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Effects of Spent Pot Liner on mitotic activity and nuclear DNA content in meristematic cells of *Allium cepa*

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ABSTRACT

Industrial waste usually contains complex mixtures of mutagenic chemicals. Spent Pot Liner (SPL) is a complex solid waste from the aluminum industry, which is composed of organics, fluoride salts, inorganic cyanides, metals, and sodium. Due to the toxicity of these compounds, this study sought to use cytogenetics and flow cytometry to assess the effects of SPL on cell cycle parameters and DNA content in meristematic cells of *Allium cepa*. Three concentrations of leachates from SPL-soil mixtures were used for the study: 0, 10, and 25%. Roots were collected and analyzed after 4, 8, 12, 24, and 36 h of exposure to the above SPL leachates. The results showed an overall mitodepressive effect accompanied by an increased percentage of condensed nuclei and genomic instability as evidenced by the presence of cellular/chromosomal abnormalities. Terminal deoxynucleotidyl transferase dUTP nick end labeling revealed nuclei with fragmented DNA, a marker of programmed cell death. This study also addressed the question of reversibility of the effects of SPL and found that 36 h of exposure to 25% SPL seemed to be the point at which the effects on the induction of apoptosis became irreversible.

1. Introduction

Industrial waste usually contains complex mixtures of mutagenic chemicals. SPL (Spent Pot Liner) is solid waste generated by the aluminum industry (Pong et al., 2000; Silveira et al., 2003; Lisbona and Steel, 2008). Its composition is highly variable and includes organic and inorganic compounds such as cyanide, fluoride salts, sodium, aluminum, cadmium, zinc, lead, iron, manganese, and copper (Silveira et al., 2003; Andrade et al., 2008). Although it is stored at controlled depots, there is no effective option to manage the excess of SPL produced, which may ultimately leach into the environment.

Environmental pollutants are well known to cause a wide variety of toxic effects. DNA damage (genotoxicity) is a common effect of many environmental toxins and specific DNA lesions or damage can trigger cell death (Kultz, 2005; Franco et al., 2009).

SPL leachate arrests the cell cycle, changes chromosomal structure, and strongly induces nuclear condensation (Andrade et al., 2008, 2010). Chromatin condensation characterizes the initial stages of apoptosis (Hengartner, 2000; Galluzzi et al., 2007). The term, "apoptosis" or "programmed cell death" defines a genetically encoded cell death program leading to specific biochemical and morphological alterations, which are distinct from necrosis or accidental cell death (Pan et al., 2001; Galluzzi et al., 2007; Franco et al., 2009). The morphological signs of apoptosis are cellular shrinkage, membrane blebbing, and nuclear condensation and fragmentation (Pennell and Lamb, 1997; Solomon et al., 1999; Vermes et al., 2000; Pan et al., 2001). Necrosis is a passive process involving the swelling of the cell and its organelles, loss of membrane integrity, and cell lysis (Robertson and Orrenius, 2000; Xiong et al., 2006). Cell death is traditionally associated with necrosis, but there is evidence suggesting that some environmental pollutants are toxic and can trigger apoptosis (Robertson and Orrenius, 2000; Franco et al., 2009). It seems that low doses of toxicants preferentially induce pathways of active cell death and only very high doses lead to necrosis (Lennon et al., 1991; Gomez-Lechon et al., 2002).

In recent years, flow cytometry has been used as a tool for the evaluation of cell cycle and cell death in plant cytogenotoxicity studies (Watanable et al., 2002; Cvikrova et al., 2003). Flow cytometry involves the use of light scatter measurements as indicators of cell size and shape, whereas fluorescence intensity allows the evaluation of DNA content and quantification of nuclei in the

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G1, S, and G2 phases of the cell cycle (Dolezel and Bartos, 2005; Dolezel et al., 2007). These parameters allow the evaluation of effects on the cell cycle and cell death.

Thus, this study was carried out to identify the harmful effects of different concentrations of SPL for different exposure periods in *Allium cepa* by evaluating cell cycle parameters and nuclear DNA content as well as the reversibility of the effects by cytogenetic and flow cytometric analyses. *A. cepa* is considered to be an efficient test organism to indicate the presence of mutagenic substances due to its proliferation kinetics and chromosomes that are suitable for this type of study ($2n = 16$, large chromosomes) (Leme and Marin-Morales, 2009).

2. Materials and methods

2.1. Preparation of SPL-soil leachates

SPL leachates were prepared according to the absorption methods described by Andrade et al. (2008). SPL-soil mixtures (20 g) containing 0, 10, and 25% of SPL were added to 200 mL of 0.01 M $CaCl_2$ solution and incubated for 12 h in a rotary shaker, followed by 12 h at rest. This procedure (12 h in shaker and 12 h at rest) was repeated twice. These solutions were then centrifuged for 15 min at 3000 rpm and the supernatants were collected as leachates for the study. Leachates from SPL-soil mixtures containing 0, 10, and 25% SPL are referred to as Environmental control, 10, and 25% SPL, respectively. The composition of the 25% SPL-soil leachate had been previously reported by Andrade et al. (2008) to show the presence of cadmium (<0.18 mg L^{-1}), copper (<0.34 mg L^{-1}), iron (75 mg L^{-1}), lead (<0.23 mg L^{-1}), manganese (<0.18 mg L^{-1}), sodium (657 mg L^{-1}), zinc (0.34 mg L^{-1}), aluminum (0.7 mg L^{-1}), cyanide (23.4 mg L^{-1}), and fluoride (47.8 mg L^{-1}). The same solutions were used in this study.

