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Inoculation With *Azotobacter vinelandii* Enhanced Chlorpyrifos Degradation and Reduced Cytotoxic and Genotoxic Effects in Soil

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Using microbial cells for bioremediation requires evaluating suitable inoculation techniques and their effects. This study applied liquid and encapsulated in alginate beads inocula of *A. vinelandii* in agricultural soil to evaluate chlorpyrifos (CP) degradation and its impact on cytotoxic and genotoxic effects. *Allium sativum* cells and *Eisenia foetida* organisms were used as biomarkers for toxicological evaluations. Changes in the mitotic index and nuclear abnormalities in *A. sativum* cells were used for toxicity determinations. The percentage survival of *E. foetida* was calculated. Ultra-high-performance liquid chromatography was used to detect CP. The initial CP concentration (250 mg/kg) decreased by 92% when inoculated with liquid *A. vinelandii* and by 82% with *A. vinelandii* encapsulated after 14 d. A 60% decrease in cytotoxic and genotoxic damage to *A. sativum* cells was detected in treatments inoculated with *A. vinelandii*. The survival rate of *E. foetida* was improved by 33% when inoculated with free *A. vinelandii* compared to contaminated soil. Encapsulation as an inoculation strategy extended the viability of *A. vinelandii* compared to free inoculation. Both free and encapsulated inocula of *A. vinelandii* effectively degrade CP in soil and decrease its toxic effects. This study contributed by identifying sustainable agricultural alternatives for the inoculation and bioremediation of agricultural soils.

Keywords: encapsulation, rhizobacteria, toxicity, pesticide, biological degradation

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INTRODUCTION

Chlorpyrifos (O, O-diethylO-3,5,6-trichloro-2-pyridyl-phosphorothioate, CP) is among the most widely used pesticides worldwide and a broad-spectrum organophosphate insecticide that produces multiple adverse effects on the environment and human health (Norén et al., 2020; Zhang et al., 2021). CP acts by inhibiting the enzyme acetylcholinesterase (AChE), affecting the nervous system of insects, mammals, fish, and humans (World Health Organization, 2015; Malla et al., 2023). CP is associated with neurological disorders, cancer, hormonal disruption, kidney damage and reproductive problems (Huang et al., 2021; Aung et al., 2022; Sun et al., 2023). Similarly, damage to other organisms, such as pollinators, annelids, and soil microbiota has been reported (Wang e al., 2020; Ju et al., 2023; Cheng et al., 2023).

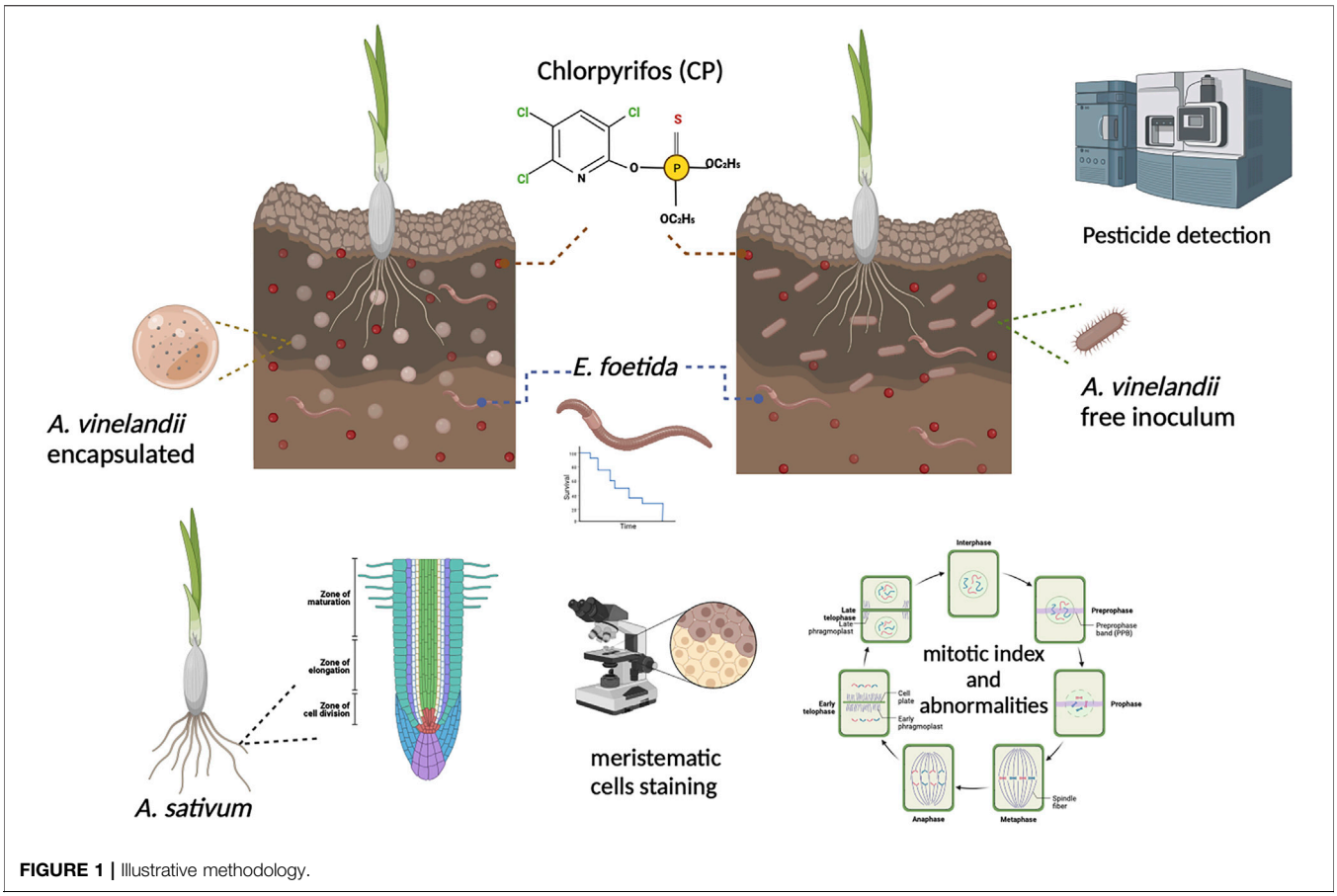

FIGURE 1 | Illustrative methodology.

Given the toxicity of CP, in 2021, some countries implemented measures to ban it, like the USA and the European Union (Hites, 2021; Pechen and Venturino, 2021). Notably, an important initiative by the European Union involves the proposal to include CP in Annex A of the Stockholm Convention, which lists the most hazardous organic substances worldwide (EPA, Environmental Protection Agency, 2022; United Nations Environment Programme, 2023). However, CP continues to be widely used without monitoring or usage restriction measures for either CP or its metabolites (Ruiz-Arias et al., 2023). Consequently, developing and implementing strategies for detecting and mitigating CP induced damage in different environmental matrices such as food, air, water, sediments, and soil are necessary.

The soil is a primary entry route for pesticides into the environment and trophic chains. CP is usually applied repeatedly in a crop cycle and in concentrations higher than recommended by the manufacturer (250 mg/ha). It can enter the soil by several routes, mainly by direct application as a preventive treatment, in solid and liquid form, drench or foliar application; by spray drift in aerial applications by light aircraft or drone to agricultural fields; by treatment of gardens and airstrips, tank washing, and through agricultural wastewater (Huang et al., 2021; Bose et al., 2021; Elzakey et al., 2023).

