Micronucleus Induction in *Vicia faba* Root Tips by Crude Oil-Polluted Soil from Ecuadorian Amazon Rainforest

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Abstract: In the past four decades, the Amazon rainforest has emerged as a crucial zone for crude oil extraction in the South American region. In the Ecuadorian Amazon rainforest, hydrocarbon blocks (called “bloques”) cover vast zones, including agricultural and livestock farms, protected natural regions and the territories of uncontacted indigenous tribes. This study proposes a micronuclei assay on *Vicia faba* following a 24 h exposure to various soil samples collected from Bloque 57 in Ecuador. Sampling was conducted between the Dayuma and Aguarico zones, approximately 30 km from Nueva Loja city. The research aimed to assess the impact of different soil samples, particularly those from areas affected by crude oil spills, to induce micronuclei and mitotic index changes in *V. faba* roots. Results: The soil pollution caused by crude oil is not the sole factor contributing to cytotoxicity and genotoxicity in *V. faba*. Most samples from areas polluted by crude oil outside the small-scale farm showed no significant difference in micronuclei rate compared to negative control and Amazon unpolluted soil. Conversely, samples from the small-scale farm displayed a statistically significant genotoxic effect. Furthermore, samples collected from open-air wastewater pools demonstrated higher levels of cytotoxicity compared to the controls and those from small-scale farms. The mitotic index was lower in seedlings exposed to wastewater in open-air pools, especially for the 20 cm deep samples. This phenomenon could be linked to bitumen-like substances and oils floating on the surface, attaching to the small roots and causing suffocation.

Keywords: oil spill; petroleum; Amazon rainforest; Ecuador; micronuclei; environmental carcinogens

1. Introduction

The Amazon rainforest represents a challenging area of study in biodiversity. With a rich diversity of flora and fauna [1], the region contributes significantly to ecological studies, and simultaneously, the diverse indigenous and peasant communities residing here offer important insights into cultural diversity. Understanding this complex interconnection between biological diversity and cultural richness is pivotal for scientific exploration and conservation efforts. However, it grapples with numerous social and environmental challenges primarily stemming from extensive agriculture, deforestation, mining, quarrying, and crude oil extraction.

In Northern Ecuadorian Amazon rainforest, petroleum extraction started in the early 1920s, reaching maximum production in the 1970s. Increasing levels of pollution caused by petroleum extraction activities in the Ecuadorian Amazon rainforest have been extensively reported [2–4] as well as issues concerning oil spills particularly affecting indigenous and settlers’ territories as contamination can extend far beyond impacting peripheral areas [5]. Arellano et al. (2017) reported over 800 oil spills and more than 1200 open pits contributing to pollution during the last decades across the Ecuadorian Amazon rainforest [6].
In the Ecuadorian Amazon rainforest, the interplay between environmental contamination and human health is a matter of significant concern not only for crude oil spills [7–9] but also for metal mining [10–12] and farming activities [2,13]. The local population heavily depends on the region’s natural resources for their sustenance, including water [4], hunting, fishing, and the collection of vegetal species for traditional medicines usage [14,15]. In particular, water and soil pollution pose a serious threat, as residents also rely on local water sources for drinking and other domestic purposes [4,16]. Furthermore, the reliance on contaminated resources exacerbates the health risks faced by the local communities, leading to a cycle of environmental degradation and public health challenges. Understanding the intricate relationship between environmental contamination and human health is essential for devising effective strategies to mitigate the adverse effects on both the residents and the ecosystem in the Ecuadorian Amazon region.

