Global Research Journal of Microbiology Vol. 4(1) pp.10 –16 January 2014.
Available online http://www.globalresearchjournals.org/journal/grjm

Full Length Research.

Atrazine degradation by wild filamentous fungi

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Accepted 27th December 2013.

Bioremediation is a promising technology for the treatment of polluted areas due to its minor cost; moreover, indigenous fungi had not been already applied to detoxify contaminated habitats. The kinetic of atrazine degradation by *Gliocladium roseum*, *Mucor alternans* and *Pycnidiophora dispersa* were assessed in soluble and soil sorbed herbicide with different organic matter content. Their half-saturation constants, Km, expressed the affinity for the substrate, being 12.5, 3.8 and 2.7 µg/ml for *G. roseum, M. alternans* and *P. dispersa*. Moreover, Vmax, uptake rate/ biomass, assumed by the asymptotes of each fungal curves were 43.5-41.0, 37.5-35.0 and 26.5-24.5 µg Atz/min^{-1}. mg for *G. roseum*, *M. alternans* and *P. dispersa,* respectivelly. The 65-75% was in soluble phase, that was preferentially degraded by fungi. Our results showed that the atrazine transformation were consistent with those in pure cultures, suggesting that the parameters derived from *in-vitro* studies may be useful to predict the herbicide detoxification in polluted sediments. Fungal kinetic allowed us to predict the atrazine degradation in natural contaminated habitats and was in relation to the herbicide levels especially in soil solutions.

Keywords: atrazine degradation - micoremediation - polluted soils - wild filamentous fungi
Abbreviations: Atz atrazine - MM mineral medium

Introduction

The use of wild fungi for the bioreclamation of polluted soils had become the focus of considerable attention due to their high detoxification potential of a great variety of toxicants and the considerable cost savings, compared with other technologies (Coccia *et al*., 2009; Romero *et al*., 2010; Sannino *et al*., 2010). Incineration or burial methods had became insecure for landfills and highly expensive when contaminants are large; mechanical and chemical techniques have limited effectiveness and are also expensive. Bioremediation is the promising technology for the treatment of contaminated sites since it is cost-effective and would lead to complete mineralization (Singh, 2006; Das and Chandran, 2011).

The fungal abilities to degrade diverse agricultural pollutants had been documented in white-rot fungi (Simões, 2003; Khiyami *et al*., 2006; Pereira *et al*., 2013b; Tortella *et al*., 2013); moreover, yeasts had been also mentionated as organic toxicants degraders, like *Pichia* spp. (Abigail *et al.,* 2013), *Trichosporon* spp. (Huang *et al*., 2012) and *Saccharomyces cerevisiae* (Marius *et al*., 2013; Gaytán *et al*., 2013).

Among filamentous fungi, the kinetic degradation of other pesticides had been confirmed in *Aspergillus niger* (Marinho *et al*., 2011; Pereira *et al*., 2013a), cladosporium-like hyphomycetes (Seifert and Hughes, 2007; Wang *et al*., 2012), *Penicillium* spp., *Rhizopus stolonifer* and mycelia sterilia (Martins et al., 2013; Ortiz-Hernández *et al*., 2013) and species were more frequently isolated in polluted soils and developed a significant higher biomass also in adverse habitats (Romero *et al*., 2001; Romero *et al*.; 2005).

At sites where the appropriate indigenous species are present, remediation may consist in optimizing the habitat conditions and environmental factors to enhance the survival and proliferation of the wild degrading species (Valentín *et al*., 2007; Megharaj *et al*., 2011).

To increase the pesticides transformation is essential for restoring soils, but different factors affected the success or failure of the bioremediation strategy, such as pH, texture, aeration, nutrients and moisture content. Although these parameters can be optimized to enhance the survival and proliferation of any particular biodegrader, successful biodetoxification was still a hard task (Mishra *et al,* 2001; Baborova *et al*., 2006). Therefore, the purpose of this study were to isolate filamentous fungi able to degrade the herbicide atrazine (Atz), to evaluate the transformation rates of wild species with different half-saturation constants, and to assess the effect of diverse organic matter content.