In soil, macro, meso, microfauna, and microbial communities, that are essential for nutrient management, availability, and biodegradation, are severely affected by CP and other pesticides (Zhu et al., 2020; Hou et al., 2023). Consequently, the symbiotic and ecological functions that facilitate plant growth are directly and indirectly damaged (Kisvarga et al., 2023). To reduce the negative impact, the biodegradation of pesticides like CP in agricultural soils can be achieved using beneficial microorganisms capable of tolerating or eliminating contaminants by adapting to adverse conditions. However, due to the chemical properties of CP, its frequent application, and environmental variability, the parent molecule and its metabolites are challenging to remove (EPA, Environmental Protection Agency, 2022).

CP is a compound derived from thiophosphoric acid with chemical formula $C_9H_{11}Cl_3NO_3PS$ and molecular weight of 350.6 g/mol (**Figure 1**). It has low solubility in water (1.4 mg/L at 25°C) and high retention in soil (360–31,000 L/kg) (Bose et al., 2021). It is generally estimated that the half-life of CP in the environment can be 100–386 d (United Nations Environment Programme, 2023). However, it may have variable ranges in different soil types (De Bernardi et al., 2022), so the estimate is discrete in terms of acute toxicity risks and the wide variability of environmental conditions (Foong et al., 2020). CP is usually resistant to hydrolysis at acidic pH, especially at low ambient temperatures and high organic matter content, which can extend its permanence up to years (Bose et al., 2021).

CP degradation pathways include abiotic (volatilization, oxidation, photodegradation) and biotic forms (biodegradation

or mineralization, either individual or cometabolic). The latter is the most effective for their complete elimination. The biodegradation of CP has been tested employing efficient bacteria of the genera *Pseudomonas*, *Bacillus*, *Cupriavidus*, *Ochrobactrum* individually (Deng et al., 2015; Akbar and Sultan, 2016; Abraham and Silambarasan, 2016; Malla et al., 2023), with fungi of the genera *Aspergillus*, *Byssochlamys*, *Ganoderma*, *Trichoderma* (Jayaraman et al., 2012; Silambarasan and Abraham, 2014; Kumar et al., 2021; Bose et al., 2021); and in consortia consisting of different bacteria and fungi species (Khalid et al., 2018; Abraham and Silambarasan, 2018; Huang et al., 2021; Elzakey et al., 2023). Such studies have been mainly carried out through *in vitro* assays with varied degradation efficiencies. Most microorganisms show impairment and decreased degradation capacity at concentrations above 200 mg/L CP, and their efficiency usually declines in soil tests (Deng et al., 2015; Conde-Avila et al., 2021).

The microorganism's ability to degrade CP is related to the production of enzymes such as chlorpyrifos hydrolase, organophosphorus hydrolase, phosphotriesterase, methyl parathion hydrolase, other metalloenzymes (Schenk et al., 2016), and laccases in the case of fungi (Kumar et al., 2021). The genes *opdA*, *opdB*, *mpd*, and *cpd* are strongly related to such metabolic capacity (Huang et al., 2021; Bose et al., 2021). The main CP degradation products are CP oxon (an even more toxic compound resulting from CP oxidation), O, Odiethyl thiophosphate (DETP), and 3,5,6-trichloro-2-pyridinol (TCP) (resulting from enzymatic cleavage of CP by hydrolysis). DETP, upon hydrolysis, yields phosphorothioic acid and ethanol. TCP is much more persistent and easier to translocate due to its solubility in water compared to the original molecule. TCP is an endocrine disruptor with a bactericidal effect, thanks to its structure with three chlorine atoms and an aromatic nitrogen ring, which most microorganisms cannot tolerate or degrade, which is a limiting step in the CP degradation pathway (Jabeen et al., 2015; Li et al., 2020; Bose et al., 2021).

Therefore, the diversity of organisms capable of fully and efficiently degrading both CP and TCP is limited, and many of its effects in agricultural soils still need to be better understood (Malla et al., 2023).

A current sustainable approach is to identify symbiotic, nutrient-fixing, and pesticide degrading microorganisms, alongside developing strategies to improve their viability and effectiveness, especially under stress conditions (Gomathy et al., 2021). A promising alternative is the use of plant growth-promoting rhizobacteria (PGPR), known for their contribution to plant growth and protection, potentially reducing or even replacing the need for chemical fertilizers and pesticides (Lewis, 2022). However, integrating PGPR into agricultural production faces several challenges, including their tolerance to contaminants in soil, variability in performance across different environments, biological interactions with native organisms, cultivation techniques, formulation processes, and overall effectiveness (Strobel et al., 2018). It has been identified that the most important challenges of effective inoculation occur in the first days (Bashan et al., 2014). Therefore, applying promising PGPR inoculation techniques and evaluating their efficacy in the degradation of toxic substances in soil is essential to generate efficient and sustainable alternatives (Conde-Avila et al., 2020; Gallego and Olivero-Verbel, 2021).

Among the PGPRs, the genus *Azotobacter* is an efficient free-living dinitrogen (N_2) fixer (Apriliya and Mulyawan, 2022). Additionally, *Azotobacter* spp. produce polymers, phytohormones, antifungals, alkylresorcinols, and nutrient chelators (Vejan et al., 2016; Mankar, Sahay, and Gothawal, 2020; Bolaños-Dircio et al., 2022). *Azotobacter* spp. have also demonstrated tolerance and ability to degrade aromatic substances such as phenols (Revillas et al., 2000), pesticides (Chennappa, Sreenivasa, and Nagaraja, 2018; Mousa et al., 2021), hydrocarbons (Devianto et al., 2020) and sequestering heavy metals using exopolysaccharides (Hindersah et al., 2023). Laboratory have revealed that some *Azotobacter* spp. exhibit tolerance to CP, with varying effects on their metabolism and respiration depending on strain and medium conditions (Chennappa et al., 2019). In contrast, our research group has identified a highly tolerant and efficient strain of *Azotobacter vinelandii* (ATCC 12837) capable of mineralizing CP, i.e., 500 mg/L in 6 h under *in vitro* conditions, superior to degradation with other *Azotobacter* spp. and other microorganisms (Deng et al., 2015; Huang et al., 2021; Conde-Avila et al., 2021). Furthermore, we have applied an alginate encapsulation strategy that not only protects the cells but also enhances their ability to promote plant growth in soil (Conde-Avila et al., 2022). Encapsulation involves confining metabolically active cells within a non-toxic polymeric matrix to improve their establishment in field conditions, maintain their viability over an extended period and enable them to tolerate higher contaminant concentrations (Conde-Avila et al., 2020).

Despite the potential of PGPRs, the effectiveness of free and encapsulated *A. vinelandii* in the degradation of CP in agricultural soil in competition with a native environment and microbiota, as well as its effects on soil cytotoxicity and genotoxicity reduction, is unknown. Some studies have been conducted on the degradation of CP by bacteria or evaluations of toxicological damage and oxidative stress on non-target organisms like *Allium cepa* cells and earthworms (Santo et al., 2023; Ju et al., 2023; Cheng et al., 2023). However, few proposals have evaluated novel encapsulated formulations of efficient strains for CP bioremediation and the reduction of pesticide toxicological damage in agricultural soils.

The objective of this study was to evaluate the degradation of CP in soil using both free and encapsulated in alginate-Na beads *A. vinelandii* and to determine effects on cytotoxicity and genotoxicity using plant meristematic cells (*Allium sativum*) and earthworms (*Eisenia foetida*) as biomarkers of damage. Insights derived from this study contribute significantly to the monitoring and comprehension of pesticide toxicity effects, thereby promoting the amelioration of soil quality and the generation of sustainable agricultural alternatives.

