CYTOGENOTOXICITY OF "SPL (SPENT POT LINER)" BY PLANT ROOT TIP BIOASSAYS

LARISSA FONSECA ANDRADE

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Dissertação apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós Graduação de Genética e Melhoramento de Plantas, para obtenção do título de "Mestre".

Orientadora
Profª Lisete Chamma Davide

LAVRAS
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APROVADA em 08 de Julho de 2008.

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2008

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RESUMO GERAL

ANDRADE, Larissa Fonseca. **Citogenotoxidade do SPL (Spent Pot Liner) por meio de bioensaios vegetais em ápice radicular.** 2008. 135p. (Dissertação – Mestrado em Genética e Melhoramento de Plantas) – Universidade Federal de Lavras, Lavras, MG*.

O SPL (Spent Pot Liner) é um resíduo sólido, tóxico, gerado nas indústrias de alumínio, durante o processo de eletrólise da alumina. Sua composição é bastante variável, incluindo sais de fluoreto, cianetos, metais e compostos orgânicos. No Brasil, mais de 35.000 t de SPL são geradas por ano. Algumas indústrias já adotaram o co-processamento do produto, que é utilizado como matéria prima nas indústrias cimenteiras. No entanto, os efeitos do SPL sobre os sistemas biológicos necessitam ser compreendidos, o que poderia auxiliar nas estratégias que buscam solucionar tal problema mais diretamente. A utilização de plantas para estudos de mutagênese tem sido reconhecida como excelente indicadora de alterações genéticas provocadas pela presença de substâncias químicas no meio ambiente e uma enorme quantidade de poluentes ambientais já foi testada. Neste contexto, metodologias que evidenciam danos no material genético, ou em estruturas celulares e tecidos são ferramentas úteis no estudo de citotoxidade de compostos sobre organismos. Além disso, as raízes são comumente utilizadas nos testes biológicos, pois são as primeiras a serem expostas aos produtos químicos ou resíduos lançados no solo ou na água. O objetivo do presente trabalho foi avaliar o efeito citogenotóxico do SPL em modelos biológicos por meio de bioensaios vegetais. Os resultados evidenciaram o efeito tóxico do SPL em função da alta freqüência de alterações cromossômicas, redução no crescimento radicular e indução de morte celular, após exposição ao SPL. Além disso, foi observado entre os modelos, relação entre aumento da concentração de SPL e aumento da freqüência de células com alterações cromossômicas (pontes em anáfase/telófase, aderência cromossômica, fragmentos e c-metáfases), além de redução no crescimento radicular. Na avaliação do efeito de diferentes concentrações em intervalos de tempo, foi obtida correlação positiva entre o aumento da concentração de SPL e o aumento do tempo de exposição em relação a características de morte celular evidenciadas pelo bioensaio do TUNEL (Terminal deoxynucleotide transferase mediated-dUTP nick-end labeling), formação de bandas decorrentes da fragmentação do DNA, alteração na razão núcleo-plasmática e aumento na atividade enzimática, via ROS, em decorrência ao estresse oxidativo causado pelo SPL. O SPL também provocou alterações na superfície radicular. Não obstante, a pós-exposição das raízes em água destilada mostrou que o dano causado pelo SPL pode ser reversível, após tratamentos em menor intervalo de tempo. É obvio pelos resultados apresentados que o SPL é resíduo tóxico e soluções são necessárias para resolver o problema do SPL que continua a ser depositado no ambiente.

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ABSTRACT

ANDRADE, Larissa Fonseca. **Cytogenotoxicity of SPL (Spent Pot Liner) by plant root tip bioassays.** 2008. 135p. (Dissertation – Mester in Genetics and Plant Breeding) – Federal University of Lavras, Lavras, MG*.

SPL (Spent Pot Liner) is a toxic solid waste generated by aluminum industry during the manufacturing of aluminum metal in electrolytic cells. It has a composition highly variable including fluoride salts, cyanides, metals and organic compounds. In Brazil, aluminum industries generate about 35,000 t of SPL per year. Some industries recycle this waste as feedstock in other industries, i.e. cement industries. However, information on the cytotoxicity of SPL is necessary to facilitate the understanding of its action on organisms in the environment and to subsidize environmentally correct solutions. The use of plants to measure potential environmental risk has been recognized as excellent to detect a wide range of genetic alterations under genotoxic contamination in the environment and a lot of chemicals elements were tested. Thus, methodologies that evidence DNA damage, alterations in cell structures and root meristem are important tools for these studies of cytotoxicity on organism. In addition, roots are frequently used on bioassays because they are first exposed to chemicals or wastes dumped on soil or water. The aim of the present work was to evaluate the citogenotoxicity of SPL in plant bioassays. The results showed the toxic effects of SPL by the increase in frequency of chromosome abnormalities, inhibition on root growth and induction of cell death in SPL-treated root tips. Moreover, it was observed among the plant tests a relation between the increases of SPL concentrations and incease on percentage of cells with alterations (anaphase/telophase bridges, stickness, fragments and c-metaphasis) and also the reduction on root growth. By evaluating the effects of different SPL concentrations in different time of exposure, was observed a concentration-exposure-depend manner in relation to cell death features. TUNEL (Terminal deoxynucleotide transferase mediated-dUTP nick-end labeling) assay, ladder pattern of DNA fragmentation, changes in nucleo-plasmic ratio and increase on antioxidants enzyme activity due to oxidative stress caused by SPL treatment were observed. SPL also provoked damage in root tips surface. Nevertheless, post-exposure root in distilled water demonstrated that SPL damage may be reversible after lower exposure times. It is obvious from the results that SPL is a toxic waste and solutions are necessary to solve the problem of SPL that is still dumped on the environment.

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CHAPTER 1

1 INTRODUCTION

Spent pot liner (SPL) is a solid waste generated by the aluminum industry during the manufacturing of aluminum metal in electrolytic cells. Initially the electrolityc cell liners comprise graphite and carbonaceous materials, but after several years of operation, the liner materials deteriorate and must be removed from the cells. In Brazil, aluminum industry generates about 35,000 t of SPL per year and it is a serious environmental pollution problem concerning the highly variable composition of this waste. Due to the concentrations of fluorides and cyanides in spent pot liner, and the tendency to leach in contact with water, the US Environmental Protection Agency (US EPA) has listed SPL as a hazardous material.

