effect on rats, and pro-oxidative, cytotoxic and recombinogenic effect on *Saccharomyces cerevisiae*. Aphids and *Chlamydomonas reinhardtii* are found to be the most susceptible to CPF. Experimental evidence supporting the suggestion that CPF damages photosynthetic pigments and chloroplasts DNA in algae. It could be concluded that the application of the proposed set of test systems/endpoints could provide concise information concerning the genotoxicity of chlorpyrifos.

Key words: Chlorpyrifos; *Myzus persicae*; Wistar rats; *Chlamydomonas reinhardtii*; *Saccharomyces cerevisiae*; toxicity.

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Introduction

Chlorpyrifos (CPF; O, O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate)) is widely applied broad-spectrum organophosphorus insecticide, mainly known to inhibit the acetylcholinesterase activity, to induce oxidative stress and to damage DNA (Hatami et al., 2019). Contradictory data exist concerning its effectiveness against aphids, which may be explained with a different population resistance (Rabea, 2009; Halder et al., 2007). Although, it was believed that it is safe for humans, data in literature concerning its toxicity on target and non-target organisms is contradictory. Many reports in the recent years confirm that low concentrations of CPF are toxic for the aquatic organisms (discussed in Huang et al., 2020).

On the other side, significant results on animals and particularly rodents present the potential of CPF to induce oxidative damage, genotoxicity, hepatic dysfunction, immunological abnormalities, neuro-behavioral and testicular damage (Yahia and Ali, 2019; Ojha and Srivastava, 2014; Elsharkawy et al., 2014). Studies on human cells revealed that CPF induces oxidative stress and possesses genotoxic and DNA damaging properties (Gao et al., 2020; Zhao et al., 2019; Li et al., 2015). Contrary, lack of cell toxicity was observed in enterocytes, peripheral blood mononuclear cells and hepatic cells exposed to CPF concentrations up to 100 µM (Tirelli et al., 2007; Oostingh et al., 2009).

Further, although CPF is considered as a non- or low-toxic to plants, several studies reported that CPF possesses phytotoxic properties presented as changes in growth, number of pods, mito-inhibitory and genotoxic effect, oxidative stress and lipid concentration (Mercado & Bayona, 2019; Fernandes et al., 2018; Sinha & Kumar, 2014).

Thus, based on these data, several regulations exist. The Priority substances under the Water Framework Directive for the protection of aquatic ecosystems (Directive 2013/39/EU) lists CPF as a priority chemical pollutant. The US Environmental Protection Agency classifies it as a moderately toxic agent (Li et al. 2015). CPF as an ingredient of variety of insecticides is banned for use in China, Hawaii and California (Huang et al., 2020). Following the regulations of the European Commission, the insecticide has been forbidden for use and should be withdrawn from the markets since 10 January 2020 (EFSA, 2019). Although, many countries reduced the use of CPF, it still remains one of the most popular insecticides. Many kinds of research on various test organisms and endpoints have been performed, but they provide partial information for the mode of action of this insecticide.

Thus, there is a great need to develop set of tests with different resolution, which may provide fast and accurate information for the potential toxic effect of pesticides on different levels – organismal, cellular, sub-cellular, and molecular.

The present study aims to evaluate the reliability of the proposed by us set of test systems/endpoints for genotoxicity screening. One of the most commonly used pesticides – chlorpyrifos was chosen for our purpose. The proposed by us set of test systems includes target and non-target organisms.

Based on the contradictory data in literature concerning toxic effect of CPF on aphids, the green peach aphid *Myzus persicae* was chosen as a target organism due to their long-term pest control treatment with CPF. Studies on these aphids are important because of several reasons: it is considered as the most economically important aphid crop pest (Bass et al., 2015); these aphids are globally spread and cause direct and indirect damages on plants, which may lead to huge economic losses to the food production (Blackman & Eastop, 2000; 2006); they become resistant to almost all classes of insecticides; these aphids transmit various plant viruses such as the potato virus Y; the

potato leafroll virus and several mosaic viruses (Bass et al., 2014; 2015).