Material and methods

Isolation and identification of atrazine degrading fungi. Filamentous soil fungi were isolated from 5 different sites of agricultural polluted soils, near La Plata, Argentina. Soil characteristics (Table 1) such texture, pH, C-organic (%), N-organic (%), P-organic (%) and PAH mg. kg soil^{-1} were determined according to Soil Survey Investigations (SSI, 2009).

Table 1: Physical and chemical properties of the 5 agricultural soil samples used to isolate the degrading fungi (x ± SD)(Soil Survey Investigations, SSI, 2009).

soil samples	1	2	3	4	5
sand:clay:lime (%)	69:18:13	72:17:11	67:15:10	75:19:15	80:20:14
pH	6.8	6.5	6.9	6.9	6.4
C-organic (%)	7.8±0.4	7.0±0.2	6.8±0.5	7.5±0.4	7.3±0.6
N-organic (%)	0.38±0.01	0.50±0.02	0.48±0.02	0.58±0.01	0.60±0.03
P-organic (%)	0.09±0.008	0.10±0.006	0.12±0.005	0.08±0.004	0.11±0.004
mg PAH kg soil^{-1}	97.5 ±2.5	120.0±7.0	148.5±5.5	133.0±8.5	150.5±9.3

The degrading fungi were isolated by spread plate technique on a mineral medium (MM, (Romero *et al.,* 2002) with 100 ppm Atz as sole carbon source and 75 ml.l^{-1} antibiotic solution (5.0 g streptomycin, 2.5 g chloramphenical, 1.0 l distilled water) to avoid bacterial growth (American Public Health Association, 1992). The samples and two control-sets, MM-sterile and MM-without Atz plates, by duplicate, were incubated at 27^{o}C, in dark, for 30 days. The same MM-Atz medium was employed to enumerate all the filamentous fungi, and the assays were realized with three degrading fungi.

Different culture media were used to induction sporulation of fungal isolates to identify the sporulating strains; the species were identified by according to morphological characters of colonies culture morphologies, spores conidia charachers as well as ascospores (Romero *et. al*., 2005), and by upper and down agar-plates color. *Penicillium s*pp. were cultivated in special media at 25ºC, non sporulating strains were grouped as mycelia sterilia.

Fungal dry biomass were estimated gravimetrically by centrifugation 10 ml of each culture suspension at 8000 rpm for 10 min; then the supernatant was discarded and the pellet dried at 90ºC for 24 h and weighed.

Degradation assays. Batch cultures with 500 ml Atz-medium were inoculated with 5.0 mg mycelia suspension of the species, and periodically subsampled to assess the insecticide uptake kinetics. Fungal dry weight were prepared by homogenizing the cultures, centrifuged at 15000 x g for 15 min, dried at 60ºC till constant weight. Sterile aliquots of two soil types, with different organic matter content, 1.6 and 14.0% (Metting, 1995), were adjusted to field capacity with MM amended with 0, 1, 10 or 100 µg Atz/g soil; then, 500 mg Atz-solid medium were inoculated with mycelial extracts and incubated at 28ºC for 48h on a rotary shaker at 120 rpm.

Concentrations of soluble Atz were determined by analyzing samples of pore water pressed from soils, and levels of sorbed insecticide were assessed by ethanol solvent extraction and analyzed by HPLC (Romero *et al*., 2005). Extraction efficiencies were 89% and 75% for low and high-organic matter soils and the data were used to adjust the analytical results.

Data analysis: the relative frequency of the fungal growth in Atz-plate medium was calculated as the number of species from each soil sample divided by the total number of sediment samples (30), and were classified as very frequent (>20%), frequent (5-20%) or infrequent (<5%, Tan and Leong (1989). The Atz-percent in soil solution was calculated by: [(µg Atz/ml pure water) x (ml of water per gram of wet soil/µg Atz per gram of wet soil)] x 100. All values were the average of 2 assays for each fungi culture corrected by control flasks (sterile soils without inoculants).