SPL is stored in controlled deposits, but the industries recognize the environmental liability of SPL and are pursuing many options for treatment and/or disposal, such as recycling as feedstock in other industry, i.e. cement industry where it is recycled as hardened paste for portland cement. Despite this, there is no effective option for the excess of SPL produced. Therefore, in a long term, more viable environmental solutions are necessary because leachates from these wastes might contaminate sources of potable water and affect human health.

At this context, information on the cytotoxicity effect of SPL is necessary to facilitate the understanding of its action on organisms in the environment and to subsidize environmentally correct solutions. Thus, cytogenetic assays, wide range used to detect the genotoxicity of environmental pollution could be applied with other bioassays to elucidate the SPL toxicity and its mechanisms in root tip of plants. These assays were carried out on the Laboratory of

Cytogenetic and Electron Microscopy at UFLA (Lavras, MG, Brazil) and Laboratory of Cytogenetic at UFJF (Juiz de Fora, MG, Brazil).

The cytogenetic analysis on root tips of *Allium cepa* and *Zea mays* demonstrated changes in cell cycle and mainly cell death (Andrade et al., 2008). Considering this, some assays including TUNEL (Terminal deoxynucleotide transferase mediated-dUTP nick-end labeling), flow cytometry, electrophoresis and enzymatic activity analysis were done in order to verify the cells features-like programmed cell death after SPL exposure. Furthermore, the germination tests in *Lactuca sativa* were also carried out to biomonitoriing the SPL phytoxicity. These studies will be shown in this work.

2 LITERATURE REVIEW

2.1 Environmental Pollution

Humans have been polluting the environment for a long time. However, the intensity and the severity of different kinds of pollution have drastically increased over the past century and their mutagenic impact has still to be estimated. The increments of pollution by the release of organic pollutants and the increase of radiation levels have affected the ecosystem and the health of living organisms, including humans. Thus, the presence of a large number of chemicals in the environment, including mutagenic agents, is a problem of major concern (Maluszynska and Juchimiuk, 2005).

Environmental mutagens can be broadly classified as physics (ionizing and ultraviolet radiation), chemical (organic and inorganic) and biological (toxins). A wide variety of organic and inorganic compounds contribute to chemical pollution; anthropogenic contamination by heavy metal ions and pesticides is an especially dangerous side effect of civilization (Kovalchuc et al., 2001). Numerous potentially mutagenic chemicals have been studied mainly because they can cause damaging and inheritable changes in the genetic material (Caritá and Marin-Morales, 2008).

Industries are indispensable components for the nation economic development. However, they have a major potential for generating hazardous waste. About 5.2 million tonnes of industrial waste are still dumped on land (The Energy Research Centre - TERI, 2003). It usually contains complex mixtures of chemicals that substantially contaminate ground and surface water reservoirs used for drinking purposes (Mumtaz, 1995; Dewhurst et al., 2002; Chandra et al., 2005), and might become hazardous to human health by inducing genetic alterations, as heavy metals are the most common toxicants of

wastewater sludge (Rank and Nielsen, 1994). The presence of complex organic-inorganic mixtures in these waste difficult the assessment of their genotoxicity (Minissi et al., 1998). There is a need for quick and accurate methods for the detection and evaluation of air, water and soil contamination and their effects on organisms.

Nevertheless, the presence of toxic agents can be detected by the changes caused in an organism with the use of bioassays. These tests are reliable, cost effective, quick, and simple (Gustavson et al., 2000). Bioassays can also be used to measure potential environmental risks (Gopalan, 1999). Then, the use of plants offers an advantage over other organisms because they can be more sensitive to environmental stress (Dutka, 1989), they are easy to manipulate and store, and furthermore, they offer a low-cost and good correlation in contrast with other bioassays (Fiskesjö, 1993). Several wastes from dye, metal, cyanide, paints, tanneries and others were studied by plant bioassays (Chandra et al., 2005; Feng et al., 2007; Caritá and Marin-Morales, 2008).

2.2 SPL (Spent Pot Liners)

In the process of aluminum production, a solid waste, SPL (Spent Pot Liner) is generated when alumina is dissolved in cryolite in electrolytic cells, called pots. A number of pots are arranged in series to form a potline. The pots contain a molten electrolyte consisting primarily of cryolite (Na_3AlF_6), but other materials are added to the electrolyte to improve the efficiency of the operation or to reduce power consumption, such as alumina, aluminum fluoride, sodium fluoride, soda ash, calcium fluoride, lithium carbonate and magnesium oxide. Initially the electrolityc cell liners comprise graphite and carbonaceous materials, which serve as the cathode, but after several years of operation, the

liner materials deteriorate and must be removed from the cells. This spent material is referred to as spent pot liner (SPL) (Silveira et al., 2002).

This removing of cathodes is a continual process. Thus, in Brazil, aluminum industries generate about 35,000 t of SPL per year. Although it is stored in controlled deposits, the industries recognize the environmental liability of SPL. In addition to containing fluoride salts, SPL contains cyanides that are formed by the infiltration of air through openings in the potshell and subsequent reaction of nitrogen with the carbon lining. Furthermore, SPL has a composition highly variable including not only cyanides and fluorides salts, but also organics and metals (Silveira et al., 2003). Leachates of SPL simuling the natural leachability of SPL in soil, showed the presence of cadmium, copper, iron, lead, manganese, sodium, zinc and also aluminum Sodium is also a SPL component and it was detected as the most abundant element in SPL leachate (Andrade et al., 2008).

The US Environmental Protection Agency (US EPA) has listed the SPL as a hazardous waste due to the concentrations of cyanides and fluorides (Silveira et al., 2002, 2003). Concerning this, aluminum industry is looking for technologies that could be used to stabilize its waste (Conner, 1997; Adaska et al., 1998). The options for treatment and/or disposal include landfill, recycling as a feedstock in other industries such as the steel, cement, aluminum, or mineral wool industries, fluidize bed combustion, cryolite recovery, pyrohydrolysis, pyrosulfolysis and others. Recycling through other industries is an attractive and proven option (Chanania and Ebby, 2000). The more attractive option is recycling SPL as part of the raw material in concrete manufacturing due to the characteristics of SPL inorganic fraction. It showed a relatively low cyanide content and can be mixed with soil, binder and water constituting the hardened paste for portland cement (Channell and Kosson, 1993; Conner, 1997; Adaska et al., 1998, Silveira et al. 2002, 2003). This technology is currently being used to

treat a wide variety of waste containing contaminants as metals, organics, organo-metallics, soluble salts, etc. and showed to be effective in reducing the mobility of cyanide and soluble fluoride salts (Conner, 1997; US EPA, 1997, 1999).

