Ecotoxicology
https://doi.org/10.1007/s10646-018-1992-7

Biodegradation of chlorpyrifos and 3,5,6-trichloro-2-pyridinol by the epiphytic yeasts *Rhodotorula glutinis* and *Rhodotorula rubra*

E. D. Bempelou[1] · J. G. Vontas[2] · K. S. Liapis[1] · V. N. Ziogas[2]

Accepted: 8 October 2018

Abstract

The possible involvement of the epiphytic yeasts *Rhodotorula glutinis* and *Rhodotorula rubra* in the biodegradation of the insecticide chlorpyrifos and its metabolite 3,5,6-trichloro-2-pyridinol (TCP), in pure cultures and in plant surfaces (tomato fruits) was investigated. Higher biodegradation rates were observed as the concentration of chlorpyrifos and the inoculum of the microorganisms were increased, while the yeasts proved to be more active at 25 and 15 °C. The presence of glucose in the mineral nutrient medium, as an extra source of carbon, delayed the biodegradation by *Rhodotorula glutinis*, while *Rhodotorula rubra* proved to be more active. The detection and quantification of the parent compound and TCP was successfully achieved using a LC/MS/MS chromatographic system. The in vitro enzymatic assays applied suggested that esterases may be involved in the biodegradation of chlorpyrifos, a fact that was further enhanced after the addition of the synergists triphenyl phosphate, diethyl maleate and piperonyl butoxide in the biodegradation trials. The decrease of chlorpyrifos residues on tomato fruits confirmed the corresponding on pure cultures, resulting in the suggestion that the yeasts *R. glutinis* and *R. rubra* can possibly be used successfully for the removal or detoxification of chlorpyrifos residues on tomatoes.

Highlights

- Biodegradation of chlorpyrifos by *Rhodotorula glutinis* and *Rhodotorula rubra*
- Biodegradation of TCP by *Rhodotorula glutinis* and *Rhodotorula rubra*
- Triphenyl phosphate inhibited the biodegradation of chlorpyrifos

Keywords Biodegradation · Chlorpyrifos · Epiphytic yeasts · Synergists.

Introduction

The broad use of organophosphates has resulted in the contamination of the environment. The excessive and frequent application of pesticides has also resulted in high levels of pesticide residues accumulated on vegetables, a matter that poses a potential health risk to consumers (Bolognesi and Moraso 2000). Accordingly, increased interest has been given to remove or detoxify pesticide residues from vegetables, using environmental friendly approaches, such as microorganisms or recombinant enzyme systems.

Chlorpyrifos, *[O, O-diethyl O-[3,5,6-trichloro-2-pyridinyl) phosphorothioate]* is a broad spectrum organophosphorus insecticide, also been used as acaricide and termicide. It is a non systemic pesticide acting through stomach or suffocation (Tomlin 2003). The main paths of its degradation in the environment are photolysis in air (Racke 1993), hydrolysis in water (Meikle and Youngson 1978; Chapman and Cole 1982 and McCall 1986a) and hydrolysis and microbial degradation in soil (Getzin 1981a and Racke et al. 1988). The half-life of chlorpyrifos in plants has been measured from 1 to 9 days, mainly by evaporation and secondary photolysis (Bauriedel 1986a). The processes by which pesticides are transformed or degradated in

✉ E. D. Bempelou
e.bempelou@bpi.gr

[1] Department of Pesticides Control and Phytopharmacy, Pesticide Residues Laboratory, Benaki Phytopathological Institute, 8 St. Delta street, Kifissia 14561, Greece

[2] Laboratory of Pesticide Science, Agricultural University of Athens, Iera Odos 75, Athens 11855, Greece

Published online: 20 October 2018

environmental compartments and the factors that modulate the kinetics are critical from both pest control efficacy and non target organism toxicity points. Both abiotic and microbial transformation of chlorpyrifos has been found to contribute significantly to its degradation. Microorganisms possess the unique ability to completely mineralize many aliphatic, aromatic and heterocyclic compounds (Bhagobaty et al. 2007).

Generally, the microorganisms able to degrade chlorpyrifos are bacteria and fungi. Very few works have been reported on the degradation of 3,5,6-trichloro-2-pyridinol (TCP). To date the degradation of the chlorpyrifos has been reported by several bacteria belonging to the genera *Bacillus*, *Lactobacilus*, *Streptococcus*, *Pseudomonas*, *Micrococcus*, *Flavobacterium*, *Arthrobacter*; *Enterobacter*, *Alcaligenes*, *Sphingomonas*, *Paracoccus*, *Brucella*, *Klebsiella*, *Serratia*, *Sphingobacterium*, *Cupriavidus*, and also by *Ochrobactrum* and *Achromobacter* (Jones and Hastings 1981; Ivashina 1986; Shaker et al. 1988; Havens and Rase 1991; Guha et al. 1997; Mallick et al. 1999; Singh et al. 2004; Yang et al. 2005; Li et al. 2007; Xu et al. 2008, Lakshmi et al. 2008; Anwar et al. 2009; Abraham and Silambarasan 2013; Lu 2013; Abraham and Silambarasan 2016 and Akbar and Sultan 2016). Regarding fungi, *Trichoderma*, *Verticillium, Aspergillus*, *Ganoderma* and *Cladosporium* have been up to now proved to be able to breakdown chlorpyrifos (Ivashina 1986; Fang et al. 2008, Silambarasan and Abraham 2013, 2014 and Chen et al. 2012), while Lal and Lal (1987) have studied the possible degradation of chlorpyrifos (1-10 ppm) by the yeast *Saccharomyces cerevisiae*, which is the only reference on yeasts.

In the present study, the capability of the epiphytic yeasts *Rhodotorula glutinis* and *Rhodotorula rubra* to degrade chlorpyrifos and its metabolite TCP in pure cultures and tomato fruits was investigated. This is the first report on the biodegradation of chlorpyrifos and TCP by those microorganisms. Furthermore, this is a first report of investigating their bio-degradative action using synergists as well as examining the phenomenon in plant surfaces. The ultimate objective of this study was to explore the feasibility of using these microorganisms, which, are naturally present in fruits and vegetables, to detoxify chlorpyrifos residues from the plant surface and furthermore to improve food safety.

Materials and methods

Isolation of the yeasts and maintenance of pure cultures

The microorganisms examined in the present study are the epiphytic yeasts *Rhodotorula glutinis* and *Rhodotorula rubra*. Both strains were isolated epiphytically from cultivated plants. *Rhodotorula glutinis* was isolated from tomato plants phyllosphere and *Rhodotorula rubra* from the phyllosphere of strawberry plants. Both strains had been characterized by the protocols of classical microbiology, according to their macro-morphology (colony color, appearance, texture, no pigment), micro-morphology (cell shape, budding) and strain physiology (fermentation in different sugars, starch formation and assimilation growth).

