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BIOLOGICAL TRANSLOCATION OF PARATHION METHYL BY *PILOBOLUS* SP. IN CORN MICROSYSTEMS

Parathion methyl is a widely used organophosphorus pesticide (OPs). OPs in agroecosystems cause severe health and environmental effects. In this study, the *Pilobolus* inoculum's ability to remove parathion methyl in microsystems corn crop was evaluated at the laboratory level. Agricultural soils containing *Pilobolus* inoculum were exposed to 50 μg/dm^3 of parathion methyl. The results showed that *Pilobolus* inoculum can eliminate parathion methyl in 80% of microsystems, and positively influences 20% of the physiological processes of corn plants. This research into the field of myco-remediation widens the use of little-studied fungal species such as *Pilobolus* to remove pesticides.

1. INTRODUCTION

The widespread use of organophosphorus pesticides (OPs) is a serious environmental pollution problem that is emerging in recent years. Parathion methyl (O, O-dimethyl O-4-nitrophenyl phosphorothioate) is one of the most hazardous OPs worldwide used. OPs are employed for disease vector control and agricultural and household pests. Parathion methyl is known to harm non-target organisms; therefore, exists a serious concern about the toxicological and environmental risks associated with parathion methyl

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residues [1, 2] which affect the central nervous system through the non-reversible phosphorylation of esterases, mainly acetylcholinesterase, responsible for the hydrolysis of acetylcholine neurotransmitters. This could result in severe health problems in the visual system, sensory function, and cognitive function, both in humans and animals [1–4].

Parathion methyl was introduced into the market in the early 1950s and has been extensively used (as an agricultural insecticide) due to its high efficiency, low bioaccumulation, relatively short half-life in the environment, and low price [5]. Because of its high toxicity, parathion methyl has been classified as an extremely hazardous insecticide by the World Health Organization and its use has been restricted by the U.S. Environmental Protection Agency [1, 5, 6].

Many methods for OPs detection have been proposed, including the Association of Official Analytical Chemists methods, and enzyme-linked immunosorbent assays. These assays require expensive instrumentation, complex sample preparation or purification procedures, and costly bio-molecular reagents. As an alternative to the expensive instrumental and laborious anti-body methods, fluorescence measurements have been successfully developed as a qualitative and quantitative screening tool for OPs detection. Quantum dots owing to the quantum-size confinement effects have unique spectral and electronic properties such as narrow emission spectra, water solubility, and high light stability, resulting in being widely used for biological detection [2, 4]. The indiscriminate use of pesticides has led to public concern about their disposal and accumulation in the environment, as well as the exposure of non-target organisms, which may be affected by their toxicity. The best-known treatments include the removal of the contaminated material and disposal in landfills, in situ incineration, or in situ biological remediation [7].

Pesticides can be degraded by microbial, chemical, and photodegradation processes in the environment. Nonetheless, microbial degradation is considered the major determining factor of the Ops' fate in the environment and is often the main process of pesticide degradation in soils, representing the safest, least disruptive, and most cost-effective treatment method [3, 7]. The biodegradation of parathion methyl by bacteria has been described [8–11]; however, the biodegradation of parathion methyl by fungi is still underexplored in comparison with bacteria studies. Fungal biomass is a promising source for the degradation of parathion methyl, utilized degrading enzymes, and shows unique properties such as high tolerance, and adaptability [12, 13]. Therefore, the main objective of this work was to evaluate the ability of *Pilobolus* sp. to remove parathion methyl in corn crop microsystems, and its influence on the growth of corn plants.

2. MATERIALS AND METHODS

An analytical standard solution of 50 $\mu g/dm^3$ parathion methyl (Sigma Aldrich) was used in corn microsystems [12].

Growth of Pilobolus fungi in the experimental systems. Dung samples were collected at San Miguel Zinacantepec municipality (19°17′–19°28′ N and 99°44′– 99°73′ W), state of México. Considering that the dung samples were fresh, they were placed in sealed containers for transportation to the laboratory. 1 kg of dung inoculated with *Pilobolus* spores was placed in three boxes 35×20 cm with red cellophane on top as infrared wavelengths increase fungi proliferation. The culture remained at 28 °C and 60% relative humidity in a greenhouse; under these conditions, the fungus began to bear fruiting structures (sporangia) after 5–6 days. The fungus was morphologically characterized by optical and stereoscopic microscopy (Fig. 1). Sporangia were collected directly from the grow boxes, and placed in a solution of 10% nitric acid to release the spores. With this solution, the corn microsystems were inoculated [6–13].

