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Evaluation of Chlorpyrifos Tolerance and Degradation by Non-Toxigenic *Aspergillus* Section Flavi Strains Isolated from Agricultural Soils

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A B S T R A C T

This study was conducted to evaluate the *in vitro* tolerance, utilization and degradation of chlorpyrifos by non-toxigenic *Aspergillus* section *Flavi* strains natives of agricultural soils. The tolerance assay showed that all strains were able to grow at the highest insecticide concentration (700 mg L^{-1}). In media supplied with chlorpyrifos, the highest values of growth rate were observed in the full CzapekDox medium (CZ) (7.2 to 8 mm day^{-1}) and the lowest values were registered in the medium where the insecticide replaced the carbon source (CZC) (3.6 to 4.4 mm day^{-1}). In media where the insecticide replaced the nitrogen (CZN) and phosphorous (CZP) source, the values of growth rate were significantly higher than in CZC and lower than in CZ. Degradation assays showed that the degradation percentage was 75% at 0.98 and 0.95 of water activity, in all concentrations (1753, 3506 and 7012 mg L^{-1}). A fast decrease of chlorpyrifos was observed within the first day of incubation, and then the biodegradation rate decreased over time. This study provides evidence on the effective growth ability and degradation of no-toxigenic *Aspergillus* section *Flavi*in presence of high chlorpyrifos concentrations and under optimal growth conditions (a_W and temperature).

Keywords

Chlorpyrifos, *Aspergillus* section Flavi, tolerance, degradation.

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Introduction

Pesticides are one of the most important substances to which man is exposed. Argentina's economy is traditionally based on agricultural production; this brings the implementation of significant quantities of pesticides that impact on health and environmental quality. Since the 70', data about the environmental impact, food and human health is informed (Brunstein *et al.*, 2009). An important change in the last years was the introduction of glyphosate-resistant transgenic soybean, which led to a significant increase in crop yields and in sowed area. These increases caused an important growth in the use of technological packages based on the utilization of

genetically modified seeds and pesticides like glyphosate, chlorpyrifos and pyrethroids (VillamilLepori *et al.,* 2013). According to the Chamber of Agricultural Health and Fertilizer (CASAFE, 2014), in the last twenty two years in Argentina the utilization of pesticides increased in 858% while the cereals and oilseeds cultivated area and crop yields had only an increase of 50 and 30%; respectively. The chlorpyrifos doses applied depend on the crop, the insect and the commercial formulation. For soybean and maize, the doses range between 0.40 and 1.60 L ha^{-1} and between 1.2 and 4 L ha^{-1}, respectively (Agrobit, 2013). Pesticide use is questioned, mainly in developed countries, due to potential accumulation of residues and degradation products, and negative effects on soil microorganisms. Toxicological risks on animals, humans and environment are being increasingly studied (Antunes *et al.,* 2010; Alvarez *et al.,* 2013).

Chlorpyrifos (O,O-diethyl-O-3,5,6-trichloro-2 pyridyl phosphorothioate) is an organophosphate insecticide for foliar or soil application. It was commonly used for both household and agricultural applications until it was banned for household use by the USEPA in 2000 (EPA, 2000). This insecticide is one of the most detected in surface and ground water in the world (Gilliom *et al.,* 2000; Domagalski and Munday, 2003) and in water and soil in Argentina (Loewy *et al.,* 2011). Their solubility in water is limited and it can be adsorbed to suspended particles and sediments. The half-life of this insecticide in soil has been estimated at around 360 days (EPA, 2000). Temperature, pH, moisture, organic carbon content and pesticide formulation can produce variations in the half-life in soil (Alvarez *et al.,* 2013).

Chlorpyrifos degradation on soil is influenced by environmental factors like pH, moisture and temperature; likewise the commercial formulation and application rates. Biotic and abiotic process contributes to the degradation. A key process is the enzymatic hydrolysis, in which the rate increases with pH and temperature; another important process is the photolytic degradation (Gebremariam *et al.,* 2012). Nevertheless, the main pathway of degradation seems to be aerobic and anaerobic metabolism (Awad *et al.,* 2011; Massiha *et al.,* 2011). Depending on the microorganisms and environmental conditions, chlorpyrifoscleavage produce two major metabolites: 3,5,6-trichloro-2-pyridinol (TCP) and dieth-ylthiophosphoric acid (DETP), while some other metabolites, are produced in very minute quantities (Bootharaju and Pradeep, 2012).

Several studies have demonstrated that the inoculation of adapted microorganisms on pesticide-contaminated soils is a good option to decontaminate them (Diez, 2010; Abo-Amer, 2011; Massiha *et al.,* 2011). There are a great diversity of microorganisms producers of organophosphates degrading enzymes in chlorpyrifos-exposed soils (Bhagobaty and Malik, 2008; Sasikala *et al.,* 2012). Several microbial species capable to metabolize chlorpyrifos as sole source of carbon have been isolated from pesticide contaminated soils, sludge or waste water by enrichment culture techniques (Ghanem *et al.,* 2007; Latifi *et al.,* 2012; Liu *et al.,* 2012; Savitha and Raman, 2012). Most investigations have focused mainly in bacteria; few of them have demonstrated that chlorpyrifos could be degraded by fungi (Yu *et al.,* 2006; Chu *et al.,* 2008; Fang *et al.,* 2008).Filamentous fungi are part of the soil microbiota and they have evolved for use more efficiently several solid substrates, growing on their surface and penetrating in their matrices. These microorganisms are capable to produce and secrete enzymes that turn

complex macromolecules to simple compounds for their growth and metabolism (Rabinovich *et al.,* 2004).