METHODS

Strain Characteristics

The strain used in this study was *A. vinelandii* ATCC 12837. Cells were cryopreserved at 70°C in sterile 4% (v/v) glycerol solution. For

both plate-based and in liquid culture propagation on, Burk-Sucrose (BS) medium was used with the following composition (g L^1): sucrose 20, yeast extract (Difco™ BS, USA) 3, K_2HPO_4 0.66, KH_2PO_4 0.16, NaCl 0.2, $MgSO_4$-$7H_2O$ 0.2, $CaSO_4$ 0.05, Na_2MoO_4-$2H_2O$ 0.0029, $FeSO_4$-$7H_2O$ 0.027 and an initial pH of 7.

Obtaining the Liquid and Encapsulated Inoculum

A. vinelandii cells were grown at 28°C in 500 mL Erlenmeyer flasks with 100 mL of BS medium at 200 rpm until a concentration of 4×10^8 CFU/mL was reached. The biomass was collected by centrifugation at 1000 × *g* for 10 min, and the supernatant was discarded. The recovered biomass was mixed with sterile distilled water and used as liquid inoculum for subsequent experiments. To encapsulate *A. vinelandii*, the biomass pellet was mixed in a sterile 2% (w/v) alginate-Na solution. An aliquot of the alginate-Na and *A. vinelandii* suspension was added by drip to a sterile 0.1 M CaCl, forming 3–5 mm diameter beads.

Experimental Conditions

The treatments used in the experiment were: Control (No inoculum/no pesticide), Control + CP, Liquid inoculum, Liquid inoculum + CP, Encapsulated inoculum and Encapsulated inoculum + CP. A completely randomized design with four independent replicates (n = 4) per treatment was used. Sterile polyethylene pots of 10 cm diameter were used as experimental units and filled with 80 g of soil for treatment preparation. A commercial formulation of CP was used (Clorver 480® EC Versa Agrochemicals, Mexico) at a concentration of 250 mg of active ingredient per kg of soil. The following factors were considered in the experiment: 1) pesticide concentration (0 and 250 mg/kg of CP) and 2) type of *A. vinelandii* inoculum (liquid, encapsulated, and control without inoculum). Inoculums were adjusted to a cell density of 4×10^8 CFU/mL for both inoculated treatments. Soil inoculation was performed with 6 mL of *A. vinelandii* inoculum in a liquid solution (inoculum diluted in 54 mL of distilled water), and its equivalent encapsulated in alginate Na beads per experimental unit. A control treatment without inoculum was included, to which 60 mL of distilled water was applied. The experiment was conducted under controlled temperature (28°C) and soil moisture adjusted according to field capacity until 50%. During the experiment, soil samples were taken in triplicate for viability determinations of *A. vinelandii* and soil pesticide tracing.

Soil Sampling

Soil was collected from an agricultural plot managed with conventional farming practices (fertilizer, pesticide and tillage use). Soil physical and chemical properties were determined as previously reported (Martínez-Aguilar et al., 2020). The soil used corresponded to sandy loam soil, with the following properties: pH 6.46; organic matter 1.94%; cation exchange capacity 13.97 meq/100 g.

Detection of CP in Soil

Pesticide extraction in soil was performed using the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) methodology (Lehotay et al., 2007; Association of Official Analytical Chemists, 2013). Samples of 5 g of soil were placed in a 50 mL centrifuge tube, then 10 mL of deionized water was added and allowed to stand for 30 min. Standard solutions of CP 99.5% N-11459 (Chem Service, United States) and its degradation intermediates TCP 99.5% (33972-BCBZ8746) (Sigma Aldrich, USA) and DETP (Sigma Aldrich, United States) were used for pesticide identification and quantification. Samples were automatically injected through an Acquity® Sample-ManagerFTN system into an Acquity® H-Series ultra-high-performance liquid chromatography (UPLC) system (Waters Corporation, United States) equipped with an Acquity® UPLC BEH Phenyl 1.7 µm, 2.1 × 100 mm column, in a volume of 5.0 µL (Waters Corporation, United States). For chromatographic conditions, mobile phase A was 5 mM pH 3.0 ammonium formate, and mobile phase B was 5 mM acetonitrile +0.1% formic acid at a constant flow rate of 0.30 mL min^{-1}, with the following gradient: starting with 90% solvent A and 10% solvent B, reaching 90% solvent B at 5.0 min remaining 10 s and returning to its first constitution at 8 min and remaining for 1 min. The total running time was 9 min. The injection needle of the autosampler was rinsed with a mobile phase after each injection. Nitrogen was used as the desolvation gas at a flow rate of 1000 L h^{-1}. The desolvation temperature was 600°C, and the source temperature was 150°C. Helium was used as collision gas at a flow rate of 1.0 µL min^{-1}. Identification and quantification were performed using ESI + (CP) and ESI - (TCP) probes on a Xevo TQ-S Mass Spectrometer (Waters Corporation, USA) and workstation with MassLynx ™ 4.1 software (Waters Corporation, USA). Ions were monitored using multiple reaction monitoring (MRM) (Conde-Avila et al., 2021).

The results of the verification of the quality and performance of the method used for the determination of CP and its metabolites were satisfactory according to the validated official method of the AOAC (2013). Details on conditions, extraction method quality, and performance parameters are available as **Supplementary Material**.

Kinetic Analysis

CP degradation was calculated according to first-order reaction kinetics (Zaranyika et al., 2020) given by the following equation:

$$Ct = C_0 e^{-kt}$$

Where C_0 is the initial CP concentration used, C_t the CP concentration at time interval t, k is the rate constant (d^{-1}), and t is the reaction time (d), in this case 14 d.

The half-life or DT_{50} (t1/2) of CP was estimated by the following equation: t1/2 = ln 2/k. The percentage of degradation was obtained by calculating the reduction in the concentration of CP, considering 250 mg/kg as 100%.

Genotoxicity and Cytotoxicity Bioassay on *A. sativum*

For the toxicity test, organic *A. sativum* bulbs with homogeneous sizes were placed in the soil of the different treatments for day one of the experiment until they roots emerged (10 per experimental unit). Once the roots had developed, they were quantified, measured, and used to obtain meristematic cells. The cut apices were processed and stained with orcein for observation under an optical microscope to assess cytotoxicity and genotoxicity (Gallego and Olivero-Verbel, 2021) (**Figure 1**). Cytotoxicity was estimated based on the mitotic index (MI) values of 100 cells exposed to the soil of each treatments. The MI was expressed as the number of dividing cells per total cells, according to the following equation:

MI = (Total number of dividing cells/Total number of cells examined) × 100.

For the determination of genotoxicity, the presence of micronuclei (MN) and abnormalities such as binucleated cells (BC), elongated nuclei (EN), caryolysis (CAR), and total abnormalities were considered according to the following equation:

Frequency of abnormalities = Total number of abnormal cells/ Total number of normal cells.

Percentage Survival of *E. foetida* and Viability of *A. vinelandii* in Soil

E. foetida individuals were provided by the Centro de Agroecología (Instituto de Ciencias, BUAP, Puebla, Mexico). They were cultured at 20°C ± 1°C in organic compost and fed with organic vegetables. Adults with a well-developed clitellum (720 g wet mass) were selected and acclimatized in the same substrate used in the experiments. Thirty ±2 adults per replicate were used in the experiments. At the end of the experiment, the surviving worms were collected and cleaned with distilled water for quantification. To estimate the percentage survival of *E. foetida*, the number of live organisms at 7 and 14 d was quantified considering the total number of adults introduced in the soil treatments.

The viability of *A. vinelandii* in soil was determined by a plate count performed on 1 g of soil sample for each treatment, in terms of colony forming units (CFU)/g soil. Subsequently, cultures were made with the recovered bacteria for identification (Conde-Avila, 2022).