So, information on the cytotoxicity effect of SPL is necessary to facilitate the understanding of its action on organisms in the environment and to subsidize environmentally correct solutions.

2.3 Cytotoxicity of SPL (Spent Pot Liners) components

2.3.1 Aluminum

The toxicity caused by aluminum (Al) in plants in acid soils is well documented. Aluminum (Al) is the third most abundant element, making up more than 8% of the earth's crust. Its toxicity becomes acute in acid soils that comprise almost 40% of the total world's arable land and limits crop productivity (Kochian, 1995; Hede et al., 2001). Al interferes with a wide range of physical and cellular processes. It has been suggested that free Al (Al^{3+}) is the most toxic of the soluble forms of Al to plant, because affects plant growth and development (Mohanty et al., 2004). Potentially, Al toxicity could result from complex Al interactions (Kochian et al., 2005). It may be located in the cell walls (Horst, 1995), in the symplasm (Liu and Jiang, 1991; Clarkson, 1995), in the plasma membrane (interaction with lipids) (Dellers et al., 1986; Jones and Kochian, 1997) or in the formation of callose (Wissemeir et al., 1992). The primary effect of Al ion toxicity is the inhibition of root growth by affecting cell elongation, especially in the distal part of the transition zone of the root apex elongation (Sivaguru and Horst, 1998; Matsumoto, 2000).

At tissue level, the meristematic, distal transition and apical elongation zones of the root apex are the most sensitive to Al, at cellular level, the targets of

Al toxicity are primarily cell wall, plasma membrane, cytoskeleton and nucleus (Zheng and Yang, 2005). At a molecular level, strong binding affinity of Al^{3+} with oxygen donor legands (proteins, inorganic phosphate, nucleic acids, carboxylic acid, phospholipids, polysaccharides, anthocyanin) results in the inhibition of cell division, cell extension, and transport (Mossor-Pietraszewska, 2001).

Studies at meristematic zone have indicated that some Al can enter the cytosol of cells within minutes following Al exposure (Vazquez et al., 1999; Silva et al., 2000; Taylor et al., 2000). Consequently, a large fraction of the Al interacts with apoplastic targets and a small fraction enters the symplasm and interacts with symplastic targets. The promptness of the root growth inhibition upon exposure to Al could be due to the inhibition of cell division (Kollmeier et al., 2000; Matsumoto, 2000; Frantzios et al., 2001). Prolonged exposures lead to Al interactions with structures within the root cell nuclei, affecting DNA composition, chromatin structure and template activity, resulting in disruption of cell division and the cytoskeleton (Silva et al., 2000). It also reduces DNA replication, increasing the rigidity of the DNA double helix (Rout et al., 2001). Moreover, Al toxicity is associated with gross changes in root morphology (Ciamporova, 2002), inducing the formation of lateral roots, inhibiting root elongation and root-hair development. This extensive root damage results in a reduced and damaged root system and limited water and mineral nutrient uptake (Pan et al., 2001, Barcelo and Poschenrieder, 2002; Ciamporova, 2002).

In addition, it has been demonstrated the occurrence of cytogenetic alterations in root meristematic cells of plants after Al exposure, including cell death (Fiskesjö, 1988; Roy et al., 1989; Liu and Jiang, 2001; Pan et al., 2001 Campos and Viccini, 2003). Several works have shown that Al stress can increase the production of reactive oxygen species (ROS) and activate oxidative

enzymes in plant and animals. This oxidative stress provoked by Al^{3+} can also induce cell death (Deslile et al., 2001; Pan et al., 2001, Boscolo et al., 2003).

Notwithstanding, though some effects off Al are known, the degree of toxicity reported in the literature varies widely depending on the plant species, the growth conditions, the Al concentrations, and the duration of the exposure (Kochian et al., 2005). Thus, given the complexity of many cellular processes involved in root growth inhibition, the precise aluminum stress signaling remains unclear (Panda et al., 2008).

2.3.2 Fluor

Fluoride is an element widely found in the atmosphere, air, water, soil, coal, food, dental and industrial uses (World Health Organization - WHO, 2004). The fluoride toxicity mechanism in plants is so far to be elucidated (Franzaring and Fangmeier, 2007). Nevertheless, it can be accumulated in plants, leading to metabolic alteration, leave damage, plant growth inhibition and development, and also affects the whole environment by polluting the air (Franzaring and Fangmeier, 2007). Plants take fluoride from the air trough the leaves, and marginal necroses represent typical symptoms of fluoride pollution and toxicity (Abdallah et al., 2006).

Furthermore, in polluted areas there were also some reports of acute fluoride poisoning, indentified by the fluoride effects in plants. They have shown the occurrence of apoptosis in several types of cells (Wang et al., 2004; Otsuki et al., 2005; He and Chen, 2006). It is possibly due to the increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) because of the presence of fluor. Thus, it may be one of features for fluoride toxicity. Sustained increase in [Ca2+]i is proposed to induce apoptosis (Trump and Berezesky, 1995). However, an intracellular Ca2+ loading also causes necrosis if it is excessive (Gwag et al.,

1999). Therefore, the type of cell death induced by fluoride may vary from cell to cell (Matsui et al., 2007).

2.3.3 Cyanide

Cyanide, a strong ligand capable of complexing virtually any heavy metal, had its first industrial use in metallurgical operations (Yu and Gu, 2007). Apart of this, cyanide is a common soil contaminant and is often found associated with present or former manufactured gas, electroplating plants and precious metal mining sites. It is known to be toxic to most organisms. At such sites, cyanide is commonly found associated with iron as ferrocyanide $[Fe^{II}(CN)^6]^{-4}$ and ferricyanide $[Fe^{III}(CN)^6]^{-3}$ (Mansfeldt et al., 2004). Although both forms are much less toxic than free cyanide (CN^-), they are potentially hazardous because CN^- can be released from these compounds by photolysis (Meeussen et al., 1992; Zimmerman et al., 2007). Regarded as a highly toxic compound and a potent metabolic inhibitor, cyanide is involved in biochemical pathways of several plants (Ebbs et al., 2003). However, reports suggest that vascular plants possess various enzymes that can detoxify or degrade toxic chemicals and are able to metabolize the free cyanide in solution to remediate cyanide contamination (Burken, 2003; Ebbs et al., 2003; Trapp et al., 2003; Trapp and Christiansen, 2003; Yu et al., 2005; Yu and Gu, 2007).