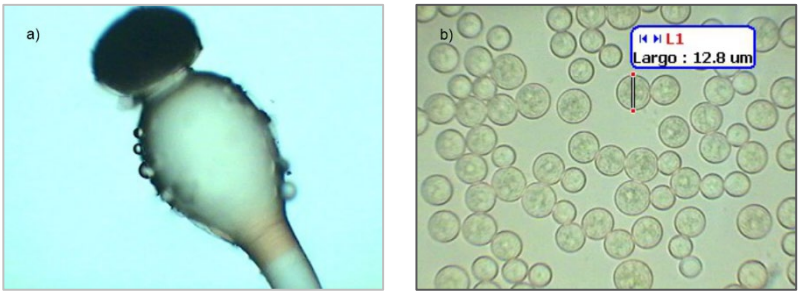

Fig. 1. *Pilobolus* fungi in the experimental microsystems: a) vesicle sporangial, b) spores

Soil collection and corn microsystems establishment. Three agricultural sites in the same area, where the cattle dung was collected, were selected. A total of 30 plots of 10×10 m, ten for each agricultural site, were sampled. In each plot, 10 soil cores were randomly collected (to obtain a representative sample per plot) at a depth of 0–20 cm. Before soil sampling, the litter was retired manually. The samples from the 10 cores were mixed and homogenized in a single sample. After the soil samples were deposited and air dried in a greenhouse for 72 h at 28 °C, ground and sieved with a 2 mm mesh (stainless steel). The soil samples were sterilized in an autoclave at 0.14 MPa pressure and 120 °C for 30 min. Finally, the soil samples were stored at –5 °C until later use.

For the microsystem establishment, 300 g soil samples were placed in 30 pots, watered with 50 cm^3 of the parathion methyl (50 $\mu g/dm^3$) solution, and inoculated with *Pilobolus* spores [3, 12]. Subsequently, in each pot, 3 seeds of the corn cacahuazintle variety were planted. Corn seeds were collected from the same agricultural area, and variety was chosen because it has a higher rate of growth compared with other varieties of corn. The seeds were germinated and grown for 15 days under greenhouse conditions (28 °C and 60% relative humidity). After 15 days, at the end of the experiment, biomass data, total length (root-aerial part, cm), and weight (complete plant, root, stem, and leaves, g) data of the corn plants were collected.

Determination of parathion methyl in soil samples, Pilobolus inoculum, and corn plants. For the determination of parathion methyl in soil samples, the spectrophotometric method was used based on the reaction of Ellman. The cholinesterase activity was determined using a volume of mouse serum, which was obtained from male Wistar mice obtained from our animal facility. Mouses were housed in a temperature-controlled room under a 12/12 h dark/light cycle with access to food and water. All experimental procedures were approved and conducted according to the Institutional Ethical Committee. Mouses were deeply anesthetized with sodium pentobarbital intraperitoneally and transcardially perfused. Blood was collected directly from the right atrium and placed in tubes, centrifuged at 2500 rpm for 20 min at room temperature, and the serum was separated and stored for later analysis.

The reaction pseudocholinesterase catalyzes the hydrolysis of acetylcholine, forming thiocholine and acetic acid. Thiocholine is released yielding a compound of yellow color, which absorbs at 405 nm (the rate of appearance of the yellow color is proportional to the enzyme activity). The serum was incubated with an extract of soil polluted with parathion methyl. The contaminated soil inhibits the mouse enzyme; therefore the hydrolysis is diminished and thiocholine is not generated (formation of the yellow complex). The amount of light absorbed at 405 nm is inversely proportional to the pesticide concentration, therefore, the greater the contamination, the lower the activity of the enzyme (yellow complex) [4, 6, 7].

For the quantification of the enzymatic activity (inhibition), 20 g of soil together with mouse serum was incubated at 25 °C for 24 h. Then 50 cm^3 of acetone was added to each sample, and they were stirred for 10 min. The serum was filtered through Wattman paper, collected in beakers, and heated in a water bath at 35–40 °C until the solvent was evaporated. 2.5 cm^3 of deionized water was added to the residue obtained and stirred for 5 min. Afterward, samples of 50 µl of this suspension were taken, placed in Ellman reaction tubes, and incubated with 40 µl of mouse serum for 10 min. Finally, their absorbances were measured at 405 nm [4, 6, 7].