Cultures were developed in mineral salts medium (MSM) containing in g/L: 12 NH_4NO_3, 8 KH_2PO_4, 2 Na_2SO_4, 4 KCl, 1 $MgSO_4{\cdot}7\ H_2O$, 0.5 $CaCl_2$, 0.26 $ZnSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7\ H_2O$ and $Na_2MbO_4{\cdot}7\ H_2O$ in 250 mL Erlenmeyer flasks. The pH was adjusted to 7.2 and cultures were incubated in an orbital incubator at 25 °C and 150 rpm.

Chlorpyrifos exposure

Cultures of yeasts were supplemented with chlorpyrifos and afterwards different parameters, namely yeast inoculum density, chlorpyrifos exposure, temperature of incubation and presence of glucose in the MSM as an additional source of carbon, were examined regarding their potential influence in the biodegradation of the insecticide, The effect of the yeast inoculum density on the degradation was examined by incubation of 10^2, 10^4 and 10^6 cells/mL of each yeast in 250 mL flasks containing 50 mL MSM and 10 µg/mL chlorpyrifos. To examine the effect of chlorpyrifos exposure on the biodegradation rates, experiments were conducted in 250 mL flasks containing 50 mL MSM supplemented with chlorpyrifos at concentrations of 10 and 25 µg/mL. The influence of temperature was tested by the conduction of degradation trials in 250 mL flasks containing 50 mL of MSM supplemented with 10 µg/mL chlorpyrifos at 15, 25 and 35 °C, respectively.

Finally, simultaneously with all the above parameters the influence of glucose in the MSM, as an extra source of carbon, was also investigated. Additional 250 mL flasks containing 50 mL MSM supplemented with glucose under each time experimental conditions tested were incubated and the influence of this factor was also estimated.

Controls were also used for the comparison of the obtained results. Uninoculated MSM with the same concentration of chlorpyrifos were used as controls. All treatments were in triplicate. Flasks were incubated in an orbital incubator at 25 °C and 150 rpm. Samplings took place immediately after treatments and at time intervals of 1-day. Aliquots of cultures were taken aseptically and analyzed for chlorpyrifos residues as described below.

Extraction of chlorpyrifos and TCP from pure cultures and tomato fruits

Two different sample preparation techniques were applied for the extraction of chlorpyrifos and TCP from pure cultures and tomato fruits.

Regarding pure cultures, subsamples of 5 mL of MSM (50 mL) were taken aseptically and filtered through a Whatman No 2 filter above a separating funnel of a 100 mL. Liquid–liquid extraction was carried out with 20 mL hexane for three times. Extracts were passed through anhydrous sodium sulfate and collected in a flat bottom flask (100 mL) and evaporated to dryness on a rotary evaporator. The dry residue was diluted to 2 mL methanol: water (30:70) and filtered through a 0.2 µm film filter to be further analyzed in the LC/MS/MS chromatographic system.

Regarding tomato fruits, an existing method (Bilthoven 1996) was used for the sample processing of tomato fruits, according to which 30 mL of acetone were added in an aliquot of 15 g of the homogenated sample in a 250 mL PTFE centrifuge bottle (Nalgene, Rochester, NY, USA) and stirred for 1 min in an ultra-turrax homogenizer at 15000 rpm. 30 mL of dichloromethane and 30 mL of petroleum ether (40–60 °C) were added following by a new stirring for 1 min. The sample was centrifuged at 4000 rpm for 2 min. 25 mL of the supernatant were evaporated to dryness on a water bath, at 65–70 °C and afterwards 5 mL of 2,2,4-trimethyl pentane/ toluene (90/10) were added. The extract was placed in an ultrasonic bath for 30 s, filtered through a 0.2 µm film filter and transferred in a vial with Teflon septa, to be further analyzed in the LC/MS/MS chromatographic system.

Chromatographic determination of chlorpyrifos and 3.3,5,6-trichloro-2-pyridinol method validation

Chemicals and reagents

The analytical standards of chlorpyrifos (98.4%) and piperonyl butoxide (92.5%) were obtained purchased from Dr Ehrenstofer (Augsburg, Germany). The corresponding of 3,5,6-trichloro-2-pyridinol (TCP) (99.1%), triphenyl phosphate (99.5%) and diethyl maleate (95%) were purchased from ChemService (West Chester, UK). Regarding solvents used, they were all obtained from Lab Scan (Dublin, Ireland). Acetone, dichloromethane, acetonitrile and hexane were of pesticide residues grade, while methanol and water of LC/MS grade and petroleum ether (40–60 °C) of analytical reagent grade. Formic acid purchased from Sigma (Greece) was also used. p-Nitrophenyl acetate (PNPA), a-naphthyl acetate, b-naphthyl acetate and 1-chloro-2,4-benzene (CDNB) were the reagents used in the enzymatic assays.

Stock solutions of 1000 and 10000 µg/mL of each analyte were made in methanol (residue analysis) or acetonitrile (enzymatic assays) and were stored in –20 °C. Composite working solutions were prepared and also stored at −20 °C. These stock solutions were used during the study for preparing the working standard solutions.

Chromatographic analysis

A Varian [2x prostar 210 (LC) and 1200 L (quadrupole MS/MS)] LC/MS/MS was used for the determination of chlorpyrifos and its metabolite 3,5,6-trichloro-2-pyridinol (TCP) with a Varian Polaris C18-A (5 cm length, i.d.2 mm and 5 µm p.s) at ambient temperature (25 ± 4 °C). Elution solvents were the mixtures of methanol/water supplemented with 1 mM $HCOONH_4$ (10/90) (Solvent A) and methanol/water supplemented with 1 mM $HCOONH_4$ (90/10) (Solvent B). A flow of 0.25 mL/min and an injection volume of 5 µL (full loop) were applied. The elution program used was gradient, starting with 90% of solvent A and 10% of solvent B, reaching the 100% of solvent B at 14 min and remaining there for 6 min and returning to its first constitution at 25 min. The MS detector was set at 1500 V voltage, ion source to 50 °C and drying gas (N_2) to 19 psi pressure and 250 °C temperature. The electrospray ionization mode was in positive mode for the determination of chlorpyrifos and in negative for TCP, using nitrogen as nebulizer gas at 50 psi in both cases. The needle voltage was 5000 V in ESI(+) and 4500 V in ESI(-), while the spray shield voltage was ± 600 V, respectively.

Method validation

The analytical methods were validated in accordance with the requirements of EU (SANTE 2015/11945). Different known concentrations of chlorpyrifos and TCP were fortified in the mineral salts medium (0.05, 0.1 and 0.5 µg/mL) and of chlorpyrifos in tomato fruits (0.05, 0.1 and 0.5 µg/mL) at least in 5 replicates for both substrates.

Enzymatic assays–synergists

Enzymatic assays

1 mL of liquid culture was added in an eppendorf tube (1.5 mL) and centrifuged in 5000 rpm for 5 min. The supernatant was removed and 1 mL of sodium phosphate buffer solution 100 mM, pH 7, was added in the tube. Following the same procedure, the final sediment was redissolved in 0.5 mL sodium phosphate buffer solution 100 mM, pH 7.2, supplemented with 0.2% Triton. The activity of esterases and glutathione-S-transferases (GSTs) was determined spectrophotometrically on an UVmax microtite plate reader, as previously described (Vontas et al. 2001). A-naphthyl and b-naphthyl acetate were used as substrates for esterases and for glutathione-S-transferases (GSTs) the corresponding was 1-chloro-2,4-dintrobenzene (CDNB) (Roditakis et al. 2009). All treatments were carried out in triplicate.