Aspergillus sp. is one of the most prevalent genera isolated from Argentinean agricultural soils (Carranza *et al.,* 2014; 2016a). Being *Aspergillus* section *Flavi* strains isolated in major frequency. The *in vitro* glyphosate tolerance was evaluated. All strains tested were able to develop at the highest concentration assayed (500 mM) regardless the water availability condition (Carranza *et al.,* 2016a). In addition, *Aspergillus* section *Nigri* strains also isolated from these soils, resulted tolerant to chlorpyrifos (Carranza *et al.,* 2014). However, there is no information about chlorpyrifos tolerance or degradation by *Aspergillus* section *Flavi* strains isolated from these soils. Therefore, the aim of the present study was to evaluate the *in vitro* tolerance, utilization and degradation of chlorpyrifos by non-toxigenic *Aspergillus* section *Flavi* strains natives of agricultural soils.

Materials and Methods

Fungal strains

Three *A.oryzae* strains (AM 1, AM 2 and GM 3) and one *A.flavus* (GM 4) previously identified by molecular methods were used (Carranza *et al.,* 2016a).These strains were previously isolated from fields destined to maize and soybean production(Carranza *et al.,* 2014). These fields have been exposed to successive applications of pesticides during the last decade. The ability of the strains tested to produce aflatoxins and cyclopiazonic acid was evaluated previously (Barberis *et al.,* 2012; Carranza *et al.,* 2014); and they resulted non-toxigenics. The strains belong to our culture collection at the Department of Microbiology and Immunology, in the National University of Río Cuarto, Córdoba, Argentina, and they are maintained in 15% of glycerol (Sigma-Aldrich, St. Louis, MO, USA) at -80°C.

The nucleotide sequences for the calmodulin and ß- tubulin gene were deposited in GenBank under accession numbers KX298157- KX306816, KX298158-KX306817, KX298159- KX306818 and KX306820- KX306819 for the strains AM1, AM2, GM3 and GM4, respectively

Chlorpyrifos

The chlorpyrifos used in this study was obtained from commercial formulation (Hor-tal®). A stock solution of 10 g $L^{-1}$of the active ingredient was prepared by dissolving the corresponding volume of the insecticide in 100 ml of sterile distilled water (vv^{-1}).

For tolerance, growth and degradation experiments, the appropriate concentration of stock solution was applied to the sterilized media (tempered at 45–50ºC) to obtain the required final concentrations. They are higher than the chlorpyrifos application rates recommended on field (2-2.75 kg ha^{-1}) (Muzio *et al.,* 2008).

Chlorpyrifos tolerance by *Aspergillus* section *Flavi* strains

For tolerance assays, the methodology used was descripted in a previous study (Carranza *et al.,* 2016a).Soybean extract agar (SEA) was used and the water activity (a_W) of the basic medium was adjusted to 0.995 and 0.980 with known amounts of glycerol (Dallyn and Fox, 1980).The media were autoclaved at 120ºC for 20 min. The stock solution of insecticide was applied to the sterilized media to obtain the required final concentrations (100, 200, 300, 400, 500, 600

and 700 mg L^{-1}).Homogenized media were poured into9-cm sterile petri dishes. Water activity was checked with an AquaLab Series 3 (Decagon Devices, Inc., Pullman, WA, USA). Controls plates without chlorpyrifos at each a_W condition were prepared. Water activity was measured at the end of the experiment in order to detect any significant deviation.

The media were needle-inoculated centrally with a spore suspension in soft agar from 7-day-old cultures on malt extract agar (MEA). Inoculated Petri dishes of the same a_W were sealed inside the same polyethylene bags. Four replicate plates per treatment were used and incubated at 25°C for 28 days. Each analysis was carried out in quadruplicate and all of the experiments were repeated twice.

The growth measurement was done as it is detailed in the study done by Barberis *et al.* (2010). Two measures of colony diameter at right angles to each other were taken daily from each replicate plate. The radius of the colony was plotted against time, and a linear regression was applied in order to obtain the growth rate as the slope of the line to the X-axis. The percentage of growth inhibition produced by chlorpyrifos was calculated in each treatment. The lag phase (h) was also determined

Growth of *Aspergillus* section *Flavi* strains in the presence of commercial chlorpyrifos formulation as sole source of carbon, phosphorus or nitrogen

For growth assays, Czapek Dox agar medium (sucrose 30g, $NaNO_3$ 3g, K_2HPO_4 1g, $MgSO_4.7H_2O$ 0.25g, KCl 0.5g, $FeSO_4.7H_2O$ 0.01g, agar 15g, distilled water 1L) at 0.995 ± 0.002 of a_W was used as basal medium. In this medium, sucrose was replaced by chlorpyrifos at a final concentration of 50 mg L^{-1}as sole carbon source. When chlorpyrifos was used as sole source of phosphorus or nitrogen, K_2HPO_4 or $NaNO_3$ were replaced by chlorpyrifos at final concentration of 10 mg L^{-1}. These concentrations were selected based on the percentages that the carbon, nitrogen or phosphorous sources should represent in the fungal media (Gao *et al.,* 2012; Rokade and Mali, 2013). The Czapek Dox agar is referred to as CZ. The growth medium in which chlorpyrifos replaces the organic carbon source is referred to as CZC. On the other hand, when the mineral phosphate or nitrogen sources are replaced by this insecticide the media are referred to as CZP or CZN, respectively. The water agar (WA) and CZ media were used as control treatments.

The inoculation, incubation and estimation of growth parameters were made as it is explained above by tolerance assays. Each analysis was carried out in quadruplicate and all of the experiments were repeated twice.