Globally, it has been observed that soil in areas adjacent to oil spills exhibits a higher concentration of complex hydrocarbon compounds and inorganic substances [17]. These inorganic substances include heavy metals such as cobalt (Co), aluminium (Al), arsenic (As), barium (Ba), mercury (Hg), manganese (Mn), nickel (Ni), chromium (Cr), zinc (Zn), copper (Cu), cadmium (Cd), vanadium (V), and lead (Pb) [18–20]. Several studies have also reported high concentrations of heavy metals in plots of cultivated land coming to oil spill-polluted areas. For example, in the Ecuadorian Amazon Rainforest region, Cd and other heavy metals have been measured in Theobroma cacao beans grown on various small farms in Sucumbios Province [21]. Research conducted by Bazarra et al. (2017) identified oil spill pollution as an anthropogenic source of Cd in soils. The same authors suggested that Cd and other metals were more likely related to the extensive farm use of chemical fertilizers and pesticides. The study calculated metal(loid) risk indexes, indicating moderate to elevated values due to these practices. The same study reported a high concentration of Ba and Mn in the different crop products and a high concentration of As and Zn in drinking waters collected from rainfall. Ba and Mb levels found in atmospheric particles from the same zones showed the effect of crude oil and agrochemical activities coupled with natural (bedrock, volcanic ashes) sources.

Plant-based tests to identify mutagenic and clastogenic effects have been available for a considerable period, and the V. faba root-tip micronuclei (MN) test has been pointed as a reliable and relatively low-cost test [22,23]. Plant assays, such as the Vicia faba MN, provide a sensitive and valuable assay for detecting clastogenic and aneugenic effects [24,25]. Short-term genetic toxicity bioassays can be used to assess the effects of environmental pollution. The evaluation of the genotoxic potential of a pollutant is carried out by quantifying genetic mutations and damage to DNA. MN emerge in the process of cell division due to chromosome breakage (clastogenic effect), resulting in a small chromosome fragment, or due to the failure of the entire chromosome migration (aneugenic effect) during the anaphase. A mild cytotoxic effect reduces the number of dividing cells and consequently decreases the number of micronuclei. In the case of severe cytotoxicity, cell division may be halted, leading to a simultaneous decrease in the mitotic index [26].

In this study, we subjected V. faba seeds to soil samples taken at different sites in Sucumbios Province (Ecuador) to assess the genotoxicity damage from soil contamination through an MN assay. The soil samples were collected in 2018 from three markedly different environments within the Ecuadorian northern Amazon rainforest. The first set of samples originated from abandoned oil wells in a pristine rainforest area, representing a scenario where natural ecosystems had been potentially affected by historical oil exploration activities. The second set of samples was obtained from a small-scale farm in the same region, which had been significantly polluted due to oil spills, reflecting a more recent and localized contamination event. The third sample was collected in a pristine forest area bordering a small village without nearby petroleum pollution. The mitotic index was used to evaluate the potential biostimulant or cytotoxic effect of the tested product.

This research is important not only for understanding the immediate effects of crude oil and agrochemical pollution but also for predicting the long-term repercussions on plant
biodiversity and ecosystem health in the Amazon rainforest. We hope this preliminary study may help future environmental conservation efforts to find strategies for sustainable land use in regions vulnerable to contamination.

2. Materials and Methods
2.1. Description of the Studied Area

Genotoxicity and cytotoxicity events occurring in *V. faba* were assessed using eight soil samples from various Sucumbios locations, specifically in Bloque 57 [27]. Among these samples, four were obtained from the extractive zone (A2-1, A2-2, 64-1, and 64-2), wherein in the 1970s, two oil wells were allocated, called Aguarico 2 and Aguarico 4, today abandoned. Nearby abandoned wells remain open-air crude waste pools in the Aguarico 4 area (samples 64-1 and 64-2), along with different oil spills in the Aguarico 2 area (samples A2-1 and A2-2). Differently, samples CB1, CB2, and CB3 were collected in the Dayuma area from a small-scale farm in Pacayacu town located inside Bloque 57. This farm was affected by a crude oil spill in 2006 and subsequently remediated in 2016, according to the owner’s account. In addition, soil derived from the Cooperativa Bellavista, a residential zone that borders the rainforest at Pacayacu, was used as a negative control representative of the unpolluted Amazon environment.

To better understand existing oil spills at Bloque 57, we used GIS data from precedent studies [28] and included them in our sampled site map reported in Figure 1.