Protein was assayed by using the Bio-Rad protein assay kit (Bio-Rad, Hemel Hempstead, Herts, UK) with BSA as a standard protein (Bradford 1976).

Inhibition studies were conducted by incubation of the homogenates for 10 min with chlorpyrifos concentrations of 14.25, 28.5, 42.75 and 57 μM in the presence of acetonitrile. The remaining activity was determined in triplicate as in the standard assays mentioned above and expressed as units (U) per mg of proteins (IU = μmol of substrate hydrolyzed per minute). The IC_{50} value was calculated for each yeast, as the concentration of chlorpyrifos needed to inhibit half (50%) of the maximum activity of enzymes (esterases and glutathione-S-transferases).

Two-way ANOVA was employed, with the studied condition (control or treatment) and the concentration of chlorpyrifos (0, 14.25, 28.5, 42,75 and 57 μM) been the two factors examined. Data analysis was conducted using the statistical package JMP (SAS Institute 2008).

Addition of synergists in the biodegradation trials

The synergists triphenyl phosphate (inhibitor of esterases), piperonyl butoxide (inhibitor of monoxygonases and esterases) and diethyl maleate (inhibitor of monoxygonases and glutathione-S-transferases) were used in the biodegradation trials. 10 μg/mL of each synergist (one in a time) were added in the biodegradation flasks and the biodegradation procedure was studied.

The sensitivity of the two yeasts in the synergists had been previously examined as follows. 250 mL flasks containing 50 mL MSM were inoculated with 10^6 cells/mL under sterile conditions, supplemented with triphenyl phosphate, piperonyl butoxide and diethyl maleate (one in a time) at concentrations 1, 5, 10 and 15 μg/mL and incubated in an orbital incubator at 25 °C and 150 rpm. As observed, none of the compounds showed adverse effects in the growth of the yeasts.

Biodegradation on tomato fruits

As a first approach to estimate the possible bio-degradative activity of the yeasts in plant surfaces, trials were conducted in tomatoes. Groups of 5 tomato fruits (*Noa* variety) were washed and disinfected with an ethanolic solution 90%, sprayed with 10^8 cells/mL of yeast (one in a time) and sprayed with chlorpyrifos at 2 mg/kg 1 day later. Equivalent groups of tomato fruits were treated only with the insecticide and were used as control samples. All treatments were in triplicate. All treatments were stored in an incubator at 20 °C, with high relative humidity and 12 h photoperiod. Samples were collected at 5 and 30 days, homogenated and then analyzed as described above.

Results

Chromatographic determination and validation results

The qualitative and quantitative determination (Fig. 1) of the two analytes was successfully achieved in the LC/MS/MS chromatographic system with the technique of multiple reactions monitoring (MRM). Electrospray ionization in positive mode ESI (+) was applied for the determination of chlorpyrifos, using the ions 350 > 198 m/z and 352 > 200 m/z, while negative mode ESI (-) was considered as better for TCP with the ion transitions of 198 > 198 m/z and 196 > 196 m/z. Confirmation was based on the criteria of retention time and ion abundance of qualitative and quantitative ions according to EU Guidelines.

The analytical methods were validated by assessing the basic parameters such as sensitivity, mean recovery (as a measure of trueness) and repeatability (as a measure of precision). After the fortification of the MSM with chlorpyrifos and its metabolite TCP, the obtained recoveries ranged from 78.6 to 95.3%, values which were accepted (SANTE/11945/2015) (Table 1). Relative standard deviations (RSDs) values ranged from 6.4 to 13%, all lower to 20%, also accepted. Validation results of tomato fruits with chlorpyrifos gave an average recovery of 91.16% and a mean RSD of 8.13% (Table 1). The limit of quantification (LOQ) was set for both substrates in the lowest validation level, 0.05 μg/mL and the limit of detection (LOD) was calculated to be 0.02 μg/mL (Table 1). The methods were found to be effective for the extraction of the tested compounds and the above results indicate their efficiency for the determination of chlorpyrifos and its metabolite TCP from the mineral salts medium and tomato fruits and ensure the accuracy of the biodegradation trials results.

Biodegradation of chlorpyrifos by Rhodotorula glutinis and Rhodotorula rubra

Effect of yeast inoculum density on biodegradation

When 10^2 cells/mL of the yeasts *R. glutinis* and *R. rubra* were supplemented in the MSM, no biodegradation was observed. However, in the inoculum density of 10^4 cells/mL and especially when the MSM was non-glucose-supplemented, strong indications were provided regarding the ability of the two yeasts to degrade chlorpyrifos. These indications were confirmed in the case of incubation of each culture with an initial inoculums density of 10^6 cells/mL, where chlorpyrifos was depleted. The metabolite TCP was detected as a degradation product on day 1 or 2 of incubation with its concentration to reach a maximum on day 5