Chlorpyrifos degradation by *A. oryzae* on synthetic medium

The ability of *A. oryzae* (AM 1 and AM 2) to degrade different concentrations (1753, 3506 and 7012 mg L^{-1}) of chlorpyrifos was tested. Broth Czapeck medium (CZ) adjusted to 0.98, 0.95 and 0.93 of a_W with the addition of glycerol was used. Aliquots (50 mL) of CZ without insecticide were added aseptically into sterilized conical flasks and were immediately inoculated with one agar plugs (3 mm) taken from the margins of actively growing cultures of each strain in the appropriate agar media. Subsequently, inoculated flasks were placed in a shaking incubator (60 rpm) at 25ºC and the fungi were allowed to grow in the absence of the insecticide for 3 days. After this period, all flasks were supplemented

with the different concentration of insecticide. The corresponding controls were included (flasks without chlorpyrifos, flasks with the insecticide but without strains). Immediately after insecticide addition (0) and at 1, 2, 5, 10, 15, 20 and 30 days, subsamples of each culture (1 mL) were removed and insecticide residual concentration was determined by HPLC. All the treatments were done by triplicate and repeated three times.

For chlorpyrifos extraction and detection, subsamples of 1 mL of the liquid medium, was mixed with 2 mL of a mixture of acetonitrile:methanol (80:20 v:v) and vortexed (30 s). The mixtures were subsequently passed through a 0.45 µm syringe filter (Sartorius Stedim Biotech GmbH Goettingen Germany) and the filtrates were used for HPLC analysis followed the methodology proposed by Karas *et al.,* (2011). The HPLC system consisted of a Hewlett-Packard 1100 pump (Palo Alto, CA, USA) connected to a Hewlett-Packard 1046 programmable fluorescence detector, interfaced to a Hewlett-Packard Chem Station. Chromatographic separations were performed on a stainless steel Supelcosil LC-ABZ C18 reversed-phase column (150×4.6 mm i.d., 5 µl particle size; Supelco, PA, USA). Chlorpyrifos was quantified by correlating peak height of sample extracts and those of standard curves. Standard curves were constructed with different levels of Chlorpyrifos (PESTANAL®, analytical standard,45395 SIGMA-ALDRICH).This insecticide was quantified by correlating peak height of sample extracts and those of standard curves. The detection limit of the analytical method was 1 ng g^{-1} of sample. Concentration of chlorpyrifos (mg L^{-1}) in the media was plotted against time (days) to calculate the percentage of degradation on each condition assayed.

Assay of spiking and recovery of chlorpyrifos

Each chlorpyrifos-free medium sample (50 mL) contained in a 250 mL Erlenmeyer flask was spiked with an equivalent of 0.1, 0.5, 1.0 and 2 mgL^{-1} of chlorpyrifos. Spiking was carried out by triplicate and a single analysis of the blank sample was carried out. The chlorpyrifos concentration was determined, using the protocol previously described.

Statistical Analysis

Data analysis of *in vitro* evaluation of chlorpyrifos tolerance, replacement effect of the carbon, phosphorus or nitrogen source by chlorpyrifos and chlorpyrifos degradation levels by *Aspergillus* section *Flavi* strains were performed by analysis of variance. All data were transformed to $\log_{10}(x + 1)$ to obtain the homogeneity of variance. Means were compared also by Fisher's protected LSD test to determine the influence of the variables assayed (a_W and insecticide concentration) in the growth rate, lag phase and residual chlorpyrifos concentration by the strains tested. The analysis was conducted using PROC GLM in SAS (SAS Institute, Cary, NC).

Results and Discussion

Chlorpyrifos tolerance by *Aspergillus* section *Flavi* strains

Figure 1 shows the effect of different chlorpyrifos concentrations on the growth rate of four *Aspergillus* section *Flavi* strains at two a_W conditions (0.995 and 0.980). Control treatments showed a significant reduction in growth rate when a_W decreased only in AM 2 and GM 4 strains ($p<0.0001$). Regarding chlorpyrifos treatments, in general, this parameter was similar or higher

at 0.995 than at 0.980 of a_W; except in some conditions where the opposite results were observed. All the strains tested were able to grow at the highest insecticide concentration assayed (700 mg L^{-1}). Particularly, at the two highest concentrations (600 and/or 700 mg L^{-1}), higher values of growth rate were observed at 0.980 than at 0.995 of a_W for the strains AM 1, AM 2 and GM 4. At 0.995 of a_W and the lowest chlorpyrifos concentrations (100 and 200 mgL^{-1}), this growth parameter remained constant. On the contrary, lower values of growth rate comparing to the control were observed at the highest insecticide concentrations assayed ($p<0.0001$). An exception was observed in GM 3 strain where at 300 mg L^{-1} and 0.995 of a_W the growth rate was significantly higher than in the control treatment. At 0.980 of a_W, reductions in growth rate when chlorpyrifos concentration increased were observed; except in GM 4 strain. The highest values of this parameter were observed at 100 and 200 mg L^{-1} of insecticide for this strain. Meanwhile, from 400 to 700 mg L^{-1}, growth rate was similar and significantly lower than the value registered in the respective control. The reductions of growth rate observed along the experiment did not achieve a 50% reduction in comparison to control treatments.

As regard lag phase, the values registered in control treatments of all strains tested were significantly higher at 0.980 than at 0.995 of a_W ($p<0.0001$) (Table 1). In general, this behavior pattern was also observed in the chlorpyrifos treatments. As expected, significant increases in lag phases with increasing insecticide concentration were observed in all strains and a_W tested. At 0.995, the duration of the lag phases of the strain GM 3 was larger than the ones of the other strains along the experiment. The most noticeable increase in this growth parameter was observed with 700 mg L^{-1} of chlorpyrifos (78%) ($p<0.0001$). Particularly, at 0.980, the lag phases with 100 mg L^{-1} of the insecticide were significantly shorter or similar than the ones observed in the respective controls in AM 1, GM 3 and GM 4 strains. An increase of 28% in this parameter was observed in the strain AM 2 with 500 and 600 mg L^{-1}.