Further studies were focused on the effect on weanling male Wistar rats. Tests performed in this work are recommended by the Organization for Economic Cooperation and Development (OECD) and widely applied in the environmental toxicology risk assessment providing relatively fast results.

Additionally, two unicellular organisms were also chosen – *Saccharomyces cerevisiae* and *Chlamydomonas reinhardtii*. These model systems have some benefits in genotoxicity testing such as fast and valuable results, inexpensive laboratory equipment and consumables (Chankova et al., 2014; Todorova et al., 2015a). *S. cerevisiae* is a suitable model for genotoxicological studies due to the high similarity with the mammals in different stress response pathways (discussed in Todorova et al., 2015a). *C. reinhardtii* is considered as a robust model for plant cell (Chankova et al., 2014), providing also evidence for genotoxicity of various pesticides in the water environment (Taylor et al., 2016).

Material and Methods

Organisms. The biological activity of CPF was tested on a target organism – laboratory reared aphids *M. persicae*. Tests for toxicity evaluation on non-target organisms were performed on *C. reinhardtii* (WT) and *S. cerevisiae* strain D7ts1.

Aphicidal activity. The aphid mortality was used as an endpoint. *Myzus persicae* (Sulz.) was laboratory reared and fed on radish plants (*Raphanus sativus*) for more than 30 generations without any pesticide exposure in a Growth Chamber GC400 at optimal 16/8 h light-dark pattern, temperature 23 ^{o}C ± 2 ^{o}C and 70% ± 5% relative humidity. *R. sativus* plants were grown at the same conditions as the aphids in the Growth Chamber GC 400. Concentration range of chlorpyrifos was: 5, 10, 25, 50 and 100 ppm based on preliminary data (unpublished data). Distilled water was used as a control. The insecticidal activity of chlorpyrifos on aphids was evaluated by „The dip leaf test method" (FAO) (FAO, 1979). The mortality was counted under magnification of stereo microscope "Zeis" 24 h hours later and the corrected mortality was recalculated by Abbott's formula (Abbott, 1925).

In vivo study

Animals and treatments. Weanling male Wistar rats (average body weight of 55±5 g) were obtained from the Animal Breeding House of the National Research Centre (NRC), Dokki, Giza, Egypt. Animals were given humane care, according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals." The Local Ethics Committee at the National Research Centre (NRC), Dokki, Giza, Egypt approved the experimental protocols and procedures.

After 7 days acclimatization to laboratory conditions, rats were randomly divided into two groups, each consisting of five rats. Group one - control, was given corn oil (1ml/kg b.wt) daily, via oral route for 28 consecutive days and adjusted weekly for body weight changes. Group 2 was given CPF at a dose 25.60 mg/kg b.wt. (1/25 LD_{50}) based on published LD_{50} (640 mg/kg b.wt.) (Tomlin, 2004).

Chromosome aberrations (CA) assay. Cytogenetic analysis was performed by the direct method of rinsing marrow of long bones, according to Adler (1984). CA was identified based on criteria established by the OECD Guideline 475, updated and adopted on 21 July 1997 (OECD, 1997a).

Mitotic index determination. Slides prepared for chromosomal aberration assay were used to calculate the mitotic index as the ratio of the number of dividing cells to the total number of cells, multiplied by 100.

The micronucleus assay. The method described by Schmid (1975) was used for analysis of micronuclei (MN) in polychromatic erythrocytes (PCEs) of rat bone marrow. The study was done in accordance with OECD Guideline 474, updated and adopted on 21 July 1997 (OECD, 1997b).

Treatment of the unicellular organisms. Cell cultures of *C. reinhardtii* (WT) with a density $1x10^6$ cells/ml and *S. cerevisiae* (strain D7ts1) with a density $1x10^6$ cells/ml in the end of exponential and the beginning of stationary phase were treated with various concentrations of chlorpyrifos (6.5-100 ppm for *C. reinhardtii* and 100-10000 ppm, for *S. cerevisiae* respectively) for 30 min. These concentrations were chosen based on our pilot, yet unpublished experiments.