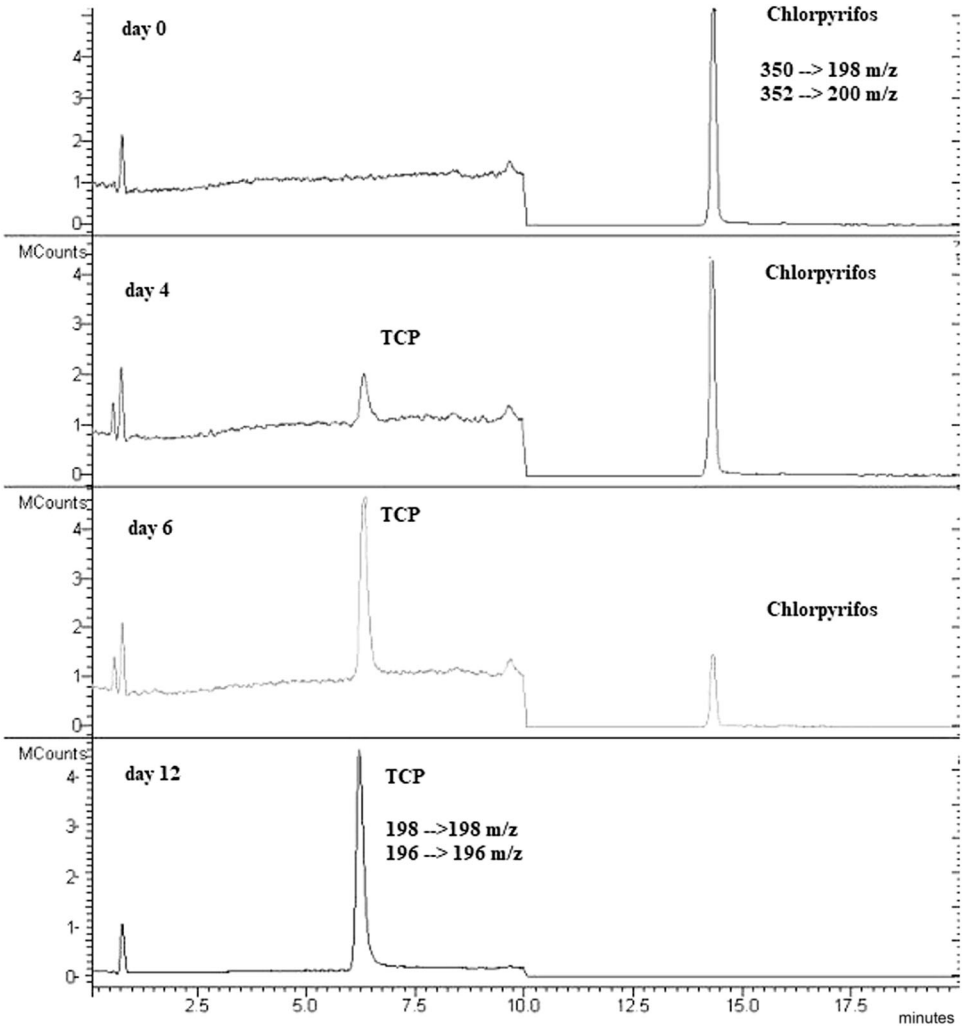

Fig. 1 Determination of chlorpyrifos and its metabolite 3,5,6-trichloro-2-pyridinol (TCP) in the LC/MS/MS chromatographic system, during a time period of 12 days, by changing the ionization mode for each analyte. Application of ESI (+) for chlorpyrifos and with ESI (+) for TCP

Table 1 Mean recoveries (R, %) and relative standard deviations (RSD, %) for the determination of chlorpyrifos and 3,5,6-trichloro-2-pyridinol (TCP) in mineral salts medium and chlorpyrifos in tomato fruits, in 5 replicates (n) at 3 fortification levels in the LC-MS/MS chromatographic system

Compound	Mineral salts medium ($n = 5$)			Tomato fruits ($n = 5$)		
	C (μg/mL)	Mean recovery (%)	RSD (%)	C (μg/mL)	Mean recovery (%)	RSD (%)
Chlorpyrifos	0.05	78.6	13	0.05	84.4	10.7
	0.1	84.4	10.4	0.1	92.4	8.3
	0.5	88.7	9.3	0.5	96.7	5.4
TCP	0.05	82.3	6.4	–	–	–
	0.1	89.6	7.8	–	–	–
	0.5	95.3	11.2	–	–	–

or 6 (respectively for each yeast) and then started to decrease (Fig. 2). Hydrolysis percentages of chlorpyrifos were less than 5% in all cases.

Effect of presence of glucose on mineral salts liquid medium

The addition of glucose in the MSM, as an extra source of carbon, effected the action of *Rhodotorula* yeasts as degraders of chlorpyrifos in a different way. As shown in Fig. 2, under the presence of glucose *R. glutinis* did not consume chlorpyrifos during the first 4 days of the trials. The consumption of chlorpyrifos in this day proved to be no-significant different from the control (t-test, $t(2) = 0.44$, $P < 0.05$) and therefore, it is assumed that the degradation actually started on day 5. Later on, the yeast decreased the insecticide rapidly and finally depleted it (day 7). TCP was detected on day 3. On the contrary in the flask without

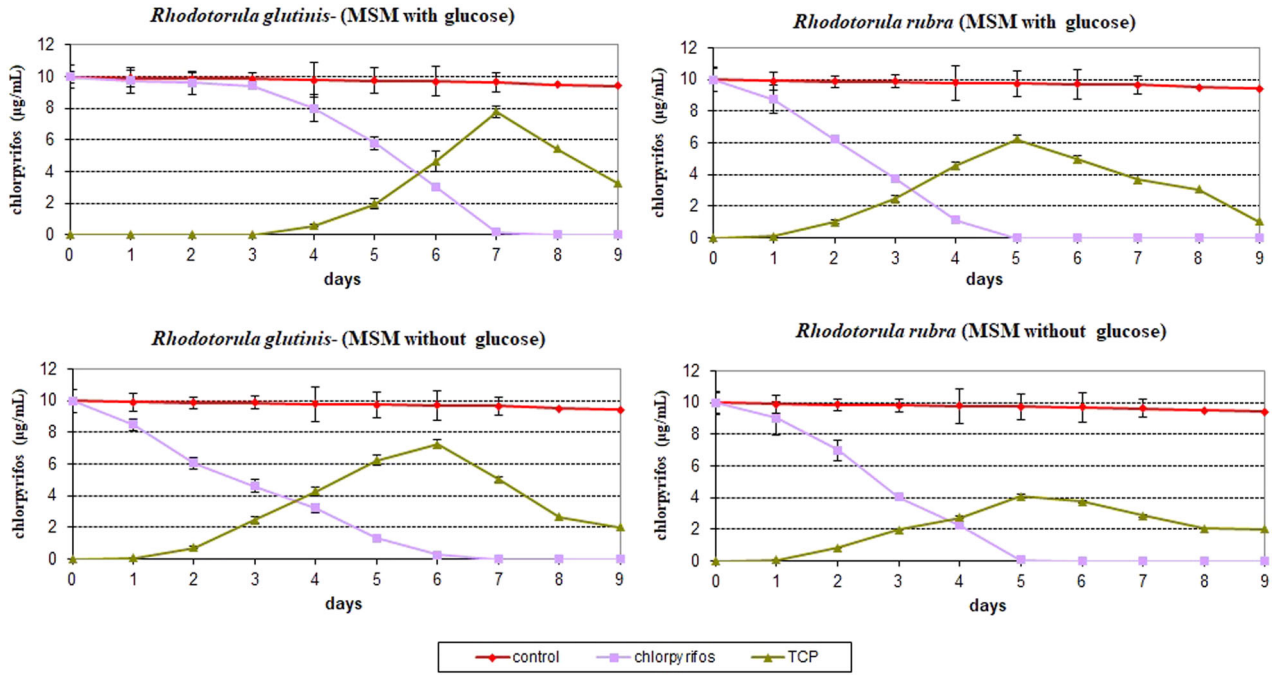

Fig. 2 Biodegradation of chlorpyrifos by the yeasts *Rhodotorula glutinis* and *Rhodotorula rubra* in MSM supplemented with 10 μg/mL chlorpyrifos and 10^6 cells/mL inoculum. Production of the metabolite 3,5,6-trichloro-2-pyridinol (TCP). Each value is the mean of three replicates with error bars representing the standard deviation of the mean

glucose degradation was observed from the beginning of the trial; chlorpyrifos was depleted up to day 6, while TCP was detected from day 2.

On the contrary, *R. rubra* showed similar action with or without the addition of glucose in the nutrient medium as an extra source of carbon. Both mediums gave positive biodegradation results from the beginning of the trials. The medium supplemented with glucose gave actually a higher biodegradation rate of 2.1 μg/mL/day comparing to 1.38 μg/mL/day on the medium without glucose. In both mediums TCP was detected on day 1.