2.3.4 Heavy Metals

Heavy metals are defined as metals with a density higher than 5g/cm³. Soils are reservoirs for heavy metals generated by industrial activities (e.g., metal finishing, paint pigment and battery manufacturing, leather tanning, mining activities, municipal waste water sludges, urban composts, pesticides, phosphate fertilizers, or from atmospheric depositions) (Adriano, 1986; Kabata-Pendias and Pendias, 1992). Although a certain level of heavy metals ions

occurs naturally in soil and water, organic contaminants are associated only with human influence.

Toxic heavy metal ions induce several cellular stress responses and can cause damage to different cellular components such as membranes, proteins and DNA. They might influence DNA via the production of free radicals or by general interference in the activity of DNA (Patra et al., 1998; Kovalchuc et al., 2001; Waisberg et al., 2003; Jimi et al., 2004). Then, SPL composition contains heavy metals such as cadmium, copper, iron, lead, manganese, and zinc that cause chromosome abnormalities and cell death, which may appear as a decline in the mitotic index (Andrade et al., 2008).

In the last several decades, cadmium (Cd) has been used as material and/or intermediate in the chemical industry and is one of the most important industrial pollutants among the toxic heavy metals. Therefore, it represent serious problem for the ecosystem and its toxicity on plant is well documented. (Fojta et al., 2006) It is rapidly taken up by plant roots (Behboodi and Samadi, 2004; Liu et al., 2005) and is stored in appoplast or vacuoles of plant cells inducing chromatin fragmentation (Zhang and Xiao, 1998; Liu et al., 2005), changes in the nucleolus (Marcano et al., 2002), apoptosis (Behboodi and Samadi, 2004) and also necrosis as response to excess of doses (Nicotera et al., 1999; Behboodi and Samadi, 2004). The inhibition of root growth is another effect of cadmium (Liu et al., 1992).

Lead (Pb) pollution occurs through a variety of human activities. Soils subjected to anthropogenic pollution have been reported to contain high Pb concentrations (Kabata-Pendias and Pendias, 2001). Similar to Cd, Pb inhibits root growth (Liu et al., 1994a, 2003; Seregin et al., 2004). Cd and Pb toxicity also results in cell cycle disturbances (Wierzbicka, 1999) and decrease in mitotic index (MI) value accompanied by reduction of cell number in metaphase and anaphase (Wierzbicka, 1988, 1989; Samardakiewicz and Wozny, 2005; Fusconi

et al., 2006). Additionally, both heavy metals induce c-metaphasis, chromosome stickness and chromosome bridges (Wierzbicka, 1988; Zhang and Xiao, 1998; Jiang and Liu, 2000; Samardakiewicz and Wozny, 2005). Pb also causes lagging chromosomes and nuclei with more condensed chromatin (Samardakiewicz and Wozny, 2005). Moreover, binucleate cells were found in root meristems after Pb treatment as a result of cytokinesis inhibition (Wierzbicka, 1989). It is also well documented that heavy metals such as Pb, Cd and Cr induced micronuclei formation (Knasmüller et al., 1998; Steinkellner et al., 1998; Matsumoto et al, 2004; Samardakiewicz and Wozny, 2005; Qian et al., 2006). Apart from that, those metals disturbed RNA and DNA synthesis (Łbik-Nowak and Gabara, 1997) and induced changes in the root cell ultrastructure (Wozny et al., 1982).

Zinc is one of the most abundant metals in nature. It is essential for plant growth and is required in very small amounts but it becomes toxic when in excess (Colling, 1981; Marschner 1995). The general symptom of zinc toxicity is a retardation of growth leading to plant stunting (Pahlson, 1990). On meristematic cells of plants, the observed effects of zinc are mitosis inhibition, chromosome abnormalities (sticky chromosomes, c-metaphases, bridges and metaphase disturbed by lagging chromosomes), alterations in root morphology and also cell death (El-Ghamery et al., 2003).

Copper is known to be an essential micronutrient for higher plants (Quartacci et al., 2000) because it is a constituent metal of several enzymes in the cytoplasm of plant cell. It is a cofactor for key enzymes of molecular processes required for growth and development, including ATP synthesis (cytochrome-*c*-oxidase) and oxygen transport (hemocyanin) (Harrison et al., 1999; Peña et al., 1999). High levels of Cu in soil can be phytotoxic, causing deleterious effects at morphological and physiological levels (Liu et al., 1994b; Ouzoundiou, 1994b, 1995; Quartacci et al., 2000). In toxic concentration Cu can lead to total inhibition of growth (Reboredo and Henriques, 1991; Ouzounidou

et al., 1992; Ouzounidou, 1994a), inhibition on root elongation and damage to root epidermal cells and root cell membranes (Wainwright and Woolhouse, 1977; Ouzounidou et al., 1995). In *Allium cepa* the excess of Cu affects root-tip cells causing chromosome aberrations (Levan, 1945; Fiskesjö, 1981, 1988) and change of nucleoli behavior (Liu et al., 1994b), both related to the occurrence of certain degree of stickness, a few c metaphasis and remarkably rapid decrease of the mitotic index with increasing Cu concentrations (Fiskesjö, 1988).

There is little information about manganese in the literature, but apparently, the most typical parameter of toxicity on root is to induce sticky chromosomes (Fiskesjö, 1988).

2.3.5 Sodium

Sodium is also a SPL component and it was detected as the most abundant element in SPL leachate (Andrade et al., 2008). Salinity induces ionic stress driven by toxic action of sodium (Bernstein et al., 1995). The osmotic and ionic stress also generates oxidative stress, which is caused by excessive amounts of reactive oxygen species (ROS). ROS can have damaging effect on cellular structures and macromolecules especially DNA. Radic et al. (2004) showed total absence of mitotic activity in root-tip cells of *Centaurea ragusina* treated with higher concentrations of salt. Concentrations of NaCl (150mM) induced high frequencies of mitotic abnormalities, such as chromosome breaks, anaphase bridges and stickness, c-metaphasis, irregular anaphases, lagging chromosomes, multipolar spindles, aneuploidy and polyploid.