Statistical Analysis

The normality and homogeneity of variance of the data obtained from continuous quantitative variables were examined using the Anderson-Darling and Levene tests, respectively. Oneway ANOVA followed by Tukey's multiple comparisons test were applied for response variables (except survival and degradation percentages). A significance level (alpha) of <0.05 was considered for all tests. The data are presented as mean values and standard deviations. For the determination of damage, the total number of abnormalities was analyzed against the control using the chi^2 test (c^2) and an alpha <0.01. Finally, the effect of CP in the treatments on the abnormalities was analyzed by applying a Pearson correlation test and a Principal Component Analysis (PCA) with the cytotoxicity and genotoxicity variables. The analyses were carried out using Prism 10 GraphPad Software LLC.

RESULTS

Detection of CP in Soil Inoculated With *A. vinelandii*

The concentrations of CP and its primary intermediate, TCP, were traced in soil samples 14 days after inoculation (dai) with free and encapsulated *A. vinelandii*. In the control treatment without pesticide, trace amounts of CP were found in the sampled soil (1.12 mg/kg), which were considered for the calculations of the rest of the treatments (**Table 1**). In the inoculated treatments and the control + CP, a decrease in the initial concentration added (250 mg/kg) was observed. In the control soil + CP, the results showed a slight elimination of CP by natural attenuation. Conversely, inoculation with *A. vinelandii* in the soil significantly decreased the initial CP concentration in both liquid and encapsulated inoculum treatments compared to the control + CP treatment (**Figure 2**; **Table 1**). Notably, when bacteria were inoculated in free form, it resulted in a shorter DT_{50} (6.8 d) compared to the encapsulated inoculum (8.5 d), both of which were significantly lower compared to the control treatment without inoculum (DT_{50} 13 d). Thus, the inoculum of *A. vinelandii* accelerated the degradation of CP in soil regardless of the formulation used.

Regarding the intermediate metabolite TCP, equal quantities were detected in the treatments containing CP, and a significant difference was observed only when compared to the control without CP. However, no accumulation of the intermediate metabolite was observed in proportion to the more substantial degradation of CP in the inoculated treatments. The calculated degradation percentage was 58% by natural attenuation and 92% or 83% after 14 d using the free and encapsulated inoculation with *A. vinelandii*, respectively.

Viability of *A. vinelandii* in the Soil

To determine the effectiveness of the inoculation method and the effects of CP, viability counts of *A. vinelandii* in soil samples were performed (**Table 2**). Initially, at 7 dai, *A. vinelandii* colonies were successfully recovered by plate count from both pesticide and nonpesticide treatments using both inoculation methods. However, the viability was higher when using the free-form inoculation (1.3–1.7 × 10^7 CFU/mL) compared to the encapsulated form application (5.1–5.8 × 10^6 CFU/mL). Subsequently, at 14 dai, the viability decreased by an order of magnitude in the free-form inoculated treatments (1–1.8 × 10^6 CFU/mL). This was lower compared to the encapsulated form inoculation (3.2–3.5 × 10^6 CFU/mL). In both conditions and sampling times, no statistical differences were observed in the

TABLE 1 | Concentration and degradation measurements of chlorpyrifos (CP) in soil 14 days after inoculation (dai) with free and encapsulated *A. vinelandii*.

Parameter	Control (No inoculum/no pesticide)	Control + CP	Liquid inoculum + CP	Encapsulated inoculum + CP
CP (mg/kg)	**1.12**^{a} (0.83)	**107.24**^{d} (20.70)	**19.89**^{b} (8.37)	**43.41**^{c} (8.16)
TCP (mg/kg)	**0.01**^{a} (0.02)	**4.16**^{b} (1.01)	**3.51**^{b} (0.95)	**3.88**^{b} (0.76)
DT_{50} (days)	**Nd**	**13**^{c} (0.60)	**6.8**^{a} (0.42)	**8.5**^{b} (0.33)
Final degradation (%)	**Nd**	**58**	**92.04**	**82.63**

The means are presented in bold, and the standard deviations of 4 independent experiments are between parentheses. Different letters in the same rows indicate significant statistical differences according to Tukey's test (alpha ≤0.05). Nd, Not determined.

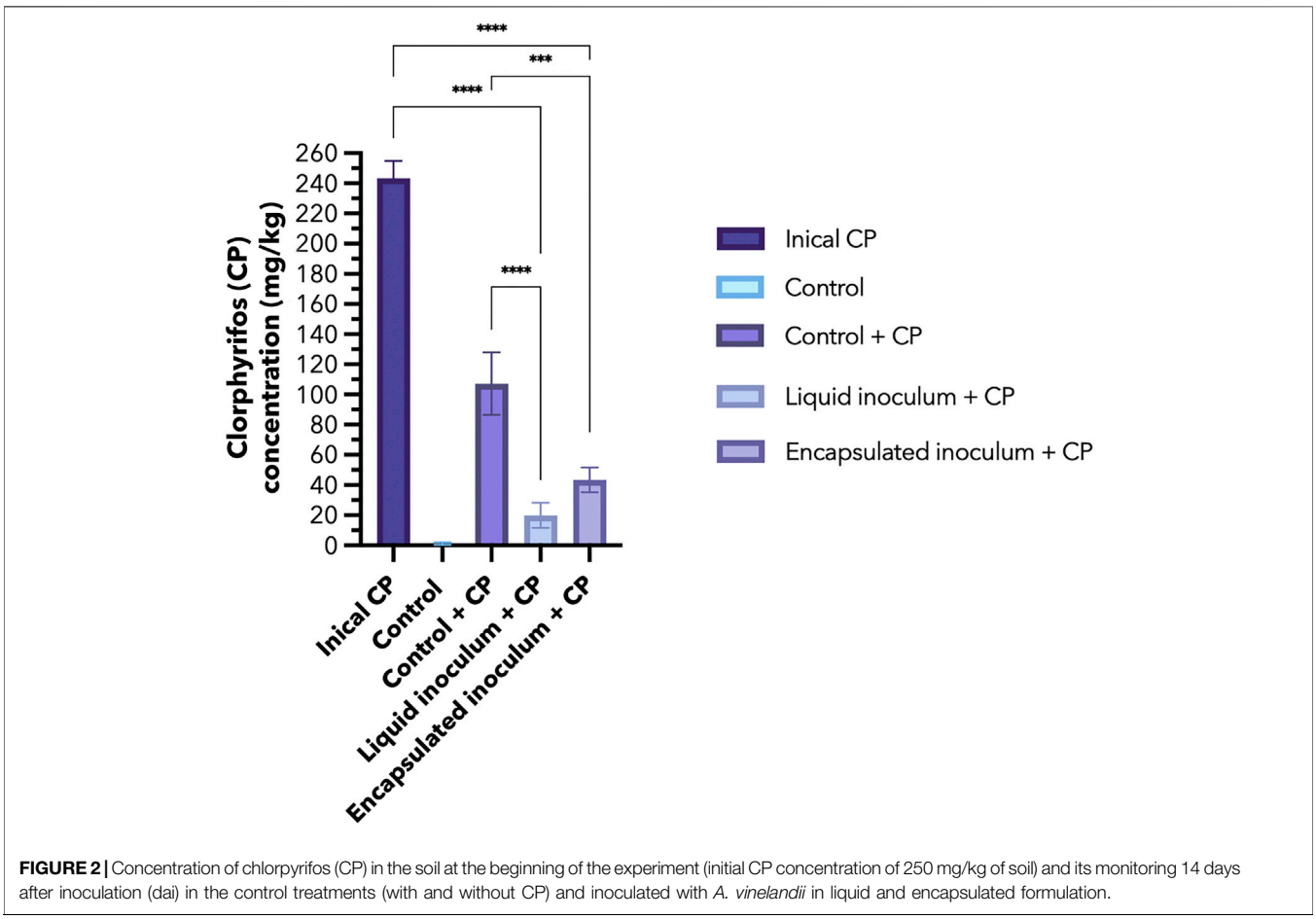

FIGURE 2 | Concentration of chlorpyrifos (CP) in the soil at the beginning of the experiment (initial CP concentration of 250 mg/kg of soil) and its monitoring 14 days after inoculation (dai) in the control treatments (with and without CP) and inoculated with *A. vinelandii* in liquid and encapsulated formulation.