Effect of chlorpyrifos concentration on biodegradation

Biodegradation trials of chlorpyrifos with different concentrations (10 and 25 μg/mL) of the insecticide were conducted in the MSM at 25 °C. Both yeasts were proved to be capable degraders of chlorpyrifos. *Rhodotorula glutinis* consumed fully 25 μg/mL chlorpyrifos at 10 days when the MSM was supplemented with glucose and at 6 days in the case of the non- glucose-supplemented MSM. As it was observed, *R. glutinis* maintained the delayed action under the presence of glucose. Biodegradation was faster and higher rates were obtained when the yeast was incubated with 25 μg/mL chlorpyrifos in both media (Fig. 3). In flasks supplemented with glucose the rate of 1.24 μg/mL/day (10 μg/mL chlorpyrifos) increased to 2.74 μg/mL/day (25 μg/mL chlorpyrifos). The corresponding values in the flasks without glucose in the MSM were 1.62 and 4.76 μg/mL/day.

On the other hand, *R. rubra* depleted chlorpyrifos in all cases presenting higher biodegradation rates in the concentration of 25 μg/mL (Fig. 3). In flasks supplemented with glucose the rate of 2.22 μg/mL/day (10 μg/mL chlorpyrifos) increased to 5.5 μg/mL/day (25 μg/mL chlorpyrifos). The corresponding values in the flasks without glucose in the MSM were 1.94 and 4.8 μg/mL/day. As it was observed the rate of biodegradation of chlorpyrifos by the two yeasts was proportionally increased with their exposure in the insecticide.

Effect of temperature on biodegradation of chlorpyrifos

Both yeasts showed similar action when they were incubated under different conditions of temperature(15, 25 and 35 °C). At 35 °C no degradation was observed after 7 days of incubation, while the degradation rates in 25 and 15 °C were 2.6 and 1.8 μg/mL/day for *Rhodotorula glutinis* and 1.88 and 1.52 μg/mL/day for *Rhodotorula rubra*, respectively. The results are considered acceptable regarding that *Rhodotorula* yeasts belong to phychrophyllic microorganisms.

Production of metabolite 3,5,6-trichloro-2-pyridinol

The metabolite 3,5,6-trichloro-2-pyridinol (TCP) was produced as a degradation product from both yeasts (Fig. 2).

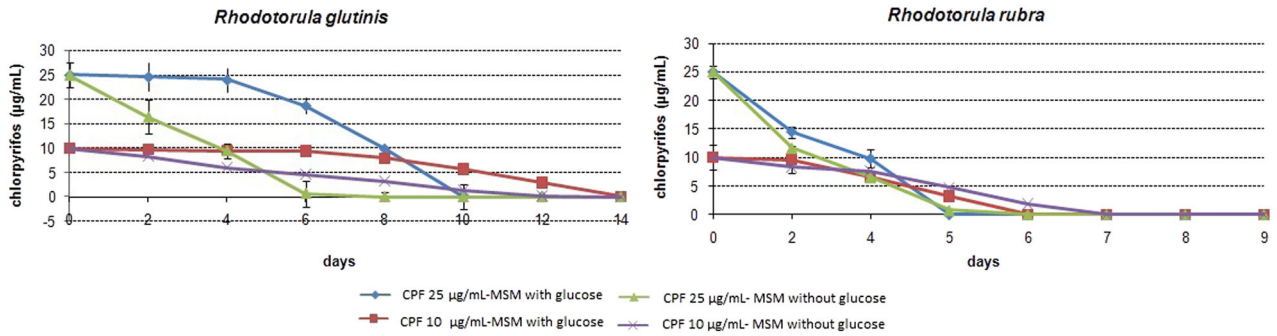

Fig. 3 Biodegradation of chlorpyrifos by the yeasts *Rhodotorula glutinis* and *Rhodotorula rubra* in MSM supplemented with 10 or 25 μg/mL chlorpyrifos and 10^6 cells/mL inoculum

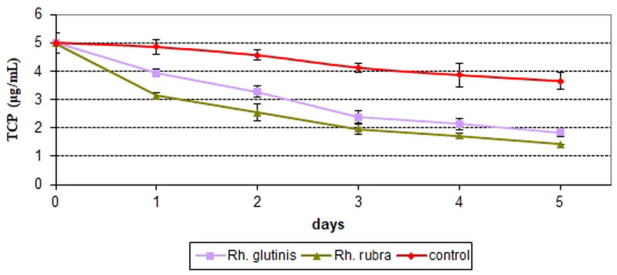

Fig. 4 Biodegradation of the metabolite 3,5,6-trichloro-2-pyridinol (TCP) by the yeasts *Rhodotorula glutinis* and *Rhodotorula rubra* in MSM supplemented with 5 TCP μg/mL chlorpyrifos and 10^6 cells/mL inoculum. Each value is the mean of three replicates with error bars representing the standard deviation of the mean

TCP was determined in all flasks of biodegradation. It was also observed that TCP levels increased proportionally to the concentration of chlorpyrifos added in the MSM. As shown TCP levels were increasing up to a maximum (day 5 to 7) and then started to decrease, a fact that gave indications of its possibly degradation by the yeasts. The above was confirmed by the results of biodegradation trials with the addition of TCP (5 μg/mL) in the MSM as a sole source of carbon. The initial concentration of TCP was decreased but not depleted (Fig. 4).

Enzymatic assays–Synergists

Enzymatic assays

As observed, the activity of esterases of both yeasts was significantly reduced by the addition of chlorpyrifos in the homogenates ($F = 330{,}38$; df = 1; $P < 0.05$), (Fig. 5). Furthermore, statistical analysis of the results showed a significant difference between the response of the control (homogenate) over the 5 concentrations of chlorpyrifos added compare to the response of the treatment (homogenate+chlorpyrifos) over the 5 concentrations of chlorpyrifos added ($F = 38.19$; df = 4; $P < 0.05$).The maximum inhibition observed was of 55.7% for *R. glutinis* and 65.7% for *R. rubra* at concentration of 57 μM. The IC_{50} (half maximal inhibition concentration- IC_{50}) values calculated were 43 μM for *R. glutinis* and 42 μM for *R. rubra*. On the other hand, chlorpyrifos did not reduce the activity of GSTs at the concentrations tested and under assay conditions described (Fig. 5). These results provide an indication that esterases mediate the observed biodegradation of chlorpyrifos by the two yeasts of the present study.

Synergists

As a first step the potential inhibition of three tested synergists in the development of the cultivations of the yeasts was tested by incubating with 1, 5, 10 and 15 μg/mL of triphenyl phosphate, diethyl maleate and piperonyl butoxide (one in a time) and no significant inhibition (< 5%) was observed. Therefore 10 μg/mL of each enzymatic inhibitor was added in the 250 mL flasks of the biodegradation trials. As it was observed after 10 days of incubation, diethyl maleate did not affect the biodegradation of chlorpyrifos by *R. glutinis* and *R. rubra* and similar results were produced after the addition of piperonyl butoxide. On the contrary, triphenyl phosphate substantially inhibited the biodegradation rate of chlorpyrifos. After the end of the experiment 97.5 and 97.2% of the initial concentration of chlorpyrifos was recovered respectively for each yeast. These results enforced the above preliminary statements that esterases are involved in the biodegradation of chlorpyrifos by the two epiphytic yeasts.