The analysis of variance of the effect of single (a_W, insecticide concentration and strains) and two and three-way interaction showed that insecticide concentration, a_W and all the possible interactions were statistically significant in relation to the growth rate. As regard lag phase, each single variable and their interactions were statistically significant ($p<0.0001$) (Table 2).

Growth of *Aspergillus* section *Flavi* strains in the presence of commercial chlorpyrifos formulation as sole source of carbon, phosphorus or nitrogen

Figure 2 shows the growth rate of four *Aspergillus* section *Flavi* strains in control media and in presence of different chlorpyrifos concentrations as the sole carbon, phosphorous or nitrogen source. All the strains showed a similar growth pattern in all media analyzed. The highest values of growth rate were observed in the full CZ medium (7.2 to 8 mm day^{-1}) and the lowest values were registered in the medium where the insecticide replaced the carbon source (CZC) (3.6 to 4.4 mm day^{-1}) ($p<0.001$). Regarding the modified media, this parameter was significantly higher in CZN than in CZP in all strain tested. Although the values of growth rate observed in CZN and CZP media were significantly higher than in CZC, they did not reach the values registered in CZ medium. The average reductions of growth rate observed in CZN and CZP media in comparison to CZ medium were 5 and 14% respectively.

With regard to lag phase, in concordance with growth rate results, all the strains had a similar behavior. The longest lag phases were observed in CZC medium ($p<0.001$), followed by CZN and CZP media. When chlorpyrifos replaced the phosphorous source, no significant differences between this media and control (CZ) were observed, except in GM 3 and GM 4 strains. GM 4 strain showed the greatest differences in this parameter between the CZ and CZP media (29.7 and 18.8 h, respectively). Regarding the other control treatment (WA), the lag phases in CZP media were significantly shorter than in WA media ($p<0.001$). On the contrary, no significant differences were observed between the lag phases registered in CZN and the ones registered in WA media, except in GM 4 strain (Table 3).

The analysis of variance of growth rates showed that each single variable analyzed (media and strains) and their interactions were statistically significant. On the contrary, media and their interaction with the strains were statistically significant with respect to lag phase (Table4).

Chlorpyrifos degradation in synthetic medium

Recovery of 93 ± 7.4 % was obtained from CZ medium at chlorpyrifos levels tested. Figure 3 shows the degradation of chlorpyrifos by two *A. oryzae* strains after 30 days of incubation at three a_W conditions. Both *A. oryzae* strains showed similar behavior at all conditions tested. Chlorpyrifos degradation was detected from the first day of incubation and the degradation percentage was 75% at 0.98 and 0.95 of a_W, in all concentrations and strains tested. Meanwhile, at 0.93 of a_W, this percentage was significantly lower in all concentrations and strains assayed (<25%). In general, a fast decrease of chlorpyrifos in the medium was observed within the first day of incubation, and then the biodegradation rate decreased over time ($p<0.001$).

The analysis of variance on the effect of single variables, e.g. strains, a_W and insecticide levels showed that all factors alone and all interactions were statistically significant ($p<0.001$) in relationship to chlorpyrifos degradation on synthetic medium.

Fungi have been successfully used to remove a great variety of xenobiotic compounds (Singh, 2008). Mycelia growth and extracellular enzymes provides them an advantage over others microorganisms like bacteria and yeasts. Several authors have reported microorganisms, both fungi and bacteria, with the potential ability to degrade chlorpyrifos (Maya *et al.,* 2012; Chishti *et al.,* 2013; Dhanya, 2014). There is scarce information about chlorpyrifos tolerance or degradation by fungal strains (Omar, 1998; Karas *et al.,* 2011; Maya *et al.,* 2012), even more by *Aspergillus* spp. strains (Silambarasan and Abraham, 2013; Hindumathy and Gayathri, 2013; Yavad *et al.,* 2014; Carranza *et al.,* 2014).

The results observed in the present work partially agree with those informed by Silambarasan and Abraham (2013). They observed that an *Aspergillus terreus* strain isolated from agricultural soil was able to tolerate chlorpyrifos concentrations up to 400 mg L^{-1} in a mineral synthetic medium. While the assayed concentrations in this study reached to 700 mg L^{-1} in AESO medium and we did not observed a decrease in fungal growth higher than the 50% in all strains and concentrations tested. In addition, these authors observed an increase in the dry weight of *A. terreus* in the medium with 300 mg L^{-1} of chlorpyrifos as

carbon source. This result is comparable to the observed in the present work due to the highest values of growth rate were registered with chlorpyrifos concentration between 300 and 500 mg L^{-1}. The differences in chlorpyrifos tolerance could be attributed to the media composition. The rich medium allowed a greatest tolerance of the insecticide.

In a previous work we evaluated the tolerance to three pesticides (among them chlorpyrifos) of *Aspergillus* section *Nigri*strains in soil-based medium (Carranza *et al.,* 2014). Unlike the results observed in the present study, significant decreases in the growth rate of *Aspergillus* section *Nigri* strains with increasing chlorpyrifos concentrations (5, 10 and 20 mg L^{-1}) were observed. As regard lag phase, in both studies significant increases in this parameter with increasing chlorpyrifos concentrations were registered.