TABLE 2 | Colony-forming units (CFU/mL) obtained from soil samples with CP inoculated with *A. vinelandii* in liquid and encapsulated form at 7 and 14 days after inoculation (dai).

Days	Control (No inoculum/no pesticide)	Control + CP	Liquid inoculum	Liquid inoculum + CP	encapsulated inoculum	Encapsulated inoculum + CP
7	Nd	Nd	**1.3 × 10^{7}** ^{a} (0.5)	**1.7 × 10^{7}** ^{a} (0.33)	**5.1 × 10^{6}** ^{b} (0.56)	**5.8 × 10^{6}** ^{b} (0.55)
14	Nd	Nd	**1 × 10^{6}** ^{b} (0.03)	**1.8 × 10^{6}** ^{b} (0.70)	**3.2 × 10^{6}** ^{a} (0.28)	**3.5 × 10^{6}** ^{a} (0.98)

The means are presented in bold, and the standard deviations of 4 independent experiments are between parentheses. Different letters in the same rows indicate significant statistical differences according to Tukey's test (alpha ≤0.05). Nd, not detected.

treatments with CP compared to the inoculated controls without CP, with the variation primarily attributed to the inoculation method. No *A. vinelandii* colonies were identified in the treatments without inoculum.

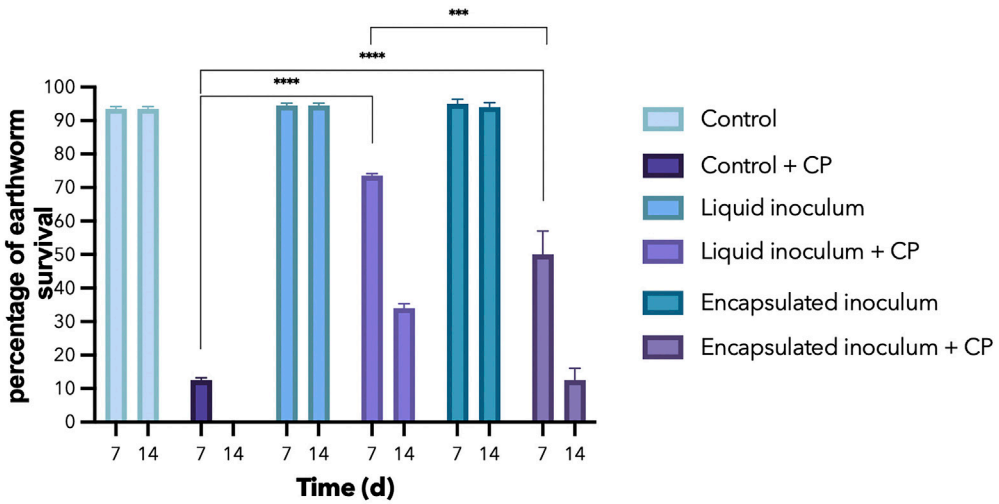

FIGURE 3 | Percentage survival of *E. foetida* at 7 and 14 days after inoculation (dai) in soil with and without CP and inoculated with *A. vinelandii* in free and encapsulated formulation.

TABLE 3 | Number and length of roots of *A. sativum* exposed to soil with and without CP and inoculated with *A. vinelandii*.

	No inoculum		Liquid inoculum		Encapsulated inoculum	
	No CP	**+ CP**	**No CP**	**+ CP**	**No CP**	**+ CP**
Root number	**17.33**^{b} (1.52)	**20.66**^{a} (2.50)	**13.66**^{b} (2.08)	**15.33**^{b} (2.3)	**16.33**^{b} (3.05)	**10.66**^{c} (1.52)
Root length cm)	**7**^{a} (1.00)	**8.5**^{a} (0.50)	**6.5**^{a} (0.50)	**6**^{a} (1.00)	**7.5**^{a} (0.5)	**8.66**^{a} (0.57)

The means are presented in bold, and the standard deviations of 10 independent experiments are between parentheses. Different letters in the same rows indicate significant statistical differences according to Tukey's test (alpha ≤0.05).

TABLE 4 | Variables of cytotoxic and genotoxic damage observed in *A. sativum* meristematic cells exposed to soil with CP and treated with inoculums of *A. vinelandii*.

Treatments	Cell in division	MN	Binucleated cells	Enlongated nuclei	Cariolysis	MI	Total abnormalities	c^2 value
Control	**76**^{c} (4.58)	**0.33**^{b} (0.00)	**0.33**^{b} (0.57)	**0.33**^{c} (0.57)	**1.66**^{c} (0.57)	**7.6** (0.45)	**2.33**^{d} (0.5)	**0.00**
Control + CP	**93.33**^{a} (4.16)	**2.33**^{a} (0.57)	**10**^{a} (0.57)	**7.33**^{a} (1.5)	**11.33**^{a} (3.5)	**9.33** (0.41)	**25.33**^{a} (1.2)	**22.65******
Liquid inoculum	**75**^{c} (3.60)	**0.00**^{b} (0.00)	**0.66**^{b} (0.57)	**1.66**^{c} (0.15)	**2.33**^{c} (1.15)	**7.5** (0.36)	**4.66**^{c} (0.5)	**1.33**
Liquid inoculum + CP	**79.33**^{c} (2.08)	**0.33**^{b} (0.57)	**0.33**^{b} (0.57)	**5.00**^{b} (0.5)	**4.33**^{b} (0.5)	**7.93** (0.21)	**9.66**^{b} (1.15)	**5.67***
Encapsulated inoculum	**78**^{c} (3.00)	**0.33**^{b} (0.57)	**0.00**^{b} (0.00)	**1.66**^{c} (0.33)	**2.33**^{c} (1.15)	**7.80** (0.3)	**4.00**^{c} (0.66)	**0.69**
Encapsulated inoculum + CP	**86.66**^{b} (3.5)	**0.33**^{b} (0.57)	**1.33**^{b} (0.57)	**4.66**^{b} (0.57)	**3.66**^{b} (1.15)	**8.67** (0.35)	**9.66**^{b} (1.10)	**5.67***

*The mean in bold and standard deviation in parentheses for three independent experiments are presented. Different letters between rows in the same column represent significant differences. Significant difference vs. negative control (c^2 test) p ****<0.0001, *<0.01.*

Toxicity of CP on *E. foetida*

The adults of *E. foetida* exposed to the soil of the control treatments without CP, both inoculated and without inoculum, did not show significant differences in the viability of the organisms at 7 and 14 dai of the experiment, maintaining levels above 95% survival. In contrast, the treatments with CP had a dramatic impact on the viability of *E. foetida* (**Figure 3**). In the control + CP treatment, within the initial 7 d, the *E. foetida* population decreased by 87.5%, and by 14 d none of organisms survived exposure to CP. In the treatments inoculated with *A vinelandii*, survival significantly improved compared to the control + CP. When free *A. vinelandii* was inoculated into CP-contaminated soils, survival rates were 74% and 34% at 7 and 14 d, respectively,