Biodegradation of chlorpyrifos on tomato *fruits*

Primary indications of biodegradation of chlorpyrifos by the yeasts *R. glutinis* and *R. rubra* can be derived as shown in Table 2 The decrease of chlorpyrifos in the treated tomatoes was significant different for both yeasts to the corresponding of the control 30 days after the application (*Rhodotorula glutinis*: t-test, $t(2) = 9.379$, $P < 0.05$ and *Rhodotorula rubra*: t-test, $t(2) = 75.44$, $P < 0.05$). The initial

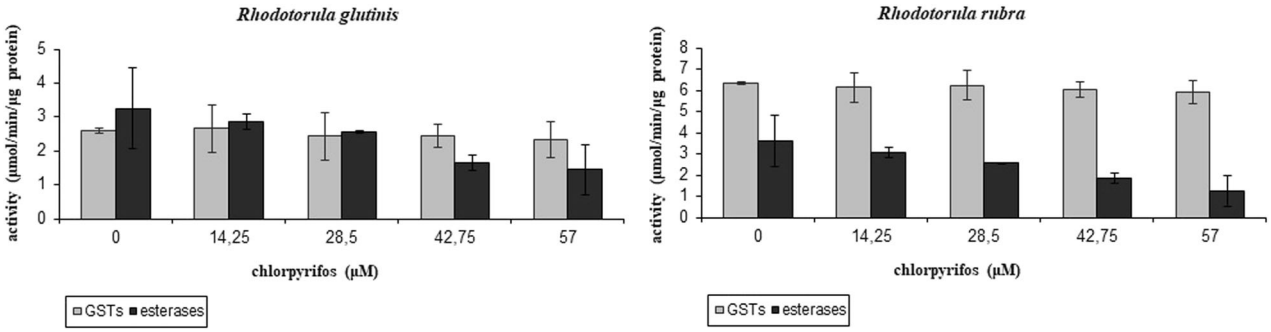

Fig. 5 Effect of chlorpyrifos on glutathione-S-transferases (gsts) and esterases activity of *Rhodotorula glutinins* and *Rhodotorula rubra*. Each value is the mean of three replicates ± relative standard deviation

Table 2 Residues of chlorpyrifos (mg/kg) on tomato fruits sprayed with 2 mg/kg chlorpyrifos and 10^6 cells/mL of the yeasts *Rhodotorula glutinis* and *Rhodotorula rubra*

Days	Concentration of chlorpyrifos C (mg/kg)					
	Rhodotorula glutinis			*Rhodotorula rubra*		
	Control	Application	% of the control in the flask	Control	Application	% of the control in the flask
5	1.75 ± 4.23	1.47 ± 0.89	84	1.75 ± 3.22	1.31 ± 1.9	89
30	1.02 ± 8.39	0.22 ± 2.01	21.6	1.02 ± 0.59	0.08 ± 2.5	7.8

Control are the fruits sprayed only with chlorpyrifos and application the fruits sprayed with chlorpyrifos and the inoculum. Each value is the mean of three replicates ± relative standard deviation

concentration of chlorpyrifos was decreased up to 78.4% of the control when *R. glutinis* was added, and up to 92.2% in the case of *R. rubra*.

Discussion

The biodegradation of the insecticide chlorpyrifos by the epiphytic yeasts *Rhodotorula glutinis* and *Rhodotorula rubra* was shown in the present report. Both organisms are naturally occurring in plants and are not pathogens.

The approach followed to examine biodegradation was based on the incubation of each yeast in MSM supplemented with chlorpyrifos under different experimental conditions.

The variation on the levels of chlorpyrifos and its metabolite TCP during time were determined by LC/MS/MS. The measurements obtained are considered indisputable, since they are based on the fully successful validation of the analytical method performed and the optimum detection and separation of both analytes on the chromatographic system. The determination of the two compounds was achieved in the LC/M/MS system using different ionization mode for each compound. However, the simultaneous detection of the two analytes in one chromatogram by changing the ionization mode of the electrospray was managed, a technique not been reported yet. Up to now the analytical methods for the determination of chlorpyrifos and TCP are based on HPLC/UV (Abu-Qare and Abou-Donia 2001) or GC/MS after derivatization (Koch and Angerer 2001). Sancho et al. (2000) reported the simultaneous determination of the insecticide and its metabolite by LC/MS/MS using different analytical columns and different mobile phases for their separation, a technique much more time and reagent consuming.

Biodegradation proved to be dependent on the inoculum density of each yeast, as almost negligible biodegradation of the insecticide occurred from low inoculums (10^2 and 10^4 cells/mL), a fact that has also been reported for *Sphingomonas sp*. strain DSP-2, *Enterobacter* strain-B14 (Li et al. 2007; Yang et al. 2006; Singh et al. 2004). Another parameter tested on biodegradation was the possibly different action of the microorganisms when the MSM was supplemented with an additional source of carbon, glucose. As shown, under the presence of glucose *Rhodotorula glutinis* showed delayed bio-degradative activity. In agreement with our results Muncnerova and Augustin (1995) and Bempelou et al. (2013) reported the degradation of benzoate and diazinon, respectively, by *R. glutinis* only after the consumption of glucose in the medium. The difference in the degradation rate of chlorpyrifos depending on the nutrient medium has also been reported for *Enterobacter strain B-14*, which delayed the consumption of chlorpyrifos under the presence of glucose or succinic acid (Singh et al. 2004).

According to Ruiz-Amil et al. (1965); Torrontegui et al. (1966); Fernadez et al. (1967) and Medrano et al. (1969) the presence of glucose in the nutrient medium prevents the assimilation or the metabolism of another substance from the cells of the yeast until the depletion of the levels of glucose. On the contrary, in the case of *Rhodotorula rubra*, the addition of glucose in the nutrient medium did not affect its ability to degrade chlorpyrifos. This has also been reported by Xu et al. (2008) during the degradation of chlorpyrifos by the bacterium *Paracoccus sp*. strain TRP. As far as the effect of chlorpyrifos concentration on biodegradation is concerned, as it was observed the higher the concentration it was, the more active the yeasts were. Similar observations have been made by Yang et al. (2005) and Fang et al. (2008) while studying the biodegradation of chlorpyrifos by the bacterium *Alcaligenes faecalis* DSP3 and the fungi *Vericillium sp*. DSP, respectively.