The results of the replacement of the different nutrient sources by the insecticide showed that all strains tested were able to develop in a synthetic medium with the pesticide as the only carbon, phosphorous and nitrogen source. Recently we informed that these *Aspergillus* section *Flavi* strains showed the ability to develop with glyphosate as the sole source of carbon, phosphorous and nitrogen (Carranza *et al.,* 2016b). With respect to growth rate, the results were comparable for both pesticides. The highest values of this parameter were observed when both pesticides replaced the nitrogen source. Nevertheless, when chlorpyrifos replaced the carbon source the lowest values of growth rate were registered; while with glyphosate this behavior was observed in WA medium, and between the media CZC and CZP the values registered were similar. Regarding the lag phase, the strains assayed need more than 29 hours to develop in a medium where the glyphosate replaced the carbon, phosphorous or nitrogen source; whereas that with chlorpyrifos this time was lower (18 hs). The duration of this growth parameter was lower in the medium where the glyphosate replaced the nitrogen source, while with chlorpyrifos this fact was observed in CZP medium. As regard CZ medium (control treatment), the lag phases in modified media (CZC, CZP and CZN) were longer than the respective control. However with chlopyrifos, the lag phases registered in CZP medium were similar to the ones observed in the control (CZ). These results indicate that the presence of chlorpyrifos produce an induction of hydrolytic enzymes that could be involved in the insecticide degradation. The adaptation time in this media appears to depend on the way of the strains tested metabolize the insecticide.

In other study it was also evaluated the growth of *Aspergillus* strains in presence of chlorpyrifos (100 mg L^{-1}) and in presence/absence of glucose as carbon source (Hindumathy and Gayathri, 2013). These authors informed that the strains showed an increase in their protein content in the medium with chlorpyrifos and without glucose, supporting the ability of these strains to use the insecticide as carbon and energy source. These results are in agreement with those informed in the present work, since they are also reporting that chlorpyrifos can be used as the only carbon source.

Several studies inform about tolerance and degradation of chlopyrifos by different fungal species both on *in vitro* conditions and on soil, being this insecticide added as the carbon source in most of the works (Fang *et al.,* 2008; Kulshrestha and Kumari 2011; Maya *et al.,* 2012; Gao *et al.,* 2012; Silambarasan and Abraham, 2013;

Hindumathy and Gayathri, 2013;Yadav *et al.,* 2015). However, there is little information about tolerance or degradation of this insecticide added as phosphorous or nitrogen source (Omar 1998). Fang *et al.*(2008) observed that a *Verticillium* sp. strain was able to develop and increase their biomass with chlorpyrifos as the only carbon source (100 mg L^{-1}) after five days of incubation.

Maya *et al.,* (2012) obtained similar results in terms of dry weight of different fungal strains with chlorpyrifos as the carbon source (in levels between 50 and 500 mg L^{-1}). In the same way, Silambarasan and Abraham (2013) also observed a significant increase in dry weight and growth rate of *A. terreus* in a mineral medium added with 300 mg L^{-1} of chlorpyrifos as the only carbon source.

Regarding chlorpyrifos degradation, Briceño *et al.* (2012) informed that two *Streptomyces* sp. strains isolated from soil were able to degrade the insecticide at levels of 90% of degradation. A complete mineralization of 50 mg L^{-1} of chlorpyrifos after 5 days using a co-culture of bacteria and *Trichosporum* spp. was reported by Xu *et al.,* (2007).

In other study, Kulshrestha and Kumari (2011) reported a high degradation (83.9%) of chlorpyrifos by a strain of *Acremonium* sp. Also, Gao*et al.* (2012) obtained similar results with *Cladosporium cladosporioides* strain. Karas *et al.*(2011) evaluated the degradation of several pesticides (among them chlorpyrifos) by three white rot fungi and one strain of *A. niger.*

All the strains assayed were able to degrade the insecticide (20 mg L^{-1}) in a soil-based medium and in straw extract medium. A fast decrease of chlorpyrifos was registered within the first two days of incubation for all strains tested. All the results before mentioned are in agreement with the informed in the present study.

Omar (1998) observed that *A. terreus* had the greatest potential to mineralize organic phosphorous and sulfur from chlorpyrifos in liquid media followed by *A. tamari* and *A. niger* among other fungal species. At 10, 50 and 100 mg L^{-1} of chlorpyrifos, the degradation percentages were 16.7, 6.3 and 3.5%, respectively when chlorpyrifos replaced the phosphorous source; and 56.3, 24.4 and 21.7% respectively when the sulfur source was replaced by the insecticide. In the present work, higher degradation percentages were registered on optimal growth conditions (0.98, 0.95 of a_W and 25°C) and comparable results (about 25% of degradation) were observed at 0.93 of a_W.

Maya et al. (2012) observed that *Aspergillus* strains had the ability to degrade faster both the insecticide (62.3 to 92.6%) and TCP (69.4 to 89.9%) at a concentration up to 200 mg L^{-1}. These results are comparable to the degradation percentages registered in the present study.

Recently, Yadav *et al.* (2015) evaluated aerobic biodegradation of chlorpyrifos by *Aspergillus* sp. in batch and continuous packed bed bioreactors. In general, degradation percentages around 90% were observed at the optimized culture conditions (oxygen saturation: 5.8 mg L^{-1}; inoculum level: 2.5 mg mL^{-1} wet weight; pH: 7; temperature: 28°C).

These percentages were registered at chlorpyrifos concentrations up to 300 mg L^{-1}; thereafter it decreased rapidly. These results did not agree with the informed in the present study since we observed lower degradation percentages at optimal culture conditions.

Table.1 Effect of different chlorpyrifos concentrations on the lag phase (h) of *Aspergillus* section Flavi strains in SEA medium under different water activity (aW) conditions at25°C.