Figure 1. Sampling points area (Aguarico2, Dayuma, Aguarico4, Bellavista) about 30 km from Nueva Loja City, between Dayuma town and Aguarico fields in Sucumbios Province. This area is part of the “Bloque 57” extractive zone. Oil spills’ data (oil spills Ec MAE) were obtained from Maestripieri N et Saqalli M, 2015 [5].

Collected soil characteristics are detailed in Table 1. All collected samples were transported to the Environmental Genomics laboratory at Genoa University and stored at −20 °C. The presence of crude oil was referenced not only from previous study findings [28] but also through inquiries with residents and an organoleptic assessment (identifying visible residues resembling bitumen and a gaslike odour). Furthermore, a systematic method was employed involving the partial separation of soil and oily residues using tap water, allowing for a physical differentiation, enabling the identification of distinct oily components within the contaminated soil samples. The combination of qualitative assessments, community engagement, and scientific separation techniques not only validated the presence of crude oil but also provided a multidimensional understanding of the contamination.
scenario. Such confirmation methods are essential in environmental research involving human health risk, ensuring the accuracy of data.

Table 1. Description of each soil sampling point and GPS coordinates. Note: Dayuma Farm GPS coordinates are not published to protect settlers’ privacy. Points Aguarico 2 and Aguarico 4 are abandoned extraction areas.

<table>
<thead>
<tr>
<th>Area</th>
<th>Point Name</th>
<th>Description</th>
<th>Crude Oil Presence</th>
<th>GPS (Lat, Long)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aguarico 2</td>
<td>A2-1</td>
<td>Superficial soil</td>
<td>Yes</td>
<td>−0.02079, −76.66181</td>
</tr>
<tr>
<td></td>
<td>A2-2</td>
<td>Soil from 30 cm deep</td>
<td>Yes</td>
<td>−0.02079, −76.66181</td>
</tr>
<tr>
<td>Dayuma</td>
<td>CB1</td>
<td>50 cm deep soil from a cocoa crop field from a small-scale farm</td>
<td>Yes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CB2</td>
<td>Superficial soil from a cocoa crop field from a small-scale farm</td>
<td>Yes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CB3</td>
<td>125 cm deep soil outside the crop field from a small-scale farm.</td>
<td>Yes</td>
<td>-</td>
</tr>
<tr>
<td>Aguarico 4</td>
<td>64-1</td>
<td>Superficial wastewater from open-air pool</td>
<td>Yes</td>
<td>−0.06332, −76.63745</td>
</tr>
<tr>
<td></td>
<td>64-2</td>
<td>Soil from 20 cm deep from the open-air pool</td>
<td>Yes</td>
<td>−0.06332, −76.63745</td>
</tr>
<tr>
<td>Bellavista</td>
<td>Amaz</td>
<td>Superficial soil from a residential area bordering the rainforest</td>
<td>No</td>
<td>−0.02501, −76.6078</td>
</tr>
</tbody>
</table>

2.2. Root Germination and Exposure Protocol

Root growth and micronucleus (MN) tests were performed according to Italian Superior Institute for Health guidelines [29]. Briefly, *V. faba* dry seeds, bought at a local store from Genoa city, were disinfected by a short immersion (5 min) in a 2% NaClO solution, followed by a vigorous rinse with 2–3 water changes. Subsequently, the seeds were soaked overnight in tap water. Germination was induced in a thermostatic cabinet at 22 ± 1 °C, in the dark, placing seeds in support covered with moist cotton. Tap water was added daily to maintain humidity. When primary roots reached at least 2 cm in length (4–5 days), about 5 mm were cut from the apex to stimulate secondary, or lateral, root growth. Seedlings were transferred in plastic grids submerged in tap water and maintained in the thermostatic cabinet for 5–7 days under the same conditions as above. Apices of secondary roots (about 5 mm from the end of the root) were used for the micronucleus assay when they reached about 2–4 cm.