The degradation product identified in all trials was the metabolite TCP whose concentration was increased up to a maximum and then started decreasing since *R. glutnis and R. rubra* proved also to be able to consume TCP as well. Chlorpyrifos-oxon, 3,5,6-trichloro-2-methoxypyridine and 2-chloro-6-hydroxypyridine have also been identified as degradation product of chlorpyrifos (Singh et al. 2006; Yu et al. 2006), but were not detected in the present study. The ability of biodegradation of TCP has also been reported for *Sphingomonas sp*. strain DSP-2 (Li et al. 2007), *Alcaligenes faecalis* strain DSP-3 (Yang et al. 2005), *Paracoccus sp*. strain TRP (Xu et al. 2008), *Verticillium sp*. DSP (Fang et al. 2008), *Bacillus pumilis* strain C2A1 (Anwar et al. 2009*), Cladosporium cladosporioides HU-01 (*Chen et al. 2012*) and Aspergillus* terreus JAS1 and *Ganoderma sp. JAS4* (Silambarasan and Abraham 2013, 2014), The majority of the microorganisms reported to be able to degrade chlorpyrifos and TCP are bacteria and fungi. No reference has been found up to now describing the biodegradation of chlorpyrifos by the epiphytic yeasts *R. glutinis* and *R. rubra*.

Later on, an effort was made in order to find the enzymes responsible for the degradation of chlorpyrifos by the two yeasts. From the in vitro enzymatic assays applied, an indication that esterases mediate the biodegradation was observed. Phosphotriesterases have been reported as responsible for the degradation of chlorpyrifos by various microorganisms, mainly soil bacteria (Serdar et al. 1982; Mulbry et al. 1986; Havens and Rase 1991; Horne et al. 2002; Singh et al. 2004 and Yang et al. 2005). As a step of further confirmation, the synergist triphenyl phosphate (inhibitor of esterases) was added in the biodegradation trials and an inhibition of biodegradation was identified. Based on those results a more robust suggestion that esterases are involved in the biodegradation of chlorpyrifos by the by the epiphytic yeasts *R. glutinis* and *R. rubra* can be made.

Finally, biodegradation was also investigated on plant surfaces and in particular in tomato fruits and the results measured in the LC/MS/MS system were in line with our in vitro biodegradation study, providing a primary indication of the phenomenon in plant surfaces. It should also be mentioned that the trials were conducted in harvested tomato fruits and therefore are considered as a worst-case scenario since the reaction between the microorganism and the insecticide was studied under completely controlled conditions without the interferences of other factors (biological, environmental) which might contribute to the disappearance of chlorpyrifos from the plant surfaces. Our findings indicate that the epiphytic yeasts *R. glutinis* and *R. rubra* could possibly be used successfully for the removal or detoxification of chlorpyrifos, residues in/on vegetables.

Acknowledgements Our study entitled "Biodegradation of chlorpyrifos and 3,5,6-trichloro-2-pyridinol by the epiphytic yeasts *Rhodotorula glutinis* and *Rhodotorula rubra*", was not funded.

Compliance with ethical standards

Conflict of interest The authors declare that they have no no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Abraham J, Silambarasan S (2013) Biodegradation of chlorpyrifos and its hydrolyzing metabolite 3,5,6-trichloro-2-pyridinol by Sphingobacterium sp. JAS3. Process Biochem 48(2013):1559–1564. https://doi.org/10.1016/j.procbio.2013.06.0341559–1564

Abraham J, Silambarasan S (2016) Biodegradation of chlorpyrifos and its hydrolysis product 3,5,6-trichloro-2-pyridinol using a novel bacterium Ochrobactrum sp. JAS2: A proposal of its metabolic pathway. Pestic Biochem Physiol 126(2016):13–21. https://doi.org/10.1016/j.pestbp.2015.07.001

Akbar S, Sultan S (2016) Soil bacteria showing a potential of chlorpyrifos degradation and plant growth enhancement. Braz J Microbiol 47:563–570. https://doi.org/10.1016/j.bjm.2016.04.009

Abu- Qare AW, Abou-Donia MB (2001) Determination of diazinon, chlorpyrifos and their metabolites in rat plasma and urine by high- performance liquid chromatography. J Chromatogr Sci 39 (5):200–204

Anwar S, Liaquat F, Khan QM, Khalid ZM, Iqbal S (2009) Biodegradation of chlorpyrifos and its hydrolysis product 3,5,6-trichloro-2-pyridinol by Bacillus pumilus strain C2A1. J Hazard Mater 168(1):400–405. https://doi.org/10.1016/j.jhazmat.2009.02.059

Bempelou ED, Vontas JG, Liapis KS, Ziogas VN (2013) Biodegradation of diazinon by the epiphytic yeasts Rhodotorula glutinis and Rhodotorula rubra. Hellenic Plant Protection. Journal 6:69–82

Bhagobaty RK, Joshi SR, Malik A (2007) Microbial degradation of organophosphorous pesticide: chlorpyrifos (mini-review) The Internet Journal of Microbiology 4(1):1–12

Bauriedel WR (1986a). *The Early Fate of ^{14}C-chlorpyrifos Applied to Leaf Surfaces of Corn, Soybean and Sugarbeat*. DowElanco, Indianapolis, IN (unpublished report). In: Reviews of Environmental Contamination and Toxicology, Vol 131,Springer-Verla New York Inc. 1993. https://doi.org/10.1007/978-1-4612-4362-5

Bilthoven (1996) Analytical Methods for Pesticide Residues in Foodstuffs, Sixth edition. Ministry of Public Health, Welfare and Sport, The Netherlands, P. Van Zoonen (Ed), 4

Bolognesi C, Moraso G (2000) Genotoxicity of pesticides: potential risk for consumers. Trends Food Sci Technol 11:182–187

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgramquantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–54

Chapman RA, Cole CM (1982) Observations on the ifluence of water and soil pH on the persistence of insecticides. J Environ Sci Health B 17:487–504

Chen S, Peng C, Hongmei L, Meiying H, Hu M, Zhong G (2012) Biodegradation of chlorpyrifos and its hydrolysis product 3,5,6-trichloro-2-pyridinol by a new fungal strain Cladosporium cladosporioides Hu-01. PLoS One 7(10):e47205. https://doi.org/10.1371/journal.pone.0047205.

Fang H, Xiang YQ, Hao YJ, Chu XQ, Pan XD, Yu JQ, Yu YL (2008) Fungal degradation of chlorpyrifos by verticillium sp. dsp in pure cultures and its use in bioremediation of contaminated soil and pachoi. Int Biodeterior Biodegrad 61:294–303

Fernadez MJ, Medrano L, Ruiz-Amil M, Losada M (1967) Regulation and function of pyruvate kinase and malate enzyme yeast. European J Biochem 3:11–18

Getzin LW (1981a) Degradation of chlorpyrifos in soil: influence of autoclaving, soil moisture and temperature. J Econ Entomol 74:158–162

Guha A, Kumari B, Bora TC, Roy MK (1997) Possible involvemrnt of plashmids in degradation of malathion and chlorpyrifos by micrococcus sp. Folia Microbiol 42(6):574–576

Havens PL, Rase HF (1991) *Detoxification of Organophosphorus Pesticide Solutions*. In: Tedder DW, Pohland FG (eds) Emerging technologies in Hazardous waste management II. ACS, Washington, DC, p 261–281

Horne I, Sutherland TD, Harcourt RL, Russel RJ, Oakeshott JG (2002) Identification of an opd (organophosphate degradation) Gene in Agrobacterium Isolate. Applied Environ Microbiol 68:3371–3376