After the experiment, parathion methyl in corn plants (25 g) and *Pilobolus* inoculum (5 g as a stock of biomass; sporangia, vesicles, and hyphae) concentrations were determined by the method of alkaline hydrolysis based on the spectrophotometric measuring the amount of *p*-nitrophenol, the product of hydrolysis of parathion methyl in alkaline conditions [4, 6, 13]. Initially, spectrophotometric characterization of the hydrolysis of parathion methyl to p-nitrophenol was performed. A 30 µg/dm^3 solution of parathion methyl was prepared (technical grade) in 1 mol/dm^3 NaOH. Its absorption spectrum at the range of 900–200 nm was taken and the maximum absorption of *p*-nitrophenol [4] at 405 nm was found. Subsequently, a calibration curve (Fig. 2) was prepared using technical grade parathion methyl at concentrations from 0.1 to 30 µg/dm^3 in an alkaline solution (1 M NaOH) to induce hydrolysis. Measurements were read 3 min after the solution was prepared [6].

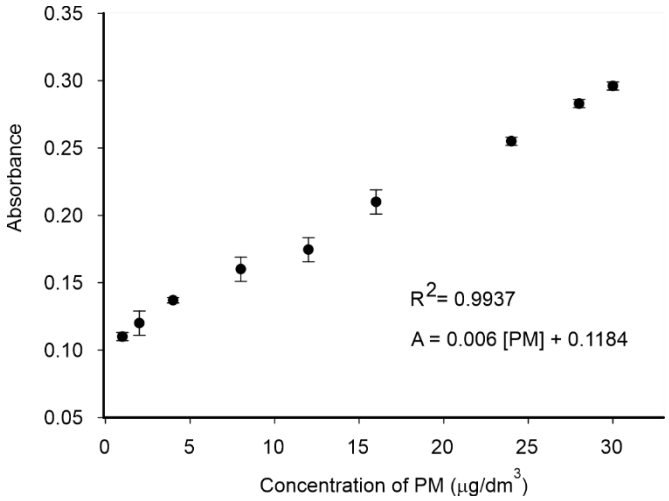

Fig. 2. Calibration curve for the parathion methyl reactive grade

Quantification of chlorophyll a, b, and carotenes in corn plants. Chlorophyll and carotenes (pigments) were evaluated in corn plants to monitor the qualitative effect of parathion methyl on the physiological processes of the plants, and the efficiency of photosynthesis, for which the method of adjusted quantifying chlorophyll a, b, and carotenes [14] was used. 1.5 g of macerated leaves of corn was placed in 15 cm^3 of acetone for 20 min. To make extraction more efficient, a closed agitation was applied, and then the pigments absorbance was measured by UV-visible spectrophotometry at the wavelengths of 662, 645, and 470 nm for chlorophyll a, b and carotenes, respectively. The formulas used for quantification of the pigments (in μg/g) are: $C_{\text{Chlorophyll a}} = 10.81A_{662} - 0.75A_{645}$, $C_{\text{Chlorophyll b}} = 19.02A_{645} - 3.98A_{662}$, $C_{\text{Carotenes}} = 3.775A_{470} - 0.21C_{\text{Chlorophyll b}}$.

3. RESULTS AND DISCUSSION

3.1. PARATHION METHYL IN SOIL SAMPLES, *PILOBOLUS* INOCULUM, AND CORN PLANTS

The soil samples without *Pilobolus* showed a maximum absorption band at 405 nm, characteristic of p-nitrophenol (Fig. 3a), which is proportional to the concentration of parathion methyl [3–6]. In contrast, in soil samples with *Pilobolus* inoculum, a low concentration of parathion methyl were detected (less than 20%). The reduction in parathion methyl (close to 80%) concentration in soil samples might be due to the biological activity of the *Pilobolus* inoculum [15]. However, despite parathion methyl having a half-life short in the environment, it may also stay persistent in the soil [3–6].

Pilobolus inoculum samples, extracted from the microsystems showed a low parathion methyl concentration or *p*-nitrophenol (Fig. 3b). Similar studies of other authors displayed

low concentrations of pesticides on the sporangia, vesicles, and hyphae of fungus [12, 15]. It suggests that some fungi such as *Aspergillus* sp. and *Penicillium* sp. can degrade parathion methyl to *p*-nitrophenol [15]; therefore, *Pilobolus* inoculum might also have the ability to degrade parathion methyl molecule, which would allow better physiological functioning of corn plants [16]. This assumption could support the low concentrations of parathion methyl found in our study; however, it will have to be evaluated in future research.