2.4 Plant bioassays

Several tests are commonly used to biomonitore pollution levels and to evaluate the effects of toxic and mutagenic agents present in the natural environment. Plants are immobile organisms. Under pollution stress, roots are

the primary point of contact with contaminants. To some extent, plants could overcome environmental stress by developing efficient and specific physio-biochemical mechanisms (Sandalio et al., 2001). The influence of environmental mutagens on a plant depends not only on the type and dose of mutagen, exposure time, and interaction with other factors, but also on the plant species, genotype, and stage of development (Gichner, 2000). Moreover, plant response to mutagenic treatment can be considered on different levels of organization: from DNA, chromosome, and genome to the whole organism (Maluszynska and Juchimiuk, 2005).

It is important for the prevention of DNA changes caused by environment to understand the biological consequences of DNA damages and their molecular modes of action that lead to repair or alterations of the genetic material. Numerous genotoxicity assay systems have been developed to identify DNA reactive compounds. The available data show that plant bioassays are important tests for detection of genotoxic contamination in the environment and the establishment of controlling systems. Plant systems can detect a wide range of genetic damages, including gene mutation and chromosome aberration (Maluszynska and Juchimiuk, 2005). Concerning this, several higher plant bioassays for screening and monitoring environmental mutagens have been established (Constantine and Owens, 1982; Grant, 1982) such as *Allium cepa*, *Hordeum vulgare*, *Arabidopsis thaliana*, *Glycine max*, *Vicia faba* and *Zea mays* etc., as good bio-indicators of genetic toxicity of environmental pollutants in recent years (Liu et al., 2005). Considering these, *Allium cepa* is the most efficient and used for this purpose (Grant et al., 1994, 1998; Caritá and Marin-Morales, 2008).

Allium cepa roots are regarded as one of the pioneering organisms used to detect the effects of chemicals on plant chromosomes in cytogenetic studies. Furthermore, the *A. cepa* test, which was developed by Levan in 1938, has been

used by the Environmental Protection Agency (US EPA) to evaluate toxicity and genotoxicity of industrial effluents (Fiskesjö, 1993; Grant, 1994, Mitteregger Júnior, 2007).

The Allium test is a simple, sensitive and rapid bioassay that has been widely used as a standard for biomonitoring environmental contaminants, toxicity of chemical compounds and evaluating potential anticancer properties (Kurás and Malinowska, 1978; Podbielkowska et al., 1981, 1995; Kupidłowska et al., 1994; Keightley et al., 1996; Majewska et al., 2003;) using various genotoxicity parameters (Kurás et al., 2006). In addition, it is a very comfortable system since it is available all year round, contains rather homogenous meristematic cells, has only 16 chromosomes, which are very long and well visible, gets stained easily and allows easy detection of chromosome aberrations. Thus, the test is a fast and inexpensive method, allowing the investigation of universal mechanisms involved in plant response to the treatment of root tip cells by the mixture of toxic wastes (DeMarini, 1991; Fiskesjö, 1993). Besides its sensitivity, it presents high correlation with other test systems. These features are essential to an accurate assessment of environmental risks, as well as to a successful extrapolation of test results obtained in exposed organisms to other species. Regarding these characteristics, Fiskesjö (1985) showed that the *Allium* test presents a similar sensitivity to that of some algal and human lymphocyte test systems. Rank and Nielsen (1994) showed a correlation of 82% between *Allium* test and carcinogenicity assays in rodents, besides demonstrating that the former was more sensitive than Ames or microscreen tests. Furthermore, studies of sensitivity amongst higher plants have also showed that *A. cepa* is more sensitive than some other species, such as *Vicia faba* (Ma et al., 1995; Migid et al., 2007).

Cytogenetic tests analyze the frequency and type of chromosome aberrations in mitotic cells and the frequency of micronuclei in interphasic cells.

Genotoxic agents cause DNA damage, which is either repaired or otherwise leads to alterations of the DNA. Chromosome aberrations are the consequence of DNA double strand break which was unrepaired or repaired improperly. Broken chromosome ends without telomeres become "sticky" and may fuse with other broken chromosome ends. The result of these chromosomal rearrangements are acentric fragments, dicentric bridges observed in mitotic cells of the first cell cycle after mutagenic treatment or micronuclei in the interphasic cell in the next cell cycle. The micronucleus test was developed parallel to chromosome aberration assays (Fiskejö, 1985). Micronuclei are extranuclear bodies of chromatin material formed as a consequence of chromosome breakage or aneuploidy. The frequency of cells with micronuclei is a good indicator of the cytogenetic effects of tested chemicals. Similarly, chromosome aberrations and micronuclei tests are conducted with other plant species such as *Vicia faba* (Sang and Li, 2004), *Crepis capillaris* (Grant and Owens, 1998; Juchimiuk and Maluszynska, 2005), *Hordeum vulgare* (Gecheff, 1996; Sang et al., 2006).

2.5 Plant Cell Death

Cell death is an important process in life cycle of an organism and is associated with various development stages. In plants it is an integral part of development and defense. It occurs at all stages of the life cycle, from fertilization of the ovule to death of the whole plant. Without it, tall trees would probably not be possible and plants would succumb more easily to invading microorganisms.

Different categories of cells can be recognized within those that undergo death (Krishnamurthy et al., 2000). One of them include those cells subjected to hypersensitive response (HR) due to pathogenesis and other abiotic stresses such as osmotic, oxidative (H_2O_2 and salicylic acid), temperature, salt, water, heavy metals, UV, nutrient deprivation, toxins, chemicals and other (Pennel and Lamb,

1997; Lamb and Dixon, 1997). This category of cells is common and cell death is used as a defence against the stress or as a means of emanating signals for the other nearby cells to build up defence/immune reactions (Mittler and Lam, 1996). Nevertheless, cell undergoes death when it is damaged and unable to function (Krishnamurthy et al., 2000).

In addition, the stress intensity actives mechanism that leads to different types of cell death: low dose leads to activation of prevent mechanisms, intermediate levels active cell death by apoptosis and higher doses inhibit DNA synthesis and lead cell to injury by necrosis (Tuschl and Schwab, 2004; Burbridge et al., 2006). It was demonstrated by McCabe et al. (1997) in carrots at study with dose-dependency. Cell death was induced by high temperatures. The authors suggested that cells undergo death after moderate stress. These cells present programmed cell death (PCD) morphological features such as condensed cytoplasm increase on space between cell wall and plasmatic membrane and also DNA cleaved into nucleosomes (McCabe et al., 1997, 2000).