The MN test was performed on the secondary root according to standard protocols. Briefly, secondary root expositions were performed in 50 mL tubes containing soil–water mixtures (1:3 v/v) for 24 h under the same environmental conditions described above. After 24 h, seedlings were delicately rinsed and left growing in tap water for 48 h. Subsequently, 4–10 cm long roots were sliced off and washed in Carnoy’s solution (25% acetic acid and 75% ethanol) for 30 min and then stored at +4 °C in the same solution until the micronuclei assay. Negative controls (Ctrl–) were obtained by exposing roots to a commercial potting soil–water mixture, as well as an unpolluted Amazon soil sample (Amazon Ctrl–). Differently, a positive reference control (Ctrl+) was obtained by exposition of 10⁻⁵ M of maleic hydrazide (≥98.0% GC, Merck 67139-100MG, CAS Number: 123-33-1) diluted in tap water. Maleic hydrazide or 1,2-dihydro-3,6-pyridazinedione is included in formulated products utilized as herbicides and plant growth inhibitors. It disrupts plant mitosis by interfering with the process, affecting plant growth and development.

2.3. Micronuclei Assay

For each experiment, at least three apices of secondary roots (about 3 mm from the end of the root) from two different seeds for each experimental point were Feulgen stained for micronucleus count. Briefly, fixed roots maintained at +4 °C were rehydrated in tap water, washed for 5 min with a 50% dimethyl sulfoxide water solution, then hydrolysed
with 1N HCl acid at 60 °C for 10 min and stained with Schiff’s reagent. The meristematic region of each root length was subsequentially positioned on the microscope slide and covered with 30 µL of glycerol. The single-cell suspension was prepared from each sample by sandwiching the root between two glass slides and applying light pressure to crush the tissue. The observations were performed in optical microscopy at 400× magnification.

Blind microscopic analyses counting about 3000 cells were carried out to identify micronucleus and mitotic cells. The micronucleus structure was recognized as discrete DNA aggregates separate from the primary nucleus with the same staining intensity and texture as the main nucleus and a diameter between 1/3 and 1/16 the size of the nucleus. In order to avoid any underestimation of the micronucleus frequency due to the impaired cell proliferation rate, the micronucleus test was performed only on root tips with a mitotic index superior to 2%. Average values of micronuclei and mitosis were related to 1000 cells for each experimental point, and the exposition results were compared to the negative control.

2.4. Statistical Analysis

The statistical analysis was performed using IBM® SPSS® Statistics Version 22 software. Differences between each experimental point were determined using the U Mann–Whitney nonparametric test. Values with a two-tail value of \( p < 0.05 \) and \( p < 0.001 \), were considered statistically significant. The statistically significant difference per micronucleated cells were determined with the chi-square test (a 95% confidence limit was used). GIS data and maps were matched using Geographic Information System QGIS®. (http://www.qgis.org accessed on 30 January 2023) Version3.32 Limma software.

3. Results

Figure 2 shows micronuclei (MN) frequencies related to 1000 counted cells after *V. faba*’s roots’ exposure to different soil samples collected from different sites, as reported in Table 1. The maleic hydrazide treatment (Ctrl+) elicited a considerable and statistically significant increase in MN formation compared to all experimental points. Roots exposed to soil samples collected from the small-scale farmers in Dayuma named CB1, CB2, and CB3 increased the frequency of MN 3.4-, 1.6-, and 1.3-fold, respectively. However, these differences provided statistical differences only in samples CB1 and CB2 compared to the negative Amazon control (Amaz). No increase in the frequency of MN was reported in pristine rainforest oil spill-polluted soil samples A2-1, A2-2, 64-1, and 64-2.

![Figure 2](image-url)  
**Figure 2.** Frequency of micronuclei (MN) in *V. faba* roots untreated (Ctr−), exposed to maleic hydrazide (Ctr+), and exposed to different soil samples. Statistical analysis: *** \( p < 0.001 \) and * \( p < 0.05 \) vs. Amazon sample.  