Strains	a_W	Lagphase (h) ± SD							
		Chlorpyrifos (mg L^{-1})							
		0	100	200	300	400	500	600	700
AM1	0.995	32.0 ± 2.7ij	33.1 ± 2.6ij	33.9 ± 3.7hi	42.6 ± 2.6ef	43.7 ± 0.6de	43.1 ± 2.0e	44.8 ± 1.5d	45.3 ± 4.8d
	0.980	37.6 ± 1.8g	35.0 ± 2.8hi	37.0 ± 1.0gh	43.4 ± 1.4e	46.7 ± 2.5c	46.3 ± 3.0cd	44.8 ± 1.2d	47.5 ± 2.4bc
AM2	0.995	31.7 ± 0.7j	32.4 ± 2.0ij	35.3 ± 1.0h	33.5 ± 1.6i	39.0 ± 2.2fg	44.0 ± 2.8de	41.9 ± 0.4f	41.3 ± 0.2f
	0.980	33.8 ± 1.5i	37.9 ± 0.7g	38.5 ± 3.4g	41.4 ± 0.3f	44.1 ± 1.2de	43.4 ± 0.3e	43.3 ± 1.7e	45.9 ± 0.7cd
GM3	0.995	30.0 ± 0.7j	33.7 ± 1.9i	39.1 ± 0.3fg	42.5 ± 1.8ef	43.7 ± 0.6de	42.9 ± 0.4e	50.4 ± 1.3ab	53.4 ± 2.4a
	0.980	35.3 ± 1.3h	35.5 ± 1.6h	42.6 ± 1.2ef	43.9 ± 0.7de	43.0 ± 0.7e	44.9 ± 1.2d	43.1 ± 3.9e	45.7 ± 0.4cd
GM4	0.995	35.1 ± 1.2hi	37.4 ± 0.8g	35.9 ± 2.0h	41.4 ± 0.9f	48.8 ± 4.0b	45.8 ± 0.8cd	47.3 ± 1.4c	44.0 ± 3.5de
	0.980	37.8 ± 2.0g	37.0 ± 2.9gh	40.4 ± 1.1fg	42.3 ± 2.2ef	45.2 ± 1.9d	44.8 ± 1.2d	46.8 ± 1.5c	48.4 ± 3.8bc

AM 1, AM 2 and GM 3: *Aspergillusoryzae* strains, GM 4: *Aspergillusflavus* strain. The letters in common are not significantly different according to Fisher's protected least significant difference (LSD) test (P < 0.0001).
SD: standard deviation. SEA: soybean extract agar.

Table.2 Analysis of variance of water availability (aW), effect of insecticide concentration (C), different strains (I) and their interactions on growth rate and lag phase on SEA medium.

Source of variation	Df†	Growth rate		Lag phase	
		MS‡	F§	MS‡	F§
I	3	0.83	5.53	92.63	23.97*
C	7	10.13	67.42*	548.01	141.80*
a_W	1	4.07	27.07*	136.87	35.42*
I x C	21	0.61	4.03*	15.86	4.10*
I x a_W	3	2.02	18.25*	34.96	9.05*
I x C x a_W	21	0.62	4.11*	14.11	3.65*

† Degrees of freedom.
‡Mean square.
§F-Snedecor.
* Significant P < 0.0001
SEA: soybean extract agar.

Table.3 Lag phases of *Aspergillus* section Flavi strains in water agar and CzapekDox agar media supplied with chlorpyrifos as the sole source of carbon, phosphorus or nitrogen.

Strains	Lagphase (h) ± SD				
	WA	CZ	CZC	CZP	CZN
AM 1	31.6 ± 1.8de	28.5 ± 0.7fg	39.4 ± 2.7a	27.6 ± 0.6gh	33.7 ± 1.0cd
AM 2	30.7 ± 1.2ef	25.1 ± 1.4h	35.0 ± 2.6bc	25.3 ± 1.1h	31.3 ± 0.5de
GM 3	32.1 ± 0.9de	30.3 ± 2.6fg	33.7 ± 0.9cd	25.7 ± 1.5h	31.6 ± 0.4de
GM 4	31.7 ± 0.8de	29.7 ± 1.0fg	36.0 ± 1.1bc	18.8 ± 1.8i	36.9 ± 3.8ab

WA: water agar. CZ: CzapekDox medium. CZC: CzapekDox medium without sucrose and supplied with chlorpyrifos at 50 mg L^{-1}, CZP: CzapekDox medium without K_2HPO_4 and supplied with glyphosate at 10 mg L^{-1}, CZN: CzapekDox medium without $NaNO_3$ and supplied with glyphosate at 10 mg L^{-1}.
Values represent the mean of four replicates. SD: standard deviation. Means from each species with the same letter are not significantly different according to the LSD test (p<0.001).

Table.4 Variance analysis of effect of media (M), strains (I) and their interactions on lag phase and growth rate of *Aspergillus* section Flavi strains in Czapek Dox agar medium supplied with chlorpyrifos as the sole source of carbon, phosphorus or nitrogen.

Source of variation	Df[†]	Growth rate		Lag phase	
		MS[‡]	F[§]	MS[‡]	F[§]
M	4	27.38	1570.27*	246.66	88.13*
I	3	2.21	126.55*	17.80	6.36
M x I	12	0.12	6.66*	20.28	7.25*

[†] Degrees of freedom
[‡] Mean square.
[§] F-Snedecor.
* Significant p< 0.001

Fig.1 Growth rate of *Aspergillus oryzae*: AM 1, AM 1 and GM3 strains; and *Aspergillus flavus* GM 4 strain on soybean extract agar at 0.995 ( ) and 0.980 ( ) of aW. Mean values based on triplicated data. Mean with a letter in common are not significantly different according to LSD test (p>0.0001).