Genotoxicity screening. Macro-colonies survival assay was performed to evaluate genotoxic potential of CPF. Survival fraction (SF) of *C. reinhardtii* colonies was calculated according to (Bryant, 1968).

Quantitative assay for superoxide anions in live cells. The experiments were performed as described in Stamenova et al. (2008). Briefly, after the treatment *S. cerevisiae* cells were incubated in 1xPBS with 125 μM XTT for 6 hours. The levels of superoxide anions were measured spectrophotometrically at wavelength 470 nm. The concentration of superoxide anions in live cells was calculated as described in Stamenova et al. (2008).

Measurement of DNA double-strand breaks (DSB) induction. Constant field gel electrophoresis (CFGE) was performed as described in Chankova and Bryant (2002), Chankova et al. (2007), Todorova et al. (2015b; 2019).

Test of "visible" mutant colonies was applied to evaluate mutagenic potential of chlorpyrifos on the unicellular algae. Changes in size, morphology and pigmentation of surviving colonies were analyzed (Shevchenko, 1979; Dimitrova et al., 2007).

Zimmermann's test for simultaneous detection of mitotic gene conversion at the trp-5 locus, reversion mutations in ilv1 locus and mitotic crossing-over. Zimmermann's test (Zimmermann et al., 1984) with *Saccharomyces cerevisiae* diploid strain D7ts1 (*MATa/a ade2-119/ade2-40 trp5-27/trp5-12 ilv1-92/ilv1-92 ts1/ts1*) was applied as described before (Todorova et al., 2015a).

Statistical analysis. Data were analyzed using Graphpad Prism5 software (San Diego, USA) and the statistical analysis was done by one-way analysis of variances (ANOVA) followed by Bonferonni post-hoc multiple comparisons test. Linear correlation, using Pearson Product-Moment Correlation Coefficient analysis (PMCC, or r) and coefficient of determination (R^2) were determined.

Results and Discussion

Effect of chlorpyrifos on aphids

Data revealed that CPF is highly toxic to aphids *M. persicae*. Concentrations equal or higher than 100 ppm resulted in 100% mortality (Fig. 1). LC_{50} was calculated to be 31.5 ppm. Our data provide evidence for the high aphicidal activity of CPF. There is a contradictory data in literature concerning the toxic effect of CPF on *M. persicae*. Halder et al. (2007) reported relatively high aphids resistance with LC_{50} =1640 ppm in the Direct Spray method and 1060 ppm – in the Leaf residue method. Conversely, Rabea (2009) calculated LC_{50} to be 12.24 ppm. Such significant variation in the values could be explained with different population resistance. Bass et al. (2015) reported that *M. persicae* could be characterized with at least seven independent mechanisms of resistance by which they are able to avoid or overcome the toxic effect of insecticides. In our study, laboratory reared aphids were used so they were not in contact with any other insecticides.

Effect of chlorpyrifos on rats

Next experiments were performed on rats. The mitotic index was used in order to determine the rate of cell division. The status of mitotic index evaluated as a percentage of dividing cells revealed around 2-fold reduction, indicating cytotoxic potential of CPF (Fig. 2).

Such result corresponds well with the suggestion that the organophosphate pesticides inhibit mitosis probably by blocking mitotic cycle during interphase (Sinha and Kumar, 2014).

Chromosomal aberrations in rat bone marrow

Table 1 presents chromosomal aberrations induced in rat bone marrow cells after the treatment with CPF.

The results show that the tested CPF dose (25.60 ppm) can induce a statistically significant increase in the percentage of CA in bone marrow cells – around 7-fold.

Induction of micronuclei in rat bone marrow PCE

Around 4-fold statistically significant increase in the total number of bone-marrow micronucleated polychromatic erythrocytes (MnPCE) was calculated after the treatment with CPF (Table 2). These data show that the tested insecticide CPF possesses clastogenic capacity. Our results are in a good correspondence with the ones reported by Yahia & Ali (2019). Serpa et al. (2019) provided evidence that concentrations corresponding to 35 ppm are able to induce around 4-fold higher levels of micronuclei in human leukocytes. Some authors reported that the primary induced DNA damages by CPF may be due to the generation of oxidative stress (Mužinić et al., 2019; Ojha & Srivastava, 2014).