Thus, the PCD, commonly referred to as apoptosis, is a genetically defined process comprising the program that cells exhibit leading to death (Doorn and Woltering, 2004). This program is related to biochemical activation of enzymes such as proteases (caspases and endonucleases), releasing cytochrome *c* from mitochondria and breaking DNA in the linker regions between nucleosomal cores, producing oligonucleosomal fragments. The ultraestructural and morphological alterations after these biochemical processes are associated with apoptosis. It is characterized by cytoplasmic shrinkage, nuclear condensation, DNA fragmentation, membrane blabbing, cytoskeleton alterations and appearance of apoptotic bodies (Savill and Fadok, 2000; Ueda and Shah, 2000; Ameisen, 2002; Behboodi and Samadi, 2004).

In contrast to apoptosis, necrosis is a non-physiological, but accidental cell death characterized as passive, which is associated with inflammation. It often

results from an overwhelming cellular insult as high concentration of toxin or metal ion, heating or freezing. These stimulus cause organelle and cell swelling, breakdown of the plasma membrane, release of lysossomal enzymes and the leakage of cell contents into extracellular milieu (Trunp, 1995; Pan et al., 2001).

Since programmed cell death (PCD) was first described in animal cells at morphological level, some characteristics of this mechanism are not found in plants. For example, in animals cells the chromatin condenses and collapses into patches, the nucleus becomes fragmented and cell parts (some of which containing fragmented nucleus) bud outwards to produce membrane coated apoptotic bodies. On the other hand, in plant cells the membrane blabbing and engulfment of the dead cell are not observed. In addition, there is some doubt over the formation of apoptotic bodies in plant cells. (Greenberg, 1996; Wang et al., 1996; Yamada et al., 2003). Today, PCD is being revealed at biochemical and molecular levels, not only in animal cells but also in plant cells, revealing the role of mitochondria in this process (Panda et al, 2008).

Exposure to various chemical agents can result in cell damage and death in plants. Numerous studies have shown the PCD features as DNA fragmentation and formation of apoptotic bodies elucidating the mechanisms of toxicity from elements like aluminum (Katsuhara and Shibasaka, 2000; Pan et al., 2001; Achary et al., 2008, Panda et al., 2008), cadmium (Behboodi and Samadi, 2004; Seth et al, 2008), organic compounds as herbicides (Robertson and Orenius, 2000) and salts (Liu et al., 2007).

Recently, it has been suggested that apoptosis and necrosis are just endpoints of a wide range of morphologic and biochemical possibilities of death (Bursch et al., 2000; Fukuda et al., 2000; Subbaiah and Sachs, 2003). Regarding this, Xiong et al. (2006), studying meristematic root tips of complete submerged maize seddlings demonstrated features in part resembling PCD and in part resembling necrosis. The conclusion was that these different types of cell death

appear to reflect a high degree of flexibility in response of cells to changes of environmental conditions (Bursch et al., 2000).

Because of these divergences, some bioassays have been realized in order to better distinguish and characterize both processes. This includes morphological analysis, flow cytometry, fluorescent dyes, TUNEL (Terminal deoxynucleotide transferase mediated-dUTP nick-end labeling) and analysis of DNA fragementation by electrophoresis and comet assay.

The morphological features of PCD as condensed nucleus and shirinky cytoplasm can be observed by cytogenetic analysis and electron microscopy.

The flow cytometry, in particular, can be used to follow the percentage of apoptotic cells present in a cell population. The detectable characteristics of PCD by flow cytometry analysis include DNA fragmentation, changes in cell size and granularity, changes in plasma membrane permeability, cell surface modification (externalization of phosphatidyl-serine) and formation of apoptotic bodies. These events are observed through the interaction of a particle with the laser beam which produces a light scatter in a forward direction (FSC that correlates with cell size) and a lateral direction (SSC that correlates with granularity and/or cell density). Necrotic death is characterized by a reduction in both FSC and SSC (probably due to a rupture of plasma membrane and leakage of the cell's content). During apoptosis there is an initial increase in SSC (probably due to the chromatin condensation) with a reduction in FSC (due to the cell shrinkage) (Nicoleti et al., 1991; Vermes et al., 2000)

Therefore, the presence of cells with DNA stainability lower than that of G1-cells (hypodiploid or sub-G1 peaks) has been considered a marker of cell death by apoptosis. The appearance of sub-diploid DNA peak is a specific marker of apoptosis; necrosis induced by metabolic damage or lysis produced by complement did not induce any sub-G1 peak in the DNA fluorescence histogram. In addition to apoptotic cells, the sub-G1 peak can also represent

mechanically damaged cells, cells with reduced chromosome number (aneuploid cells in a heterogeneous tumor population) and isolated apoptotic bodies (Nicoleti et al., 1991).

The Comet assay was used to detect DNA damage as consenquence of the process of apoptosis in animal cells and then it was adapted to plant cells. The method consists of "single cell gel electrophoresis" where the DNA migration from the nuclei is showed by a picture like a Comet (Mc-Kelvey et al., 1993; Lin et al., 2007). This test allows not only the detection of single and double stranded DNA breaks in the nucleus, but also the measuring of the level of DNA migration through an agarose gel in an electric field. Computerized image analysis system measures the amount of DNA in the head and in the tail, and the length of the tail. Measurement of Comet tails is an important parameter as it represents free DNA fragments and shows damage in individual cells (Klaude et al., 1996).The tail moment (TM) can be calculated to express DNA damage (Gichner, 2003; Maluszynska and Juchimiuk, 2005). The eletroforetical analysis can also be carried out with total DNA in a common agarose gel. Cells undergoing apoptosis are supposed to produce a patternof DNA ladders. These are useful tools to investigate the capacity of DNA repair of damage induced by different types of mutagens and various damage levels in different cell types (Menke et al., 2001).

Moreover, the TUNEL reaction (TdT-mediated deoxy-uracil nick end labeling) is used for analyzing DNA fragmentation by labeling the 3-OH ends of the DNA strand breaks. This method is based on the ability of terminal deoxynucleotidyl transferase (TdT) to attach a fluorescein-conjugated deoxy-uracil to the 3'-OH end of cut DNA (Behboodi and Samadi, 2004).