The mitosis frequency, reported in Figure 3, showed a lower rate when compared to negative controls, reaching a statistically significant decrease only in the CB1 sample. The linear regression in Figure 4 reported an inverse correlation between the number of mitotic cells and micronuclei cells. The equation was \( y = 270.95 - 1.197x \), with a significant
determination coefficient ($p < 0.05$) and $R^2 = 0.0595$. The representative microphotographs in Figure 5 show different phases of mitosis and micronucleated cells.

![Mitosis](image)

**Figure 3.** Frequency of mitosis per 1000 cells in *V. faba* roots either untreated (Ctr−), exposed to Maleic Hydrazide (Ctr+), or exposed to different soil samples. Statistical analysis: * $p < 0.05$ vs. Amazon sample.

![Representative microphotographs](image)

**Figure 4.** Representative microphotographs (stained with Feulgen stain) of soil-exposed secondary roots apices. Magnification: 400×.
Figure 4. Representative microphotographs (stained with Feulgen stain) of soil-exposed secondary roots apices. Magnification: 400 ×.

Figure 5. Linear regression analysis shows the correlation between the micronucleus count and mitosis crude rate.

4. Discussion

The present study used petroleum-contaminated soil from the Sucumbios region to investigate the genotoxic effects in *V. faba*. Petroleum is a complex mixture of polycyclic aromatic hydrocarbons such as benzene (with a clastogenic and aneugenic effect) and heavy metals like nickel and vanadium. Previous studies have confirmed that polycyclic aromatic hydrocarbons (PAH) are among the most hazardous environmental contaminants due to their toxic, carcinogenic, and mutagenic effects [30]. Therefore, genotoxicity tests are essential tools for the ecotoxicological risk assessment of petroleum products. Furthermore, different studies show that petroleum compounds cause DNA damage, triggering carcinogenic processes due to a metabolic biotransformation [31].

The MN test is an uncomplicated method to investigate the mutagenic effects triggered by complex mixtures that characterize most environmental samples. The formation of MN may result from the activity of an aneugenic agent, which breaks small fragments of chromosomes, or from a clastogenic agent that causes the loss of the whole chromosome [32]. Data regarding the impact of soil pollution by oil spills on vegetation that grows in Amazonian sites are very limited and no previous study has ever analysed the mutagenic effects of soils in these areas in a vegetal model. However, it has been observed that indigenous vegetation near oil spills can show leaf biochemical alterations, for example showing a low chlorophyll level with higher intracellular water content values, as well as an adaptation response and a species-specific selection [33].

Results in our study suggest that crude oil pollution in soil is not the exclusive cause of cytotoxicity and genotoxicity in *V. faba*. A nonsignificant increase in the frequency of micronuclei was observed when roots were growing in soil contaminated only by crude oil. This could be due to the short environmental half-lives of PAHs, ranging from weeks for the low-molecular-weight fractions to years for the high-molecular-weight components [34]. Polycyclic aromatic hydrocarbons dispersed in soils are estimated to decay over time mainly by fungal and microbial metabolism. However, PAH persistence is maintained, even at small concentrations, for several years because of declining bioavailability [35]. The natural degradation of crude oil generally includes three aspects: physical, chemical, and biological degradation.

A study conducted by Yang Id (2018) demonstrated that crude oil had the ability to infiltrate soil up to a depth of 55 cm in areas where crude oil transportation and exploitation led to discharges into the soil, thereby compromising the integrity of the surrounding ecosystem. Moreover, these discharges had a profound impact on the soil’s bacterial community, causing distinct transformations at various layers. On the other hand, the same...
authors suggested that the amount of carbon in the oil-contaminated soil reached higher values in the surface layer while it was almost similar to uncontaminated soils at a depth of 55 cm in semideserted and deserted soils [36]. The oil concentration in the deeper soil layers depends on chemical–physical interactions between the soil and crude oil. The factors mainly involved in the penetration process are the soil composition and the concentration of toxic fluids [37]. In addition, the most significant number of bacteria inhabiting the lower soil layer triggers the natural attenuation of crude oil contaminants in soils. An evident limitation of these hypotheses is the lack of geochemical analysis of the soil samples, and they should only be confirmed after identifying the aforementioned chemicals.