Effect of chlorpyrifos on Chlamydomonas reinhardtii – model for plant cell

Data revealed dose-dependent decrease in the cell survival (Fig. 3A). The lethal dose causing 50% mortality (LD_{50}) was calculated to be 36.56 ppm.

Data available in literature reveal that CPF cytotoxicity is high in microalgae when prolonged treatments are performed: *Chlorella pyrenoidosa* - 29.64 ppm (24h) to 11.46 ppm (72h) and from 27.80 ppm (24h) to 25.80 ppm (72h) in *Merismopedia* sp. (Chen et al., 2016). Our results provide new evidence for the acute toxicity within 30 min treatment.

Following the EU-Directive 93/67/EEC (Commission of the European Communities, 1996) concerning the different toxic classes according to their EC50-values, CPF fall into the class "10-100 mg/L (harmful to aquatic organisms)" for *C. reinhardtii*. Our data confirm that classification.

Next, in order to examine other damages related to the high genotoxicity, the mutagenic potential was evaluated. Data revealed no correspondence between high genotoxicity and the mutagenicity. Results obtained are in a support of concentrations dependent mutagenic effect of CPF – weak mutagenic potential of 50 ppm, and strong mutagenic activity of 100 ppm (Fig. 3B). No statistically significant mutagenic effect was obtained using doses lower than 50 ppm. Interestingly, small-sized or pigment mutant colonies were the only mutant type observed suggesting possible inhibition of cell division, damages in the chloroplast DNA and pigment content (Shevchenko, 1979).

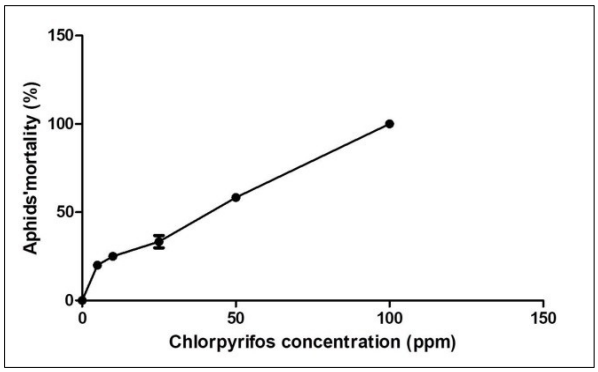

Fig. 1. Aphids' mortality after the treatment with various concentrations chlorpyrifos in the range of 5 – 100 ppm. Where no error bars are evident, they are equal or less than the symbols.

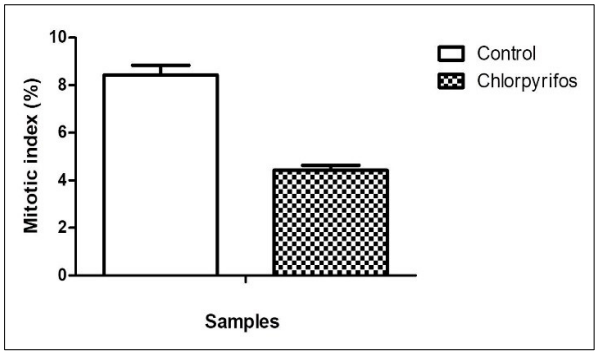

Fig. 2. Mitotic index calculated after the application of chlorpyrifos. Where no error bars are evident, they are equal or less than the symbols.

As CPF inhibit the cell division in rats, the small size mutant colonies obtained on *C. reinhardtii* confirm once again this mechanism

of action. Further, Chen et al. (2016) discussed that although, organophosphorus insecticides are considered low toxic to plants, data exist that they can damage the photosynthetic pigments (Chen et al., 2016). Our data concerning increased level of pigment mutant colonies that are considered as a result of damages in both chloroplasts DNA and photosynthetic apparatus of *C. reinhardtii* are in agreement with this finding and could be serving as a suitable marker for an evaluation of the potential genotoxic effect of organophosphorus pesticides on plants.