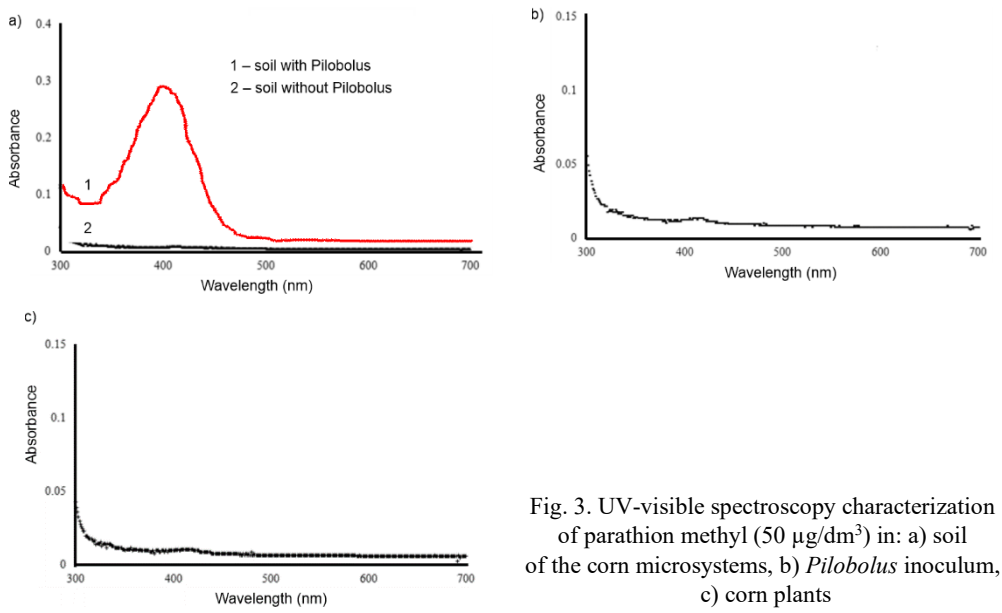

Fig. 3. UV-visible spectroscopy characterization of parathion methyl (50 µg/dm^3) in: a) soil of the corn microsystems, b) *Pilobolus* inoculum, c) corn plants

A very low, practically close to zero concentration of parathion methyl or *p*-nitrophenol in the leaves of corn plants was detected after the *Pilobolus* inoculation (Fig. 3c). Other research has also evaluated the presence of the pesticide in the roots, stem, and leaves, where low concentrations of parathion methyl has been detected [6, 9]. In addition, corn plants may accumulate pollutants such as heavy metals and pesticides in their tissues [16, 17], bringing with it a severe problem for human health. Therefore, the *Pilobolus* inoculation in corn plants might have a positive impact on the physiology of plants, not allowing the accumulation (possibly due to the degradation of this molecule) [18–20].

3.2. THE IMPACT OF PARATHION METHYL ON CHLOROPHYLL A, B, CAROTENES AND BIOMASS IN CORN PLANTS

Significant differences in chlorophyll a, b, and carotenes concentration were found among corn plants exposed and unexposed to parathion methyl. In both treatments (exposed and unexposed to parathion methyl) corn plants have a 9% higher concentration of chlorophyll a, b, and carotenes when *Pilobolus* inoculum was applied (Fig. 4a), which

suggests that *Pilobolus* inoculum has a positive impact on the physiology of corn plants exposed to parathion methyl [12, 14–18, 21–23].

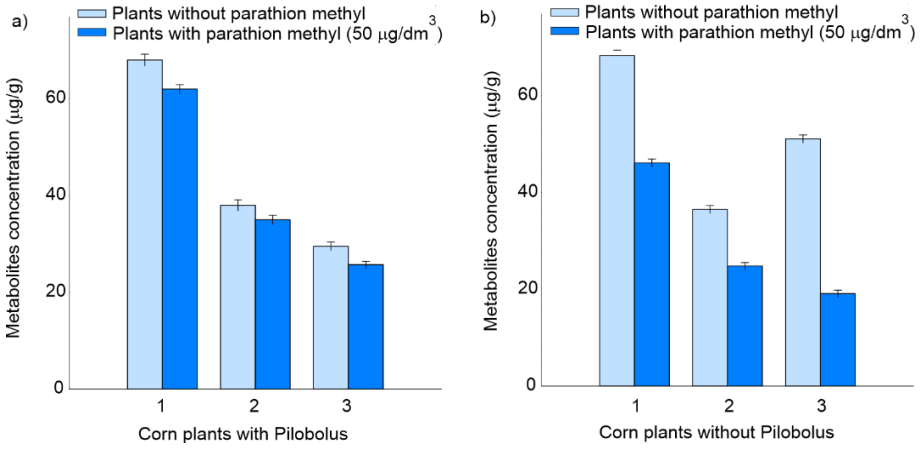

Fig. 4. Chlorophyll a (1), b (2), and carotenes (3) concentration in corn plants with (a) and without (b) *Pilobolus* inoculum