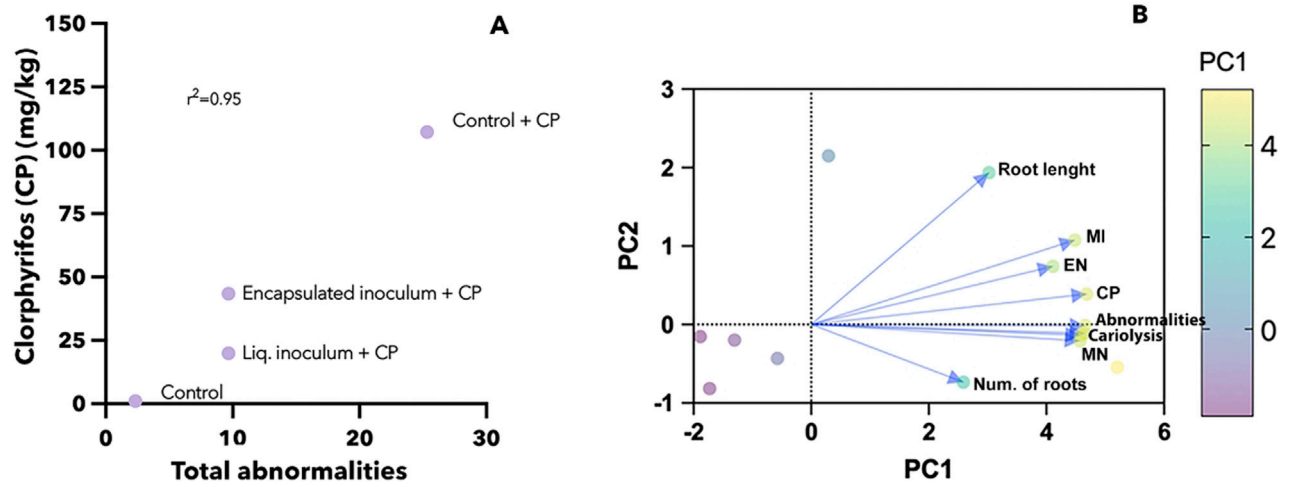

FIGURE 4 | (A) Correlation graph between the concentration of CP in the inoculated treatments and the total abnormalities in cells of *A. sativum*. **(B)** Biplot graph of the Principal Component Analysis of the toxicity variables of the treatments. CP, chlorpyrifos; MI, mitotic index; EN, elongated nuclei; MN, micronuclei, abnormalities, cariolysis, root lenght, number of roots.

while with encapsulated inoculum, survival rates were 50% and 13% at 7 and 14 dai, respectively.

Cytotoxicity and Genotoxicity on *A. sativum*

The root length and root number following the exposure of *A. sativum* to soil with CP and treated with *A. vinelandii* are presented in **Table 3**. There were no significant differences in the root length across any of the treatments. In contrast, the number of roots was significantly higher in the control soil with CP (20.66 ± 2.50), followed by the treatment with CP and inoculated with the bacteria in encapsulated formulation (10.66 ± 1.52).

The effects of CP and inoculation with free and encapsulated *A. vinelandii* on the MI in *A. sativum* root meristematic cells after 14 d of treatment are summarized in **Table 4**. When counting the number of dividing cells, an increase was found in the positive control with CP, followed by the treatment with CP inoculated with *A. vinelandii* in encapsulated formulation. However, significant differences were observed in the rest of the treatments compared to the control. Similarly, treatments without pesticide and the one inoculated with *A. vinelandii* in liquid formulation + CP showed an MI statistically similar to the negative control without CP (7.6 ± 0.45). The highest MI values were obtained in the control + CP treatment (9.33 ± 0.41), followed by the treatment with encapsulated *A. vinelandii* (8.67 ± 0.35). Thus, MI was increased in treatments in which higher concentrations of CP were detected in the chromatographic analysis (**Table 1**).

Finally, to determine the genotoxic damage caused by CP and the effect of *A. vinelandii* as a treatment for its degradation, the abnormality assay of *A. sativum* is presented in **Table 4**. Four types of aberrations, i.e., MN, BC, EN, and CAR, were observed in the dividing meristematic cells. Regarding MN and BC, differences were only observed in the control treatment with CP, where the concentration of CP, according to chromatographic analysis, remained above 100 mg/kg even after 14 d. Similarly, EN and CAR were higher in the control treatment with CP but decreased in the treatments inoculated with *A. vinelandii*, in liquid and encapsulated formulations. In summary, the number of abnormalities was significantly higher in the control + CP treatment (25.33 ± 1.2) than in the rest of the treatments. It significantly decreased in the treatments inoculated with *A. vinelandii* (9.66 ± 1.1). The treatments without CP showed a lower total number of abnormalities compared to the control + CP. The total abnormalities correspond in a dependent manner to the concentration of CP detected in each treatment at 14 d (**Figure 2**; **Table 1**).

Additionally, to determine the level of correspondence between CP concentration and its toxic effects in *A. sativum*, a positive correlation (r^2 = 0.95, p = 0.02) was found between total abnormalities and CP concentration in the soil of the treatments (**Figure 4A**). Finally, the analysis of c^2 confirmed the genotoxic effects caused by CP and a significant decrease of damage when *A. vinelandii* inocula were used, in contrast to the Control + CP treatment where only native microbiota were present. When analyzing the different toxicity variables using PCA, two factors explained 92.43% of the variance. We identified as the main factor the variables of CP concentration and abnormalities that explained 79.46% of the total variability (**Figure 4B**). As the second factor, the variables of root development represented 12.43% of the variance. The above indicates a more significant effect of genotoxicity on cytotoxicity that decreases as a function of CP concentration in the different treatments, especially those inoculated with *A. vinelandii*, since in the soil with native microbiota (control + CP), the damage persisted despite the compound's degradation (**Table 1**; **Figure 4A**).

DISCUSSION

CP Degradation in Soil Inoculated With *A. vinelandii*

In this study, we show for the first time the degradation of CP in soil inoculated with free and encapsulated *A. vinelandii*. Previously, it has been documented that some *Azotobacter* strains have been able to degrade CP *in vitro*. Chennappa, Sreenivasa, and Nagaraja (2018) showed evidence that *Azotobacter tropicalis* and *Azotobacter salinestris* effectively degraded CP in the range of 85%–97.6%, whereas in our work the most effective treatment eliminated 92% in 14 days, but in soil conditions.

In a previous study, we reported that the same strain used in this work, *A. vinelandii* ATCC 12837, exhibited *in vitro* degradation of 99.6% of a commercial CP formulation (500 mg/L) with a DT_{50} of 6 h without affecting its respiration rate, growth, or the accumulation of intermediary metabolites such as TCP, DEPT, or CP oxon (Conde-Avila et al., 2021). In our current study, when inoculated into the soil with initial concentrations of 250 mg/kg, the percentage of degradation was 92.04% and 82.63% using free and encapsulated inoculation with a DT_{50} of 6.8 and 8.6 d, respectively (**Table 1**). Importantly, no accumulation of intermediary metabolites was detected, only presence of TCP. That suggests the participation of degradation and mineralization pathways previously described, where CP is hydrolyzed to TCP, followed by reductive dechlorination until its mineralization (Conde-Avila et al., 2021; Bose et al., 2021; Huang et al., 2021). This is significant as it demonstrates the potential of *A. vinelandii* to tolerate and efficiently degrade the high CP concentrations commonly found in certain agricultural soils.