DNA damaging capacity of CPF was evaluated based on the DSBs induction. DSB levels measured in treated samples were comparable with those in the control - untreated cells (Fig. 4). No correspondence between cell survival and DSB induction was found. Our finding might be due be to specific mode of action of CPF in plants. Chen et al. (2016) has suggested that CPF can damage chloroplast DNA.

Table 1. Effect of chlorpyrifos on chromosomal aberrations in rat bone marrow cells. *Legend:* Value is mean ± S.E.; n = 5 rats/group. Values are shared the same superscript letters not differ significantly at $p < 0.05$.

Group	Percent chromosome aberrations				Total number of aberrant cell (%)		No. of aberrations per cell	
	Gaps	Breaks and/or Fragment	Deletions	Multiple aberrations	Including gaps	Excluding gaps	Including gaps	Excluding gaps
Control	1.02±0.01	1.46±0.06	0.18±0.001	0.21±0.002	2.87±0.08^{b}	1.85±0.014^{b}	0.0287±0.0001^{b}	0.0185±0.0001^{b}
CPF	4.32±0.06	5.87±0.08	0.97±0.005	6.58±0.008	17.74±0.53^{a}	13.42±0.04^{a}	0.1774±0.020^{a}	0.1342±0.010^{a}

Table 2. Clastogenic potential of chlorpyrifos in the bone marrow measured as induction of micronuclei in bone-marrow micronucleated polychromatic erythrocytes. Legend: Value is mean ± S.E.; n = 5 rats/group. The number of the scored cells was 2000 cells/ animal. Mn: micronucleus, MnPCE: micronucleated polychromatic erythrocytes, PCE: polychromatic erythrocytes.

Group	No. of micronucleated polychromatic erythrocytes (MnPCE)			
	PCE with one Mn	PCE with two Mn	PCE with more than two Mn	Total MnPCE
Control	5.60±0.074	0.60 ± 0.001	0.20 ± 0.001	6.40 ± 0.14^{b}
CPF	17.4 ± 0.18	7.80 ± 0.19	3.20 ± 0.11	28.40 ± 1.76^{a}

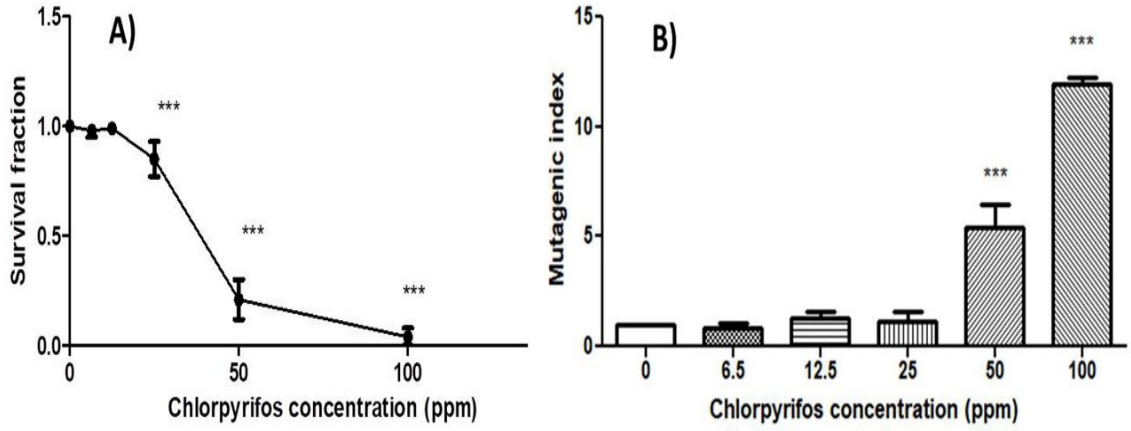

Fig. 3. Survival fraction (SF) of *C. reinhardtii* treated with chlorpyrifos at concentrations'

range from 6.5 to 100 ppm (A) and mutagenic activity of CPF presented as mutagenic index (B). Where no error bars are evident, they are equal or less than the symbols.