Results

All the filamentous fungi that growth in the Atz-agar plates with the herbicide as sole C source were enumerated, but the degradation assays were realized with degrading fungi selected on the basis of their prevailing growth on subsequent plating in Atz presence, with significantly uptake activity and frequent in the polluted soil samples. *Aspergillus fumigatus (A.f.), Aspergillus niger (A.n.), Cladosporium cladosporioides (C.c.), Cladosporium herbarum (C.h.), Fusarium oxysporum (F.o.), Gliocladium roseum (G.r.), Mucor alternans (M.a.), Penicillium chrysogenum (P.c.), Penicillium thomii (P.t.), Penicillium verrucosum (P.v.), Pycnidiophora dispersa (P.d.), Trichoderma harzianum (T.h.)* and mycelia sterilia (I) and (II) grew in Atz-agar plates. *C. herbarum*, *F. oxysporum* and mycelia sterilia (I) were infrequent and all the others frequents species (Fig. 1).

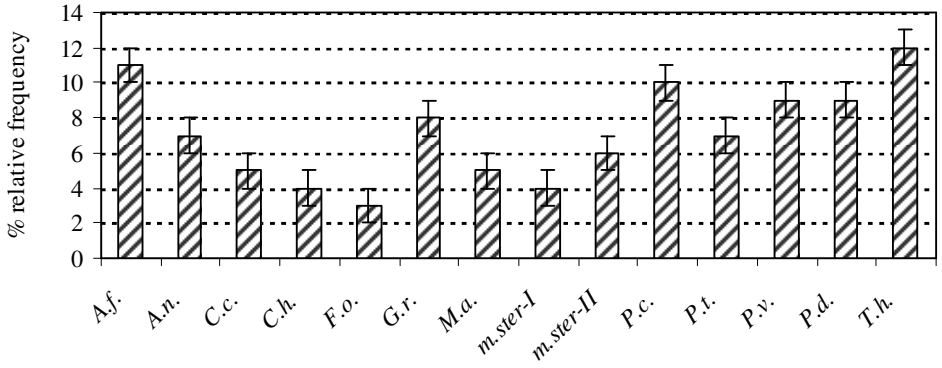


Figure 1: Relative frequency (%) of the Atz-degrading fungali species (*Aspergillus fumigatus (A.f.), Aspergillus niger (A.n.), Cladosporium cladosporioides (C.c.), Cladosporium herbarum (C.h.), Fusarium oxysporum (F.o.), Gliocladium roseum (G.r.), Mucor alternans (M.a.), Penicillium chrysogenum (P.c.), Penicillium thomii (P.t.), Penicillium verrucosum (P.v.), Pycnidiophora dispersa (P.d.), Trichoderma harzianum (T.h.)* and mycelia sterilia (I) and (II))

Gliocladium roseum (*G.r.*), *Mucor alternans* (*M.a.*) and *Pycnidiophora dispersa* (*P.d.*) were showed a significant growth with Atz as C source, and selected for the kinetics evaluation of the herbicide. The hyperbolic model was in accordance with the first-orden uptake kinetic, being the relationship that better fitted to the filamentous fungi cultures in Atz presence. Their half-saturation constants, Km, expressed the affinity of the fungi for the substrate uptake, being the Km values 12.5 µg/ml for *G. roseum*, 3.8 µg/ml for *M. alternans* and 2.7 µg/ml for *P. dispersa* (Fig. 2). Km, the intercept value (ug/ml) of the hyperbolic curves when $Y = Y_0$, were calculated from the X-eje or Atz-level (ug/ml) when Y = 0.