Adverse affectations on rhizospheric microorganisms involved in N_2 fixation are frequently reported when CP is used in soil at doses from 4 to 350 mg/kg (Bhende et al., 2022; Raj and Kumar 2022; Cheng et al., 2023; Amani et al., 2018; Martínez-Toledo, Salmeron, and Gonzalez-Lopez, 1992). Additionally, a decreased in the abundance of cbbLR and cbbLG genes involved in carbon (C) fixation on *Acidobacteria*, and other affectations on fungi, *Actinomycetes*, *Pseudomonas*, and azotobacterias (Amani et al., 2018). In contrast, in this study, *A. vinelandii* remained viable in soil after 14 days using both inoculation methods. Notably, liquid application favored CP degradation and bacterial viability at 7 dai. The higher percentage of degradation observed with free inoculation can be attributed to the higher number of free cells or CFU in the soil at this early stage (Jabeen et al., 2015; Malla et al., 2023). In encapsulated inoculation, degradation was slightly slower, but viability was better after 14 dai (**Table 2**). This is consistent with known characteristic of encapsulation in matrices like sodium alginate, which tends to retain microorganisms and release them gradually (Strobel et al., 2018). This gradual release favors sustained bacterial viability and the prolonged effects on CP degradation.

The high degradation rates observed both *in vitro* (Conde-Avila et al., 2021) and in soil conditions in this study for *A. vinelandii* can also be attributed to the near-neutral pH in our experiments (pH 6.46). Neutral pH values favor the activity of enzymes related to aromatic ring cleavage, such as organophosphate hydrolases and oxidative stress response enzymes (Duraisamy, Muthusamy, and Balakrishnan, 2018; Farhan et al., 2021). Similarly, Malla et al. (2023) reported that *Bacillus cereus* AKAD 3-1 removed 94.52% of CP (80 mg/kg) by transforming it into TCP and O, O-diethyl O-hydrogen phosphorothioate and completely mineralizing them into non-toxic by-products within a pH range of 5-7 employing 6.4–7.4 mL inoculum under *in vitro* conditions (Malla et al., 2023). This is consistent with the results of this research, where the soil pH was 6.46. However, other soil conditions (acidity, higher percentage of organic matter, changes in temperature, among others), and the use of commercial formulations may have contributed to the reduced degradation rates observed in prior *in vitro* experiments (Deng et al., 2015; Aasfar et al., 2021).

It should be noted that degradation occurred in the treatment without inoculation in this study (**Figure 2**). This can be attributed to abiotic conditions and the native microbiota, which may exhibit tolerance and capacity for co-metabolic CP degradation in agricultural soils where organophosphorus pesticides have historically been applied (Abraham and Silambarasan, 2018). This is the case for the soil employed in our study since we identified traces of CP in the sampled agricultural soil (**Table 1**). It would be interesting to consider a microbiome analysis of soils impacted by CP where there is the possibility of degradation by native microbiota. However, it is usually challenging to adapt them to other environments. Other studies have emphasized that stimulation and adaptation of parameters such as pH and humidity are required to favor the elimination of CP (Huang et al., 2021), as happened in this experiment since it would not have occurred with the same efficiency without intervention. Additionally, in this study, the control treatment + CP did not eliminate the acute toxic effects on the bioindicator organisms because the concentration remained always above 100 mg/kg, and the degradation rate was two times slower and about 50% less effective compared to treatments inoculated with *A. vinelandii*. Thus, a stimulation treatment and adding a microorganism such as *A. vinelandii* can act effectively and synergistically, reducing the contaminant and the acute toxic effects not eliminated by the native microbiota. In sum, regarding concentration tolerance, efficiency, and the elimination of toxicology effects, *A. vinelandii* is an excellent candidate for CP degradation and the reduction of its acute effects, considering sandy soil conditions and a pH close to 7.

Toxicity of CP on *E. foetida* in Soil Inoculated With *A. vinelandii*

Ecotoxicological effects of CP on various organisms have been previously reported, including bees, fish, microbiota, and earthworms (Organization for Economic Cooperation and Development, 2004; Ali et al., 2008; Vischetti et al., 2020; Hou et al., 2023). Our results indicate that the impact on earthworms is directly related to the CP concentration in the different

treatments (**Figure 3**). The application of CP induced lethal effects, especially in the control + CP treatment, where none of the organisms survived the exposure. This result can be associated with the dose used in the experiment since it has been reported that pesticide doses from 10 to 100 mg/kg typically cause mortality in earthworms (Wang et al., 2015; Ali et al., 2022). Likewise, Krishnaswamy et al. (2021) observed a similar mortality rate and reported alterations in the gut microbiome of the earthworm species *Eudrilus euginae*. Acute exposure to 180 mg/kg CP caused mortality of *E. euginae* earthworms like those from this investigation with *E. foetida*. In our study, applying 250 mg/kg in soil resulted in concentration-dependent mortality, with 100% mortality in the control + CP treatment. Even after 14 dai, the residual CP concentration remained above 100 mg/kg.

When *A. vinelandii* inoculum was used in the soil, the survival of *E. foetida* improved significantly (**Figure 3**). Particularly, with liquid inoculation, the survival percentages were notably higher at 7 and 14 dai. In the encapsulated inoculation, survival also improved but remained slightly lower than in the free inoculation. The difference in survival rates can be attributed to the contrasting residual CP concentration in the different treatments. It is consistent with the rapid degradation of CP by the inoculated bacteria in both liquid and encapsulated form (DT_{50} of 6 and 8 dai). On the other hand, although natural degradation occurred in the soil control + CP, the period of exposure and concentration of the pesticide were both twice as high (**Table 1**). These results are consistent with those reported by Zhou et al. (2007), who demonstrated that CP causes acute toxicity (LC_{50}) at concentrations ranging from 91.87 to 118.5 mg/kg in soil, leading to adverse effects on the growth and reproduction of *E. foetida* dependent on the concentration and exposure period. Damage to earthworms originating from pesticides such as CP is associated with physiological and developmental abnormalities by acting on the inhibition of enzymes such as AChE and butyrylcholinesterase (BChE) in acute exposure (Arguedas et al., 2018). In addition, both CP and TCP cause severe oxidative stress and irreversible DNA damage in *E. foetida* (Zhu et al., 2020; Hou et al., 2023).

Survival results may vary between species or due to the conditions or products employed. v.g., Ju et al. (2023) similarly observed a concentration-dependent effect of CP on the growth of *Lumbricus terrestris* but no significant effect on survival or reproduction, suggesting greater sensitivity of *E. foetida* over other species. De Bernardi et al. (2022) reported effects on weight, reproductive activity, and behavior of *E. foetida* in soil with CP. They showed a pesticide dose-dependent damage trend but did not report bioaccumulation effects. However, bioaccumulation could explain the damaging effect even in treatments where the concentration of CP decreased over time. CP has the capacity to bioaccumulate in biota, enhancing the toxicity effect on earthworms and other organisms at non-lethal doses. The value of a substance's octanol-water partition coefficient (K_{OW}) is related to its adsorption capacity or bioaccumulation potential in biota. A low K_{OW} value (<1) indicates solubility, easy biodegradation, and therefore low bioaccumulation. On the contrary, a high K_{OW} value (>5) indicates higher adsorption capacity in fatty tissues, soil, and sediments. The K_{OW} of CP is 4.7, which indicates that it is a substance with high bioaccumulation capacity; however, it is a little-explored topic that could provide more information on pesticide effects on *Eisenia* spp. at non-lethal doses (Hites, 2021).