Effect of chlorpyrifos on Saccharomyces cerevisiae – model for animal cell

The effect of CPF was also evaluated on *S. cerevisiae*. Based on the results for the cell survival, LD_{50} was calculated to be 66.05 ppm (Table 3).

Further, the pro-oxidant potential was also evaluated. Data revealed induction of superoxide anions in yeast after the treatment with all the concentrations (Table 3). Statistically significant dose-dependent increase was calculated. Around 100-fold increase in the ROS levels was observed after the treatment with the highest tested concentration.

CPF at all the concentrations tested was also found to induce reverse mutations in *S. cerevisiae*, suggesting strong mutagenic effect. Interestingly, no effect of the concentration was observed after the treatment with concentrations 1000 and 10000 ppm suggesting possible reach of a threshold. Further, CPF was found to possess well-expressed recombinogenic effect increasing the mitotic gene conversion in a dose-dependent matter. On the other side significant increase in the percentage of total aberrants was observed after the treatment with the highest concentration tested – 10000 ppm (Table 3).

Next, the DSB induction was studied. Interestingly, all the concentrations tested resulted in around 2.5-fold increase of DSB levels (Fig. 5). No statistical differences among the concentration' effect was calculated. These results indicate that the DSB induction is not related to the dose range used by us for CPF.

The results reported in table 4 revealed that statistically significant positive correlation exist only among the increase in ROS levels with this in mitotic gene convertants ($P < 0.05$) and total aberrants ($P < 0.01$).

Table 3. Frequency of survival fraction, gene conversion in *trp5* locus, reversion in *ilv1-92* allele and mitotic crossing-over in *ade2* locus, induction of superoxide anions after the treatment of *S. cerevisiae* D7ts1 with various concentrations chlorpyrifos. *Legend:* Values are mean ± SD from at least three independent experiments.

CPF (ppm)	Survival fraction[1]	Convertants/ 10^5 cells[1]	Revertants/10^6 cells[1]	Aberrants (%)[1]	ROS pM/cell[1]
0	1 ± 0	0,20 ± 0,08	0,011 ± 0.01	0,023 ± 0,012	0.003 ± 0.001
100	0.515 ± 0.058***	0.81 ± 0,05^{ns}	0.042 ± 0.005^{ns}	0.018 ± 0.008^{ns}	0.024± 0.001***
1000	0.123 ± 0.028***	1.65 ± 0.41*	0.406 ± 0.083***	0.047 ± 0.013*	0.036± 0.002***
10000	0.072 ± 0.011***	5.26 ± 1.41***	0.433 ± 0.062***	0.287 ± 0.018***	0.297 ± 0.004***

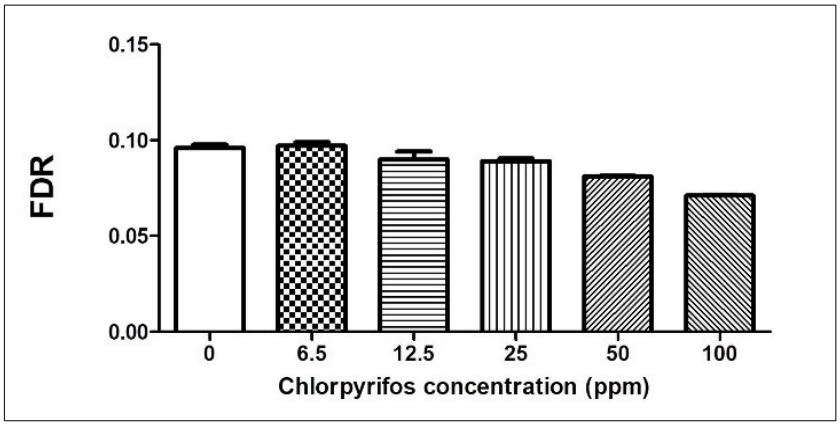

Fig. 4. Induction of double-strand breaks in *C. reinhardtii* treated with chlorpyrifos at concentrations' range from 6.5 to 100 ppm. Where no error bars are evident, they are equal or less than the symbols.