Contrary to our expectations, the roots exposed to samples of the area identified as Dayuma showed a high frequency of micronuclei as a sign of genotoxic damage. In this area, there was an oil spill in 2006, with subsequent environmental remediation in 2016. Therefore, these results may suggest that the genotoxic effects were also attributable to the agricultural activity in the sampling area. However, an explicit limitation of this thesis is the absence of a record of agrochemical compound usage in agricultural areas. Further studies could investigate whether molecules derived from the most used agrochemical compounds exist and have entered the ecosystem. The mitotic index was lower in seedlings exposed to wastewater in open-air pools, especially for the 20 cm deep samples. This phenomenon could be linked to bitumen-like substances and oils floating on the water surface visibly present during the treatment. The bitumen-like substances attached to the small roots, probably causing suffocation through nutrient deficiency, osmotic, or oxidative stress [38]. Studies on crude oil pollution on vegetation have confirmed that water stress is the first stress due to the hydrophobic properties of the oil that cause a difficult water uptake [39]. Despite the strong stress, survival is possible as plants have the capacity to respond to stressors at morphological, anatomical, and cellular levels, altering their growth and development processes. These responses have the potential to impact ion transport mechanisms and metabolic interactions within the plant [40].

The genotoxic tests indicated that CB soil samples at different depths caused different DNA response. In particular, compared to negative control soil, the soil sampled on the surface layer increased the micronucleus 1.65-fold, while the soil sampled at 50 cm of depth increased the micronucleus 3.39-fold. On the other hand, no genotoxic effect was found in the soil samples taken at 125 cm of depth. The genotoxic effect could also be due to pesticides used during agricultural processes. Additionally, the observed low mitotic rate presented a significant value only in sample CB1. This might suggest a toxicological impact owing to the presence of pollutants in the soil. These pollutants can potentially disrupt the growth of indigenous flora and cultivated crops.

Moreover, the owner of the small farm reported having resided there with his family for over 10 years. This long-term residency establishes a possible correlation between the occupant’s health and the studied soil. Consequently, any effects identified in the soil can be linked to potential health implications for the inhabitants. Considering the extended duration of their residence, it becomes crucial to assess the impact of the studied soil on their well-being comprehensively. The low number of residents, limited healthcare facilities in the area, and lack of epidemiological data pose a significant limit, and they were the main reason for this study to avoid a health assessment using the data of the studied soils.

5. Conclusions

This work represents a pilot experiment to study the potential mutagenic effects of crude oil spills in different scenarios to understand the necessity of a comprehensive analysis of the aftermath of crude oil spills in the Ecuadorian Amazon, especially in agricultural areas where other products derived from agricultural activity can impact production and the health of the inhabitants. The persistence of these spills in the forest ecosystem has resulted in the infiltration of petroleum components into the soil and freshwater reservoirs through rain. Oil spills’ contamination is not limited to the nearby area as it is well known that heavier components tend to deposit into the sediment where they can contaminate
human water sources, causing health effects ranging from skin irritation to cancer [41] or consumed by other organisms entering in the human food chain [42].

Our results suggest a variation in soil quality after pollution, even after declared remediation processes. This underscores the necessity of implementing agricultural best practices, which, if not appropriately managed, are potentially endangering farmers’ economies and health. Further research should explore the complexities of crude oil spills, focusing on long-term environmental impacts and devising efficient remediation techniques. Collaborative efforts among researchers, policymakers, and local communities are crucial to developing pragmatic and sustainable solutions. As of the writing of this article, no other studies utilizing the *V. faba* MN assay had been conducted with petroleum-contaminated soil. This lack of prior research makes it challenging to draw direct comparisons with our results. However, the absence of comparable studies highlights the novelty of our investigation, emphasizing the need for further research in this area. Our study addresses this critical gap, offering valuable insights for future studies aiming to assess the environmental impact and develop effective mitigation strategies in the mentioned areas.

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