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CHAPTER 2

Cytogenetic alterations induced by SPL (Spent Pot Liner) in meristematic cells of plant bioassays

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1 RESUMO

ANDRADE, L.F.; CAMPOS, J.M.S.; DAVIDE, L.C. Alterações citogenéticas induzidas pelo SPL (Spent Pot Liner) em células meristemáticas de modelos vegetais. In: ____. **Citogenotoxidade do SPL (Spent Pot Liner) por meio de bioensaios vegetais em ápice radicular.** 2008. 135p., p. 38-62. Dissertação (Mestrado em Genética e Melhoramento de Plantas) – Universidade Federal de Lavras, Lavras, MG.

O SPL (Spent Pot Liner) é um resíduo sólido gerado na indústria de alumínio durante a eletrólise nas cubas de redução de alumina na fabricação do metal alumínio. Informações sobre o efeito do SPL são necessárias para facilitar a compreensão da ação do mesmo nos organismos e para dar subsídios a soluções ambientais mais corretas. Neste contexto, o objetivo do presente trabalho foi comparar o efeito do SPL em células meristemáticas de *Allium cepa* e *Zea mays* além de discutir os mecanismos envolvidos na citotoxidade do SPL. Para os dois modelos vegetais, *A.cepa* e *Z.mays*, foi observado uma forte inibição no crescimento radicular nas concentrações mais altas de SPL. Para análise citogenética, os resultados mostraram uma redução no índice mitótico e aumento nos diferentes tipos de anormalidades com o aumento da concentração do SPL. Observamos pontes, fragmentos cromossômicos, cromossomo pegajoso, anáfase multipolar, segregação tardia e morte celular. Em geral, foi possível observar um aumento das diferentes anormalidades à medida que aumentou a concentração do SPL. É óbvio a partir dos resultados obtidos no presente trabalho que o SPL é citotóxico para as células das plantas teste (*A. cepa* e *Z.mays*).

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2 ABSTRACT

ANDRADE, L.F.; CAMPOS, J.M.S.; DAVIDE, L.C. Cytogenetic alterations induced by SPL (Spent Pot Liners) in meristematic cells of plant bioassays. In: ____. **Cytogenotoxicity of SPL (Spent Pot Liner) by plant root tip bioassays.** 2008. 135 p., p 38-62. Dissertation (Mester in Genetics and Plant Breeding) – Federal University of Lavras, Lavras, MG.

Spent Pot Liners (SPL) are solid waste generated by the aluminum industry during the manufacture of aluminum metal. Information on the cytotoxicity effect of SPL is necessary to facilitate understanding of their action on organisms and to subsidize environmentally correct solutions. Thus, the aim of the present investigation is to compare the effect of SPL on meristematic cells of *Allium cepa* and *Zea mays* and also to discuss the mechanisms of SPL cytotoxicity involved. A strong inhibition on root growth in higher SPL concentrations has been observed in both *A. cepa* and *Z. mays.* For cytogenetic analysis, the results showed a reduction of mitotic index and increase of different abnormalities as the SPL concentration increased. We observed bridges, chromosome fragments, stickness, multipolar anaphase, later segregation and cell death. In general, it was possible to observe an increase of different abnormalities as the SPL concentration increased. It is obvious from the results of the present investigation that SPL is cytotoxic on meristematic cells of plant tests (*A. cepa* and *Z. mays*).

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3 INTRODUCTION

The presence of a large number of pollutants in the environment, including mutagenic agents, is a problem of major concern (Juchimiuk and Maluszynska, 2005). Industry waste is a serious environmental pollution problem.

Spent Pot Liner (SPL) is a solid waste generated by the aluminum industry during the manufacture of aluminum metal in electrolytic cells (Silveira et al., 2002). SPLs have a highly variable composition (e.g. cyanide, fluoride, organics and metals), but the components of the greatest environmental concern are cyanides and soluble fluoride salts (Silveira et al., 2003). The US Environmental Protection Agency (US EPA) listed SPL as hazardous waste due to the concentrations of these fluoride and cyanide salts and the tendency to leach in contact with water (US EPA, 1991; Chanania and Eby, 2000). Leachates from these wastes might contaminate sources of potable water and affect human health (Chandra et al., 2005).

In Brazil, aluminum industries generate about 35,000 t of SPL per year. It is stored in controlled deposits, but the industries recognize the environmental liability of SPL and are pursuing many options for its treatment and/or disposal, such as recycling as feedstock in other industries (e.g. cement industry). Although this is an attractive option, the classification of SPL as hazardous waste discourages other industries from utilizing SPL. Therefore, in the long term, more viable environmental solutions are necessary (Silveira et al., 2002).

However, information on the cytotoxicity effect of SPL is necessary to facilitate the understanding of its action on organisms in the environment and to subsidize environmentally correct solutions. Many plant species have a wide range of applications as indicators of cytogenetic and mutagenic effects of environmental

agents (Juchimiuk and Maluszynska, 2005). Cytogenetic tests in plants are relatively inexpensive and can easily be handled. Due to the size of their chromosomes, higher plants are suitable to cytological analysis and they have shown good correlation with other bio-testing systems (Fiskesjö, 1985).

Plant roots are extremely useful in biological testing. The root tips are often the first to be exposed to chemicals dispersed naturally in soil or in water (Fiskesjö, 1988). Therefore, the observation of the root tip constitutes a rapid and sensitive method for environmental monitoring. *Allium* root tips, for example, have been utilized for the study of biological effects caused by chemicals since Levan introduced the first *Allium* test in 1938 (Levan, 1938). This species has been utilized for studies concerning some environment pollutants such as hazardous industrial wastes (Chandra et al., 2005), wastewater (Nielsen and Rank, 1994; Rank and Nielsen, 1994; Smaka-Kincl et al., 1996; Odeigah et al., 1997; Grover and Kaur, 1999), municipal sludge (Srivastava et al., 2005) and monitoring of pesticides in water (Fatima and Ahmad, 2006).

Although *Zea mays* has been considered as a species of large economical importance and very well cytogenetically characterized, there are few reports concerning cytotoxicity in this species. Thus, the objective of the present work is to compare, by cytogenetic tests, the effect of SPL exposure on meristematic cells of *A. cepa* and *Z. mays* and also to discuss the mechanisms of SPL toxicity involved.

4 MATERIAL AND METHODS

4.1. Plant bioassays

Bulbs from the onion (A. cepa, 2n = 16) of the line Alfa-tropical (BAG-UFV) and seeds of maize (Z. mays, 2n = 20) of BRS 3060 (Embrapa-Milho and Sorgo) were used as materials for cytotoxicity analysis.