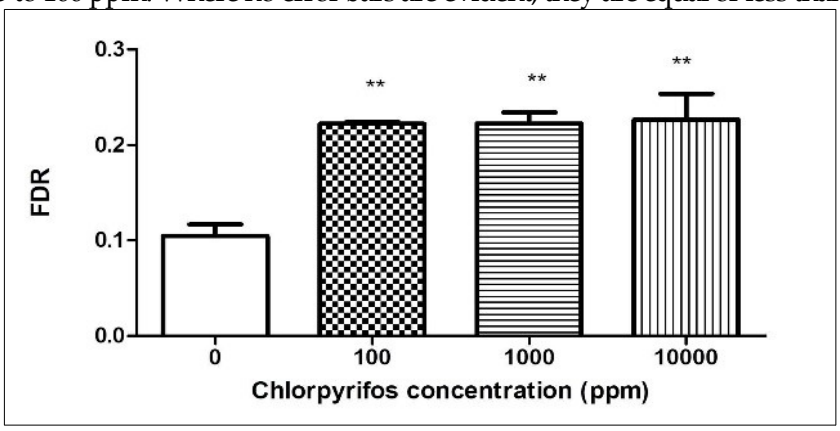

Fig. 5. Induction of double-strand breaks in *S. cerevisiae* treated with chlorpyrifos at concentrations' range from 100 to 10000 ppm. Where no error bars are evident, they are equal or less than the symbols.

Table 4. Correlation analysis of the genetic events induced after the treatment of *Saccharomyces cerevisiae* with various concentrations chlorpyrifos. *Legend:* Values represent the R^2 for linear correlation. Correlation coefficient (R) higher than 0.900 denotes a strong positive correlation. SF: survival fraction; MGC: mitotic gene conversion; TA: total aberrants; ROS: reactive oxygen species; RM: reverse mutations; DSB: double-strand breaks.

	SF	MGC	TA	ROS	RM	DSB
SF	–	-0.748	-0.609	-0.631	-0.912	-0.897
MGC	-0.748	–	0.982*	0.986*	0.787	0.552
TA	-0.609	0.982*	–	0.997**	0.687	0.396
ROS	-0.631	0.986*	0.997**	–	0.677	0.450
RM	-0.912	0.787	0.687	0.677	–	0.638
DSB	-0.897	0.552	0.396	0.450	0.638	–

The levels of DSBs remain similar despite the concentration. Additionally, concentrations equal or higher than 1000 ppm were not found to follow the dose response pattern. Thus, it could be speculated that the recombinogenic activity of CPF could be proportionally related to the well-expressed pro-oxidant activity. The cytotoxic, DNA damaging and mutagenic activity may be due to some direct actions most probably of the CPF metabolites or by reaching threshold levels of the CPF action.

Conclusions

The present study provides experimental evidence for the sensitivity of different organisms to the toxic action of chlorpyrifos. Aphids and *C. reinhardtii* are found to be very susceptible to CPF action suggesting aphicidal and phytotoxic effect. Large scale of effects was revealed: very pronounced clastogenic in rats, genotoxic and mutagenic in *C. reinhardtii* and *S. cerevisiae*, well-expressed pro-oxidative, recombinogenic and DNA damaging in *S. cerevisiae*. Additionally, mutagenic and damaging DNA effect was observed for concentrations lower than the recommended dose. New data are provided in a support of current knowledge that organophosphorus insecticides considered as low toxic to plants, can damage photosynthetic pigments and chloroplasts DNA inducing small sized and pigment mutant colonies in *C. reinhardtii*.

In short, using proposed by us set of test systems/endpoints wide spectrum of chlorpyrifos bioactivity was revealed - aphicidal, phytotoxic, genotoxic, mutagenic, recombinogenic, clastogenic and DNA damaging. From our understanding this set of test-systems/endpoints could be successfully used in a genotoxicity screening of traditional, new synthesized and natural products. This set could be successfully applied to obtain concise information concerning the genotoxicity of different pesticides and/or natural products.

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