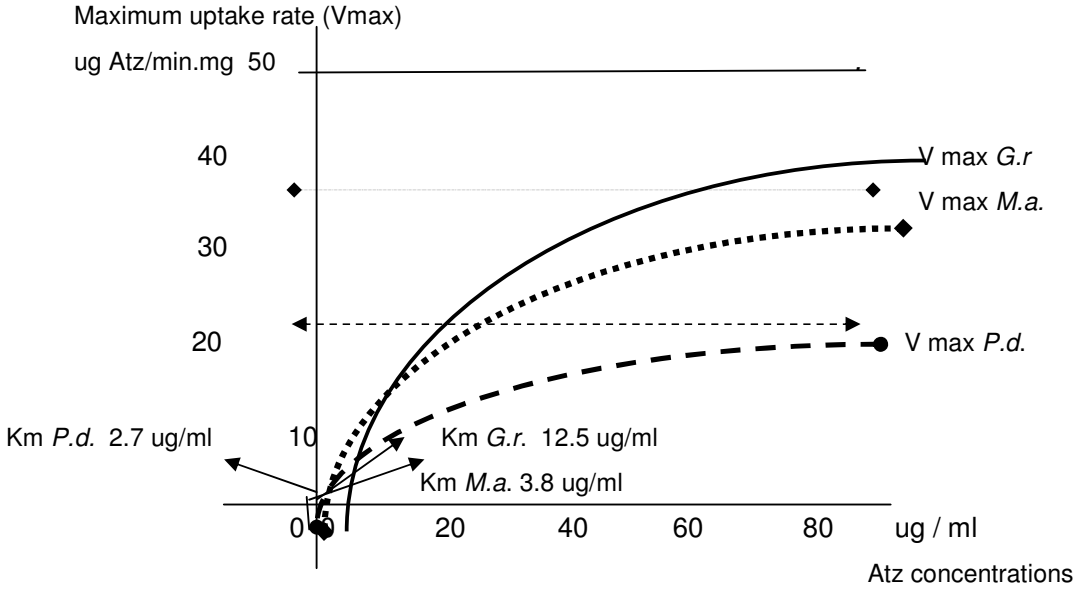


Figure 2: Kinetic curve obtained with the fungi cultures at different Atz levels (*Gliocladium roseum* (*G.r.*), *Mucor alternans* (*M.a.*) and *Pycnidiophora dispersa* (*P.d.*))

The low-substrate-concentration-adapted species had been thought of having small Michaelis constants; moreover, to understand oligotrophic strategy of fungal response to substrates in low-environmental-levels, like most of the pollutants, the specific affinity of the strains to each toxicant was important. The reciprocal relationship between affinity and Michaelis constant and the traditional idea that good oligotrophs showed

logarithmic form of Km and specific affinity, a^o_A. So, to understand the fungal adaptation to low-level of pollutants and oligotrophy the relationship $V_{max} = a^o_A$ Km (equation 1) were applied, where a^o_A was the specific affinity of each fungi species for the xenobiotic, and Vmax the maximum uptake rate of the contaminant by the fungi. This equation was converted to logarithmic form, and our data and available results from the

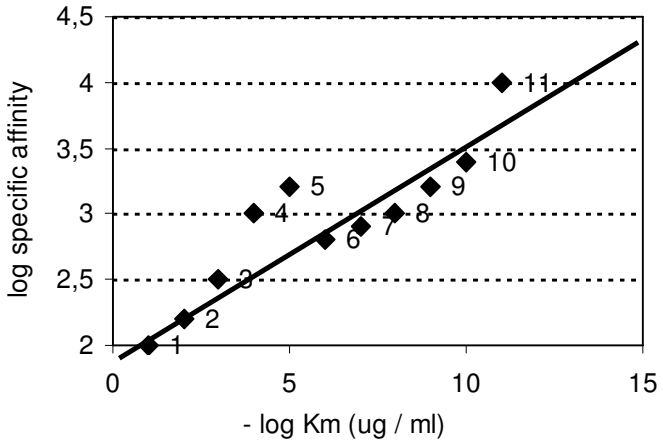


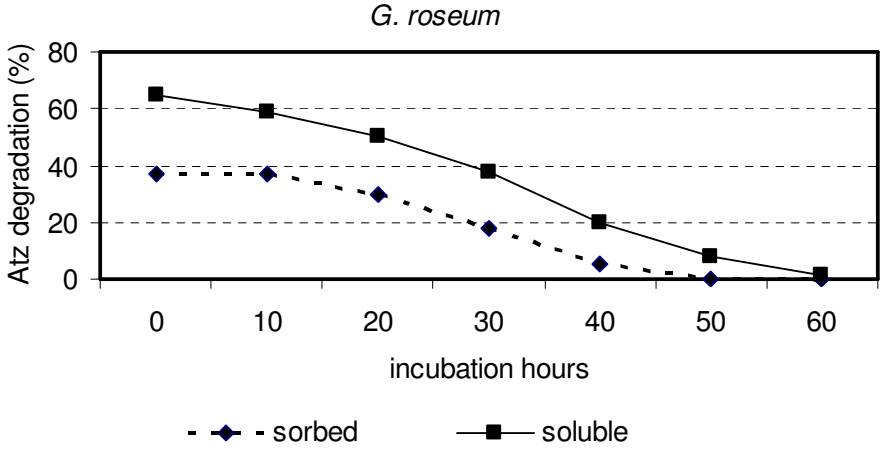
Figure 3: Plot of log a^{o}_{A} = log Vmax - log Km (equation 2). Shown data for 3-methylcatechol (point 1), asparagine (point 2), fructose (point 3), methylphenol (point 4), p-chlorophenol (point 5), glucose (point 6), glycerol-3-phosphate (point 8) and toluene (point 9) from reference (Button, 1985); point 7, 10 and 11 from this paper.