2.2. Plant bioassays

Bulbs of the commercial onion (*A. cepa*, $2n = 16$) were used as materials for cytotoxicity analyses. The onions were pre-exposed to distilled water for root emergence and later, treated with distilled water (negative control) or SPL leachates at different concentrations (Environmental control, 10, and 25%) for 4, 8, 12, 24, and 36 h. The Control (0 h) consisted of roots collected after pre-exposed to distilled water for root emergence. At least 32 bulbs were used for each treatment and after each treatment; a set of 16 bulbs was left in distilled water for 24 h for recovery. Recovery time is used to evaluate the recovery potential of the cells after exposure to SPL. After that we started to evaluate the persistence of cell alterations in the next cell cycle.

2.3. Cytogenetic analysis

After treatment, root tips (1 cm) were collected and fixed in fresh cold methanol: acetic acid (3:1 v/v) solution. The fixed root tips were hydrolyzed in 1N HCl at 60 °C for 10 min and stained with Schiff reagent for 1.5 h. The meristem was chopped into several tiny fragments, crushed, covered, and frozen in liquid nitrogen. The slides were analyzed under a light microscope to score about 5000–7000 cells per treatment. The treatments were arranged in a completely random design for four repetitions. Each repetition consisted of four slides, the root of one onion used for each slide. The following parameters were analyzed: mitotic index (MI), the percentages of micronucleated (MN) and chromosomally aberrant (CA) cells, and the frequency of condensed nuclei (CN) in interphase.

2.4. Flow cytometry

Meristematic cells exposed to different SPL concentrations were subjected to flow cytometry for cell cycle analysis. The treatments were arranged in a completely random design for four repetitions. Each repetition corresponded to the analysis of three samples and each sample was obtained by the analysis of three meristems. Fresh plant meristems were chopped with a razor blade in LB01 iced buffer (1 mL) so as to release the nuclei (Dolezel et al., 1989). The chopped tissue was aspirated through two layers of cheesecloth with a plastic pipette, filtered through a 50 μm nylon filter, and collected in a polystyrene tube. This suspension of nuclei was stained with 25 μL of a 1 mg/mL solution of propidium iodide (PI) and 5 μL of RNase was added to each sample. The samples were stored in a dark refrigerator and at least 10,000 nuclei were analyzed after 1–2 h in each sample in a FacsCalibur cytometer (Becton Dickinson). Each flow cytometric histogram was saved using Cell Quest software and analyzed with WinMDI 2.9 software. The frequency of nuclei in G1, S, and G2 phases and of sub-G1 particles was scored. Variations in forward scatter (FSC) were also scored for G1 cells to identify the level of condensation of the nuclei.

2.5. TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling)

In order to examine the question of reversibility of nuclear condensation, TUNEL was performed to examine the induction of DNA fragmentation. Bulbs that had been treated with Environmental control, 10, and 25% SPL leachates for 36 h, with and without recovery, were subjected to TUNEL analysis. Three slides were prepared for each treatment and DNA strand breaks were detected using the In Situ Cell Death Detection Kit-Fluorescein (Roche) by following the procedure recommended by the manufacturer. Percentages of labeled nuclei out of at least 10,000 nuclei per treatment were calculated after fluorescence microscopic analysis.

2.6. Statistical analysis

Data from all parameters analyzed in the above assays (cytogenetic, flow cytometry and TUNEL) were taken and their significance determined by the Scott–Knott test ($P < 0.05$).

3. Results

3.1. Cytogenetic analysis

Incubation with SPL was found to change the structure of both the chromosomes and the cell nuclei. Interphase nuclei became smaller with increasing exposure periods (24 and 36 h) during incubation with 25% SPL leachate (Fig. 1A–C). The longest period (36 h) of exposure to 25% SPL leachate induced cytoplasm shrinkage, vacuolization, and a high number of condensed nuclei (Fig. 1C). Chromosomal structure changed after SPL exposure. Highly condensed (c-metaphases) (Fig. 1D) and sticky chromosomes (Fig. 1E) were observed. Chromosomal aberrations such as micronuclei, bridges and fragments were also observed (Fig. 1F–H).

The quantitative effects of different concentrations of SPL on cytogenetic parameters such as MI, CA, MN, and CN are shown in Table 1.

Significant differences were seen between MI of the control (distilled water) and of 25% leachate-treated bulbs for all exposure periods (Scott–Knott $P < 0.05$) (Table 1). The inhibition in MI after the lowest exposure period (4 h) by 25% leachate was about 75% and became complete for the longer exposure periods of 24 and