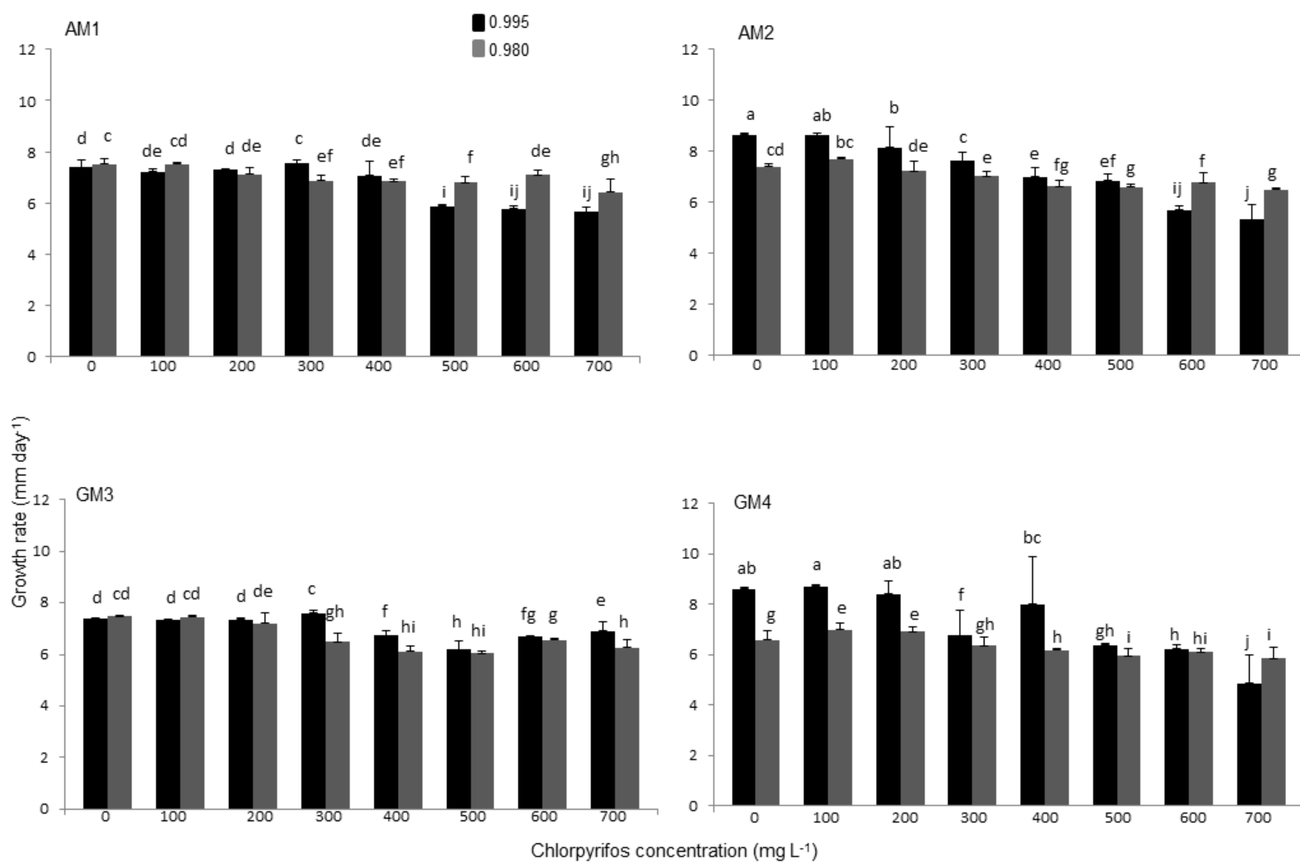


Fig.2 Growth rate of *Aspergillus* section Flavi strains on water agar WA ( ), CzapekDox medium CZ ( ), CzapekDox medium without sucrose and supplied with chlorpyrifos as the sole carbon source CZC ( ), CzapekDox medium without K2HPO4 or NaNO3and supplied with chlorpyrifos as the only phosphorous CZP ( ) or nitrogen source CZN ( ).Mean values based on triplicated data. Mean with a letter in common are not significantly different according to LSD test (p□ 0.001).