Jones AS, Hastings FL (1981). *Soil microbe srudies*. In: Hastings F.L., Coster J.E. (eds). *Field and laboratory evaluations of insecticides for southern pine beetle control*. USDA. Southern Forest Experimental Station, Forest Service, SE, USA, 21, p 13–14, 35

Ivashina SA (1986) Interaction of Dursban with soil micro-organisms. Agrokhimia 8:75–76

Koch HM, Angerer J (2001) Analysis of 3,5,6-trichloro-2-pyridinol in Urine Samples from the General Population Using Gas Chromatography- Mass Spectrometry after Steam Distillation and Solid Phase Extraction. J Chromatogr B 759:43–49

Lakshmi CV, Kumar M, Khanna S (2008) Biotransformation of chlorpyrifos and bioremediation of contaminate soil. Int Biodeterior Biodegrad 62:204–209

Lal S, Lal R (1987) Bioaccumulation, metabolism and effects of DDT, fenitrothion and chlorpyrifos on Saccharomyces cerevisae. Arch Environm Contam Toxicol 16:753–757

Li X, He J, Li S (2007) Isolation of a chlorpyrifos-degrading bacterium, Sphingomonas sp. strain Dsp-2 and cloning of the mpd Gene. Res Microbiol 158:143–149

Lu P, Li Q, Liu H, Feng Z, Yan X, Hong Q, Li S (2013) Biodegradation of chlorpyrifos and 3,5,6-trichloro-2-pyridinol by Cupriavidus sp. DT-1 Bioresour Technol. 127:337–42

Mallick K, Bharati K, Banerji A, Shakil NA, Sethunathan N (1999) Bacterial degradation of chlorpyrifos in pure cultures and in soil. Bull Environ Contam Toxicol 62:48–54

Meikle RW, Youngson CR (1978) The hydrolysis rate of chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridyl)-phosphorothiorate] and its dimethyl analog, chlorpyrifos methyl, in dilute aqueous solution. Arch Environ Contam Toxicol 7:13–22

McCall PJ (1986a) Hydrolysis of Chlorpyrifos in Dilute Aqueous Solution. DowElanco, Indianapolis, IN

Medrano L, Ruiz-Amil M, Losada M (1969) Effect of glucose on pyruvate utilization by Rhodotorula glutinis. Arch Microbiol 66:239–249

Muncnerova D, Augustin J (1995) Assimilation of benzoate by rhodotorula rubra, rhodotorula glutinis and rhodosporidium toluroides, as affected by glucose or xylose. World J Microbiol Biotechnol 11:240–241

Mulbry WW, Karns JS, Kearney PC, Nelson JO, McDaniel CS, Wild JR (1986) Identification of a plasmid-borne parathion hydrolase gene from flavobacterium sp. by southern hybridization with opd from pseudomonas diminuta. Applied Environ Microbiol 51:926–930

Racke KD (1993) Environmental fate of Chlorpyrifos. Rev Environ Contam Toxicilogy 131:1–151

Racke KD, Coats JR, Titus KR (1988) Degradation of chlorpyrifos and its hydrolysis products 3,5,6-trichloro-2-pyridinol, in Soil. J Environ Sci Health B 23:527–539

Roditakis E, Grispou M, Morou E, Kristoffersen JB, Roditakis N, Nauen R, Vontas J, Tsagkarakou A (2009) Current status of insecticide resistance in Q biotype Bemisia tabaci populations from Crete. Pest Manag Sci 65(3):313–22

Ruiz-Amil M, Torrontegui G, Palacian E, Catalina L, Losada M (1965) Properties and functions of yeast puryvate carboxylase. J Biol Chem 240:3485–3492

Sancho JV, Pozo OJ, Hernandez F (2000) Direct determination of chlorpyrifos and its main metabolite 3,5,6-trichloro-2-pyridinol in human serum and urine by coupled-column liquid chromatography/electrospray-tandem mass spectrometry. Rapid Commun Mass Spectrom 14:1485–1490

SANTE/11945/2015. Commission of the European Union, Doc No SANTE/11945/2015. Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed

SAS Institute (2008) SAS/STAT guide for personal computers, version 8 Ed SAS Institute, Cary, p 333

Serdar CM, Gibson DT, Munnecke DM, Lancaster JH (1982) Plasmid involment in parathion hydrolysis by pseudomonas diminuta. Applied Environ Microbiol 44:246–249

Shaker N, Abo-Donia S, El-Shaheed YA, και Ismail (1988) Effect of lactic acid bacteria and heat treatments on pesticides contaminated milk Egypt J Diary Sci 16:309–317

Silambarasan, S. and Abraham, J. (2013). Ecofriendly method for bioremediation of chlorpyrifos from agricultural soil by novel fungus Aspergillus terreus JAS1. Water Air and Soil Pollution, 224(1). https://doi.org/10.1007/s11270-012-1369-0

Silambarasan, S. and Abraham, J. (2014). Efficacy of Ganoderma sp. JAS4 in bioremediation of chlorpyrifos and its hydrolyzing metabolite TCP from agricultural soil. Journal of Basic Microbiology 54(1). https://doi.org/10.1002/jobm201200437

Singh BK, Walker A, Morgan JAW, Wright DJ (2004) Biodegradation of chlorpyrifos by enterobacter strain-B14 and Its use in bioremediation of contaminated soils. Applied Environ Microbiol 70 (8):4855–4863

Singh BK, Walker A, Wright DJ (2006) Bioremedial potential of fenamiphos, chlorpyrifos, degrading isolates: influence of different environmental conditions. Soil Biol Biochem 38:2682–2693

Tomlin C, (2003) *The Pesticide Manual*, 13th edition, British Crop Protection Council. UK (Omega Park, Alton, Hampshire GU34 2QD)

Torrontegui G, Palacian E, Losada M (1966) Phosphoenolpyruvate carboxykinase in gluconeogenesis and itsrepression by hexoses in yeasts. Biochem Biophys Res Commun 22:227–231

Vontas JG, Small GJ, Hemingway J (2001) Glutathione S-transferases as antioxidant defense agents confer pyrethroid resistance in N. lugens. Biochem J 357:65–72

Xu G, Zheng W, Li Y, Wang S, Zhang J, Yan Y (2008) Biodegradation of chlorpyrifos and 3,5,6-trichloro-2-pyridinol by newly isolated paracoccus sp. Straun TRP. Int Biodeterior Biodegrad 62:51–56

Yang L, Zhao YH, Zhang BX, Yang CH, Zhang X (2005) Isolation and characterization of A chlorpyrifos and 3,5,6-trichloro-2-pyridinol degrading Bacterium. FEMS Microbiol Lett 251:67–73

Yang C, Liu N, Guo X, Qiao C (2006) Cloning of Mpd gene from a chlorpyrifos-degrading bacterium and use of this strain in bioremediation of contaminated soil. FEMS Microbiol Lett 265:118–125

Yu YL, Fanf H, Wang X, Wu XM, Shan M, Yu JQ (2006) Characterization of a fungal strain capable of degrading chlorpyrifos and its use in detoxification of the insecticide on vegetables Biodegradation 17:487–494