The Atz metabolic rates were assessed by Vmax, maximum uptake (ug/min. mg), that were the asymptotes of the equation (1) for each fungal curves. The obtained rates were 43.5-41.0 µg Atz/min^{-1} mg for *G. roseum*, 37.5-35.0 µg Atz/min^{-1}.mg for *M. alternans* and 24.5-26.5 µg Atz/min^{-1}. mg for *P. dispersa*.

The relationship of the Atz-uptake with sampled sites properties, like texture, pH, C-organic, N-organic, P-organic and aromatic hydrocarbon concentratrions as index of organic pollutants, were not significant ($P > 0.05$). By other hand, Atz transformation significantly related to the Atz-sorbed to the soil particles and to the levels that remained in solution.

The Atz degradation rates in low organic matter soil, 1.6%, at field capacity with either *G. roseum, M. alternans* or *P. dispersa* were monitored, being 25-35% of Atz- sorbed to soil particles at the herbicide levels of 10 and 80 µg Atz/g soil, respectively, being the remainder herbicide in soil solution, 75-65%.

Different rates for soluble and sorbed herbicide were observed. *G. roseum* degradation rates were 100-fold and 60-fold higher than the *M. alternans* and *P. dispersa* ones, being the pore-water Atz metabolized at the first 20, 33 and 48 h, respectively (Fig. 4). Partitioning between soluble and sorbed phases at 1 µg Atz/g soil was 0.4 to 0.6, respectively, and pore-water Atz were degraded quickly and to a greater extent by *G. roseum* than by the others fungi.