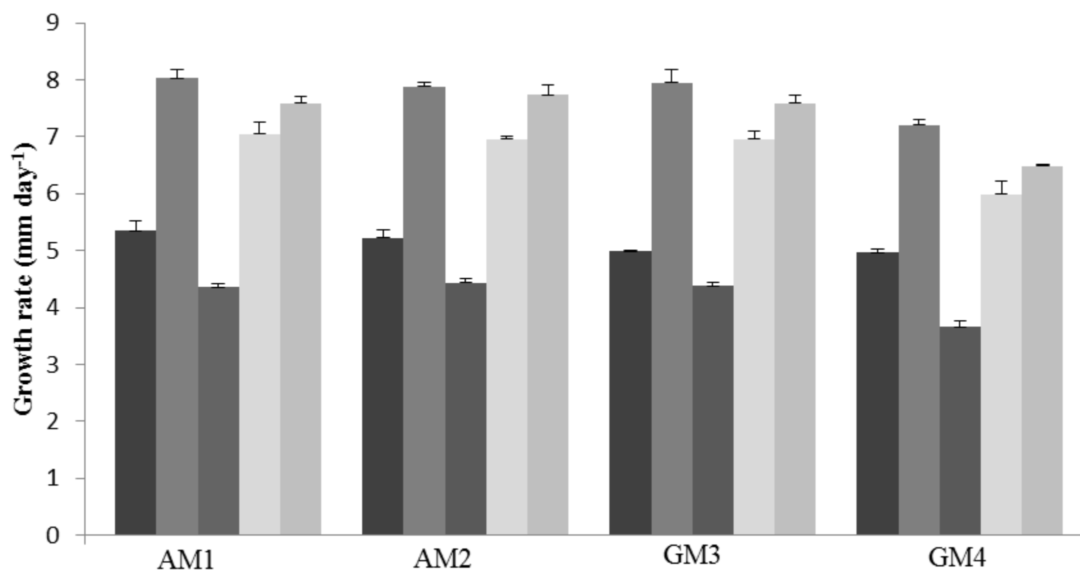
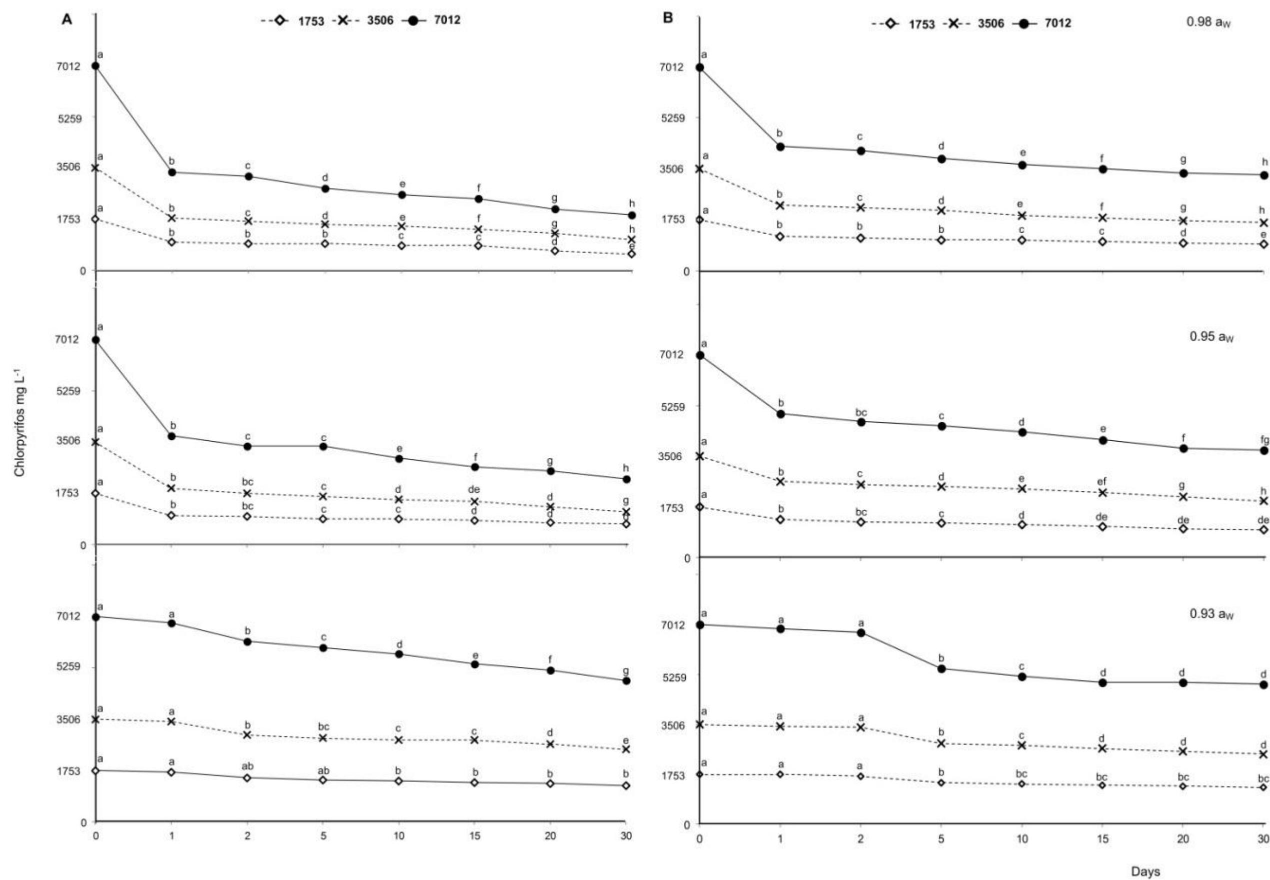


Fig.3 Chlorpyrifos degradation on broth Czapeck medium (CZ) adjusted to 0.980, 0.950 and 0.930 of aW by *A. oryzae* AM 1 (A) and AM 2 (B) strains. Mean values based on triplicated data. Mean with a letter in common are not significantly different according to LSD test (p>0.001).



Hindumathy and Gayathri (2013) studied chlorpyrifos degradation (100 mg L^{-1}) in a mineral synthetic medium by both bacteria and fungi (*Aspergillus* species) with or without the addition of glucose. These authors observed that both bacterial and fungal strains were able to degrade the insecticide at high percentages alone (73.3 and 58% respectively) and with the addition of the carbon source (84 and 76% respectively). These results partially agree with the registered in the present study. A 75% of degradation at 0.98 and 0.95 of a_W in all concentrations and strains tested was observed. These water availability conditions and 25°C are the optimal conditions for the growth of the strains tested. Meanwhile, at 0.93 of a_W(a stress condition) and optimal temperature, this percentage was reduced significantly (<25%) in all concentrations and strains assayed. It is important to mention that in the present work we observed that the water availability, at optimal temperature, affect the degradation of the insecticide. This variable was not considered in the other studies.

These non-toxigenic *Aspergillus* section *Flavi* isolated from agricultural soils have the ability to tolerate high levels of chlorpyrifos (70 times higher than the dose used at field fumigation). They can use the insecticide mainly as phosphorous and nitrogen source, and to a lesser extent as carbon source. The degradation studies showed that the *A. oryzae* strain has good chlorpyrifos degradation ability under optimal environmental conditions (a_W and temperature) for growth. As cleavage of chlorpyrifos depend on the microorganisms and environmental conditions, these studies indicate the need to detect the degradation products and to perform *in situ* degradation tests (microcosm conditions and soil).

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