Corn plants exposed to parathion methyl without *Pilobolus* inoculum showed significant differences in chlorophyll a, b, and carotenes concentration with respect to those unexposed (Fig. 4b). Corn plants unexposed to parathion methyl had a 30% higher concentration of chlorophyll a, b, and carotenes to exposed plants which suggest that parathion methyl presence in corn plants microsystems have a negative influence in corn plants physiology [6, 12–15]. Previous experiments have shown that corn plants exposed and unexposed to parathion methyl without fungal inoculum have a low concentration of chlorophyll a, b, and carotenes, compared to our results [6, 12–15].

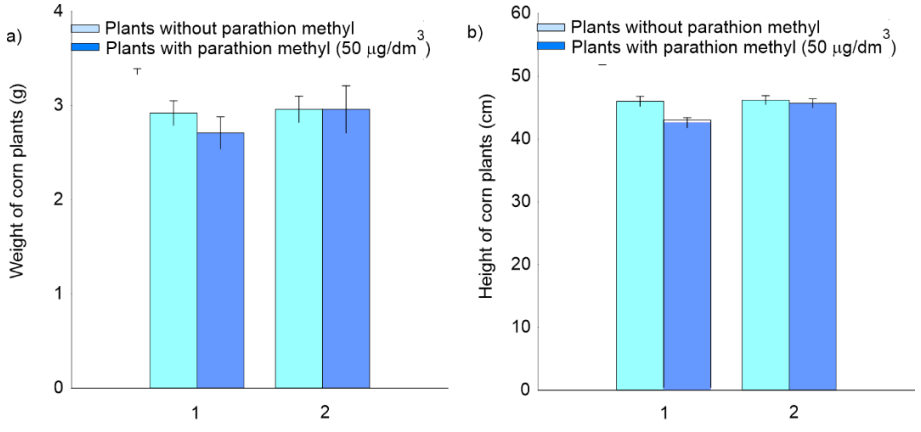

Fig. 5. Weight (a) and height (b) of corn plants without (1) and with (2) *Pilobolus* inoculum

The weight of corn plants exposed and unexposed to parathion methyl showed significant differences when *Pilobolus* inoculum was not applied; by contrast, corn plants inoculated with *Pilobolus*, did not show such differences (Fig. 5a) similarly as in papers where by other authors where plants exposed to parathion methyl had a lower weight than unexposed plants [18, 23–25]. Therefore, parathion methyl has a negative effect on the weight of corn plants, which is directly related to the concentration of chlorophyll a, b, and carotenes. The similarities in weight among plants exposed and unexposed to parathion methyl in the treatments with *Pilobolus* inoculum might be attributed to the positive influence of the fungi on the concentration of chlorophyll [21–23].

Corn plants exposed to parathion methyl have the smallest height than unexposed plants in the treatments without *Pilobolus* inoculum (Fig. 5b). This is directly related to the negative influence of the pesticide on the chlorophyll a, b, and carotenes concentration [10, 14–17]. By contrast, the treatment with *Pilobolus* inoculum did not show significant differences in the height of corn plants; therefore, the fungus might positively influence plants' growth and physiology [3, 4, 6, 23, 24].

4. CONCLUSIONS

Pilobolus efficiency in removing parathion methyl in corn microsystems is 80% and does not allow the accumulation of this pesticide in corn plants and soils. *Pilobolus* influences physiological plant efficiency due to the considerable increase in concentrations of chlorophyll a, b, and carotenes in corn plants in the presence of this fungus. The fungus is tolerant to high concentrations of (50 μg/dm^3) parathion methyl. *Pilobolus* can develop easily in the dung of cattle and for this reason, might be applied in situ. This research contributes specifically to the area of knowledge of mycoremediation. To our knowledge, it is the first evidence of the translocation of OPs by coprophilous micromycetes.

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