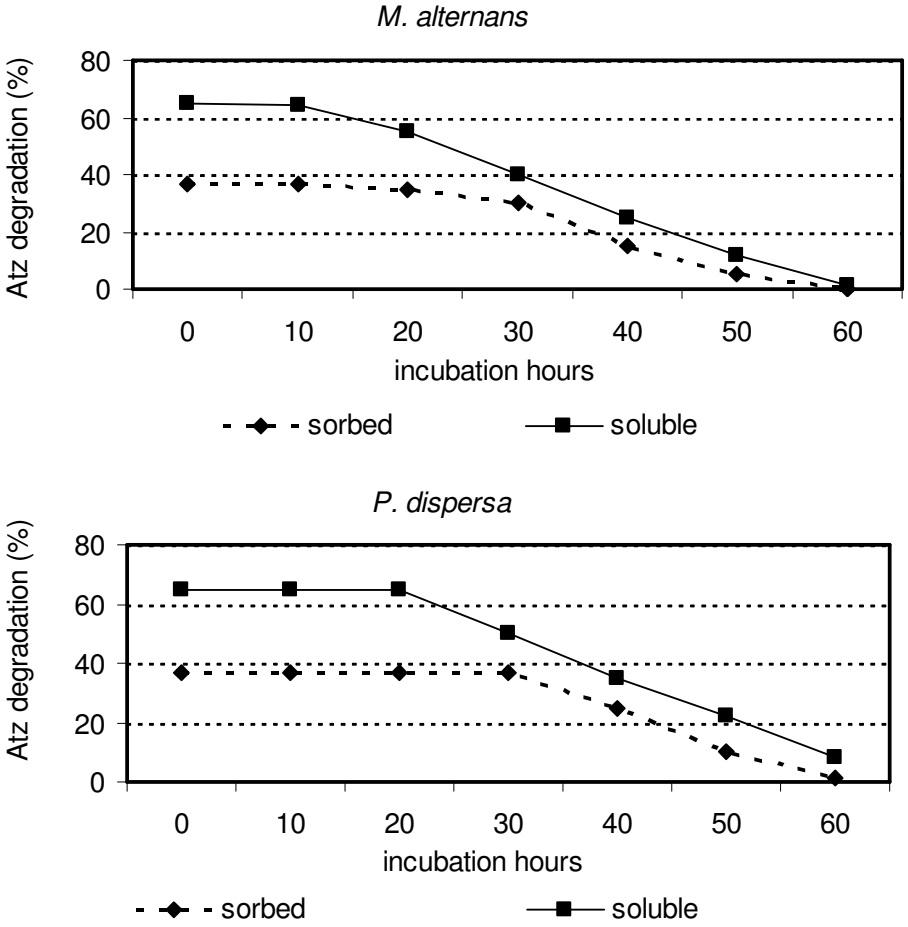


Figure 4: Atz degradation (Atz percentage, 100 ug Atz / g soil) during the assays [(a) *G. roseum*, (b) *M. alternans*, (c) *P. dispersa*)], at field capacity in low organic matter sediments.

In high-organic matter soil, 14%, and field capacity amended with 100 µg Atz/g soil, the fraction sorbed to particles was 75% and the pore water concentrations was ca. 30µg/ml. Soluble and sorbed Atz were simultaneously metabolized in *G. roseum* presence; 70h after inoculation, pore water-Atz was below the detection limit while 30% of the sorbed herbicide was still not uptaken. In the *M. alternans* and *P. dispersa* assays the 43 and 50% of the sorbed Atz remainded not degraded, and pore-water herbicide decreased to the detection limit just at 120 and 136 h incubation time. There was negligible Atz degradation in low-organic-matter soil during the incubation without inoculum, control flasks, although the indigenous fungi was in the order of 0.8 mg (dry weight)/g soil; nevertheless, in high-organic matter soil with autochthonous fungi biomass ca. 1.2 mg/g, a slightly transformation was observed. Although, the ability of indigenous soil strains to transform *in-situ* a wide variety of xenobiotics had been documented, in this case a limit capacity was obtained; thus, providing that introduced species with higher degradation abilities need to be assessed.

Results were significantly different in *G. roseum* than in *M. alternans* and *P. dispersa* experiments, with a quick 10-fold loss in Atz levels within the first 2 days followed by a lower decreased rate.

Even when metabolically and physiologically competent fungi were available, either indigenous or added, detoxification dependented on acceptable rates and extents of the biodegradation. The activities were function of the microbial constants in conjunction with toxicant bioavailability, according to the Michaelis-Menten model; thus, the half saturation constant (Km) and the chemical availability defined the degradation rates.

Discussion

The Km obtained in this study with fungi were similar and as effective as those observed with Atz-degrading bacteria taking into account that bacteria was the first and best microorganisms used in bioremediation technologies (Garcia-Valcarcel *et al*., 1998; Grimm *et al*., 2004). Similar adaptation to herbicides was found in the yeast *Saccharomyces cerevisiae*, but mediated by genes (Simões, 2003; Gaytán *et al*., 2013) and some filamentous fungi, like *Cladosporium* spp., *Rhizopus* spp. and *Penicillium* spp. (Morgana *et al*., 2012).

Therefore, soil fungi, yeasts and filamentous species were as efficient as bacteria to remove toxicants from polluted habitats (Soares *et al*., 2011; Jecu *et al*., 2013). Furthermore, the soil properties, such us percentages of sand, silt, clay, cation exchange capacities, organic carbon and nitrogen contents affected the bioavailability of the organic components presented in soils (Radosevich *et al*., 1997; Jenks *et al.,*1998). In our case, high organic matter increased the herbicide sorbed to the soil particles, that was the Atz fraction with minor fungal degradation activity, in accordance with other researches (Park *et al*., 2003; Megharaj *et al*., 2011).

In conclusion, these data indicated that fungal kinetics were a function of the herbicide concentrations especially in soil solutions. Whereas the Km values of the species were not comparable, the V values, uptake rate per biomass, were rather similar. The results showed that the fungal degradation in soil solution were consistent with those in pure cultures, suggesting that the kinetic constants derived from culture studies may be useful to predict the pollutant transformation in soils.

Acknowledgements

This work was supported by grants from the National Council of Scientific and Technological Research - CONICET; SECYT and from the National University of La Plata (UNLP), Fac. Ciencias Veterinarias, Micología Médica e Industrial, Argentina.

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