Cytotoxic and Genotoxic Effects on *A. sativum*

Toxicity tests with *Allium* spp. are validated by the United Nations Environment Programme (UNEP) and the United States Environmental Protection Agency (USEPA) (Bonciu et al., 2018), and are relevant for extrapolations to animal, human and environmental health (Saxena, Gupta, and Murthy, 2010; Ullah et al., 2018; Pereira et al., 2020; Onuminya and Eze, 2019). *Allium cepa* and *A. sativum* are the most used species to test pesticide's toxicity *in vitro* (Rosculete et al., 2019; Onuminya and Eze, 2019; Camilo-Cotrim et al., 2022; Ghisi et al., 2023). Despite this, research evaluating soil conditions and the effect of commercial pesticide formulations is scarce (Soodan, Katnoria, and Nagpal, 2012; Datta et al., 2018; Fatma et al., 2018; Mota et al., 2022). The effects caused by organophosphorus pesticides in *Allium* spp. are usually a reduction of root length and MI (Türkoğlu, 2012; Sinha and Kumar, 2014; Fatma et al., 2018), occurrence of various chromosomal aberrations and genetic damage (Gallego and Olivero-Verbel, 2021; Sheikh et al., 2020).

In this study, CP increased MI in the roots of *A. sativum* in comparison with the negative control. While a lower MI indicates impairments in plant cell growth by G2 blockade or enzymatic inhibition preventing mitosis (Ciğerci et al., 2015; Mesi and Kopliku, 2015; Gallego and Olivero-Verbel, 2021), an increase in MI is related to an overstimulation of cell division, causing uncontrolled proliferation and even the formation of a more significant number of chromosomal and nuclear abnormalities (Sheikh, Patowary, and Laskar, 2020), as in the results of this research (**Table 4**; **Figures 4A, B**). Increased cell division may result from reduced time required for DNA repair (Ghisi et al., 2023) or cytosolic signal transduction, causing cell proliferation and increased abnormalities (Hasegawa, Shimonaka, and Ishihara, 2012; Sheikh, Patowary, and Laskar, 2020). Generally, the genotoxic effect of pesticides usually occurs at concentrations lower than those necessary to produce a cytotoxic effect, similar to what was observed in this research, so it is recommended to accompany both evaluations in ecotoxicity studies (Mesi and Kopliku, 2015). In our case, genotoxicity caused by CP was evident, as dividing meristematic cells showed increased and distinct concentration-dependent abnormalities (**Table 4**; **Figures 4A, B**). Abnormalities such as CAR, BC, EN, and MN were identified in the CP treatments, especially in the control + CP, and decreased in the treatments inoculated with *A. vinelandii*, both in liquid and encapsulated inoculation. The increase in the number of abnormalities and a higher MI than the control due to exposure of *A. sativum* to CP confirms an accelerated and uncontrolled division where DNA damage cannot be efficiently repaired by cellular mechanisms and results in genetic damage observable through various abnormalities (Ghisi et al., 2023).

DNA damage caused by pesticides has been related to their oxidative effect, primarily resulting from the generation of reactive oxygen species (ROS) within plant cells. These ROS cause lipid peroxidation and damage the DNA chain, thereby obstructing processes like replication, repair, recombination, and transcription. Consequently, it causes various aberrations, including MN formation, membrane deterioration, and, ultimately, cell death (Vischetti et al., 2020; Acar et al., 2022). This has been confirmed by the activation of the plant antioxidant defense system, which manifests as an increases in superoxide dismutase (SOD) and catalase (CAT) enzyme activity in response to the presence of pesticides (Fatma et al., 2018; Kalefetoğlu Macar, 2020; Acar et al., 2022). SOD catalyzes free radical forming (O_2) and hydrogen peroxide (H_2O_2), while CAT contributes to oxidative stress protection of cells by scavenging H_2O_2 (Acar et al., 2022). The extend of the oxidative response may vary depending on the toxicity or type of pesticides because their stereochemical structures play an important role in recognition and reaction with DNA (Türkoğlu, 2012; Onuminya and Eze, 2019; Acar et al., 2022). Organophosphorus compounds such as CP have two potential electrophilic sites: alkyl and phosphoryl groups. *In vitro* studies have shown that pesticides have the potential to weakly methylate DNA bases and act as a potent alkylating agent by forming bonds between DNA strands that prevent their replication, thereby destroying actively dividing cells and generating genotoxicity (Ghisi et al., 2023). Moreover, CP can produce clastogenic effects by competing with specific essential ions for enzymes involved in mitosis, favoring the appearance of aberrations and abnormalities (Sinha and Kumar 2014). In our study, CP influenced CAR's appearance in *A. sativum* cells with increased CP concentration in soil to a greater extent. This abnormality indicates the dissolution of nucleus due to DNAase activity, which can appear due to various reasons, such as lack of ATP or oxidative stress.

The presence and concentration of CP resulted in a significantly higher number of BC in the treatment groups. Previously, BC have been identified in the presence of pesticides caused by cytokinesis arrest in the cell cycle that can lead to cell death after prolonged exposure (Acar et al., 2022; Ghisi et al., 2023). For EN, this abnormality is usually caused by the inhibition of DNA biosynthesis during the S phase of the mitotic cell cycle (Firbas and Amon, 2014; Olaru et al., 2020), or by the interaction of the pesticide with nucleoproteins involved in spindle elongation, microtubule dynamics, and chromosomal movement. Enzymes related to such biological processes include DNA and RNA polymerase, DNA gyrase, and kinases (Ciğerci et al., 2015).

Finally, the occurrence of MN indicates defects in mitotic segregation or errors in DNA replication that generate acentric chromosome fragments or inactive centromere (Tartar et al., 2006; Fernandes, Mazzeo, and Marin-Morales, 2007) and contribute to genomic instability due to loss of information in cells that continue replication (Sinha and Kumar 2014).

Notably, despite the CP´s cytotoxic and genotoxic effects, in treatments where *A. vinelandii* was inoculated, MI decreased to a level similar to that of the negative control, and total abnormalities decreased in relation to the lower concentration of CP. This suggests that the rapid degradation of CP in soil treated with *A. vinelandii,* mitigated the damage caused by CP in *A. sativum*. In particular, the decrease of EN and CAR compared to the control, as well as the consistent occurrence of MN, BC, and IM resembled the patterns observed in the negative control.

CONCLUSION

The results of this study provide toxicological data from the evaluation of the use of *A. vinelandii* in the degradation of commercially formulated CP in agricultural soil. Applying *A. vinelandii* inoculums as a bioremediation treatment effectively reduced the concentration of CP in soil and its toxic effects. Consequently, adopting of a bioremediation approach with the rhizobacterium *A. vinelandii* represents a sustainable and effective alternative for the rapid degradation of CP in agricultural soil, reducing acute effects without the formation of toxic metabolites. Toxicological monitoring in soil biota is essential for generating alternatives to pesticide contamination, such as CP. In this context, *A. sativum* is a relevant and practical bioindicator for *in situ* soil health monitoring in organic and conventional farming systems. This is important since assessing the impact on soil biota and bioindicators is vital to avoid underestimating the short- and long-term pesticides' effects. The development and implementation of this and other novel techniques for PGPR inoculation, as well as the evaluation of their effectiveness in the degradation of pesticides in soil is recommended. The results of experiments such as those presented here are essential for food security, as they contribute to the monitoring and understanding the effect of pesticides to improve soil quality and generate alternatives for sustainable agriculture.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The manuscript presents research on animals that do not require ethical approval for their study.

AUTHOR CONTRIBUTIONS

Conceptualization, Investigation, Data curation, Formal analysis, Visualization and Writing - original draft: VC. Methodology, Supervision, Funding acquisition, Resources, Review: CM. Supervision, Writing Review and editing: YN. Investigation, Data curation: PB. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GENERATIVE AI STATEMENT

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/sjss.2025.14033/full#supplementary-material

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