4.2. Preparation of SPL/soil leachates

For simulating the adsorption of the SPL in soil and leachability of this pollutant, six concentrations of SPL were prepared (0%, 5%, 10%, 15%, 20% and 25%) of SPL(mg)/soil(mg). These mixtures were added to 200mL of $CaCl_2$ 0.01M 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 supernatant was collected. These leachates were used throughout the study. Total cyanide and fluorides were evaluated using a selective ion electrode and total aluminum was determined by plasma emission spectroscopy. Additionally, the pH and electrolytic conductivity were determined for leachates (Table 1). Only the SPL 25% sample was analyzed for metal composition using inductively coupled plasma emission spectrometry.

4.3. Root growth analysis

Bulbs/seeds were exposed directly to SPL solutions at room temperature. The root lengths were evaluated 12, 24, 36, 48, 60, and 72 h after the exposure to treatments. The treatments were arranged in a completely random design with

four replications for each concentration/exposure time. Each replication was obtained from the average of six measurements of root growth, i.e. there were 24 root growth measurements for each concentration/exposure time.

4.4. Cytogenetic analysis

For cytogenetic analysis the bulbs/seeds were pre-exposed to distilled water, for root emergence, and later submitted to SPL solutions for 24 h. After exposure, eight root tips (ranging from 0.5 to 1.0 cm) were collected from each replication, washed in distilled water and fixed in fresh cold methanol: acetic acid (3:1) solution. Slides were prepared by the air drying technique with enzymatic maceration (Carvalho and Saraiva, 1993), using Pectinex (NOVO NORDISKTM)/sodium citrate buffer (pH 3,5) (1:10) incubated at 34 ºC for 2:30 h in *A. cepa* and 3:30 h in *Z. mays*. Observed under the stereomicroscope, the root tips were chopped into several tiny fragments and five to six drops of fixative solution were added in. Slides were quickly dried and stained with 5% Giemsa in phosphate buffer (pH 6.8), followed by washing with distilled water, and air dried on a heating plate at 50 ºC. The mitotic index (percentage of cells in division) and chromosome abnormalities were scored in a minimum of 8000 cells per treatment. The aberrations were characterized and classified in the following categories: bridges, fragments and later segregation (number of anaphase cells with these alterations/total number of anaphase cells analyzed); stickness and c-metaphase (number of metaphasic cells with these alterations/total number of metaphase analyzed) and dead cells (number of dead cells (pycnotic nuclei)/total number of cells analyzed).

4.5. Statistical analysis

The means of root growth, mitotic index and chromosomal and mitotic aberration data were taken and their significance was determined by the Tukey test ($p<0.05$).

Table 1 SPL leachates analysis

	pH	Electrolytic conductivity (dS m^{-1})	Aluminum (mg L^{-1})	Cyanide (mg L^{-1})	Fluoride (mg L^{-1})
Control	6.5	0.3	0.2	0	0.01
SPL 5%	7.0	0.5	1.9	1.6	13.4
SPL 10%	7.4	2.5	2.2	6.8	45.6
SPL 15%	8.0	4.5	0.5	12.3	23.5
SPL 20%	8.5	7.8	0.6	16.7	33.4
SPL 25%	9.5	9.3	0.7	23.4	47.8

5 RESULTS

5.1. SPL 25% sample analysis

The basic chemical properties of SPL 25% sample showed the following results: cadmium: <0.18mgL^{-1}; copper: <0.34mgL^{-1}; iron: 75mg L^{-1}; lead: <0.23mgL^{-1}; manganese: <0.18mgL^{-1}; sodium: 657mg L^{-1}; zinc: <0.34mgL^{-1}.

5.2. Root growth

The results presented in Table 2 show that root growth in both test plants was reduced by all the concentrations applied as compared with the control values. The inhibition of root growth was greater with increasing concentrations of SPL and treatment duration. Significant differences were detected even in the lowest SPL concentration of 5% in 48 h of time exposure in *A. cepa* (control = 2.22 cm, SPL 5% = 1.46 cm; Tukey p<0.05). For this plant, root growth was not observed for the SPL 25%. For SPL 20%, root growth was observed only in 72 h. In the *Z. mays,* the behavior of root growth was similar to *A. cepa.* The difference can be noticed in the 36 h of time exposure, where the root growth for the SPL 10% treatment can be observed (a significant difference in relation to the control; control = 1.71 cm, SPL 10% = 0.23 cm; Tukey po0.05). Additionally, in this plant, for the SPL 20% and 25% treatments, root growth was observed after 48 h of time exposure, distinguished from *A. cepa.* Comparing *A. cepa* and *Z. mays* for 72 h of exposure time and root growth in the SPL 25% (high concentration), the inhibition of growth in relation to the control was 100% and 92%, respectively (*A. cepa*: control = 6.61 cm, SPL 25% = 0 cm; *Z. mays*: control = 7.12 cm, SPL 25% = 0.56; Tukey p<0.05).

Table 2 Root growth (cm) in *A. cepa* and *Z. mays* after SPL exposure in different times (h).

Allium cepa							
Treatments	**8h**	**12h**	**24h**	**36h**	**48h**	**60h**	**72h**
Control	0.11±0.02	0.23±0.04	0.56±0.02	1.30±0.16	2.22±0.21	4.33±0.21	6.61±0.09
SPL 5%	_	_	0.43±0.02	1.00±0.12	1.46±0.12*	3.00±0.21*	4.89±0.12*
SPL 10%	_	_	_	_	0.33±0.07*	0.45±0.03*	0.89±0.05*
SPL 15%	_	_	_	_	0.23±0.02*	0.37±0.05*	0.65±0.05*
SPL 20%	_	_	_	_	_	_	0.12±0.01*
SPL 25%	_	_	_	_	_	_	_
Zea Mays							
Treatments	**8h**	**12h**	**24h**	**36h**	**48h**	**60h**	**72h**
Control	0.09±0.01	0.21±0.02	0.43±0.04	1.71±0.22	3.00±0.16	5.89±0.23	7.12±0.22
SPL 5%	_	_	0.33±0.05	1.56±0.21	2.60±0.14*	3.22±0.12*	5.00±0.14*
SPL 10%	_	_	_	0.23±0.04*	0.50±0.06*	1.00±0.14*	1.12±0.13*
SPL 15%	_	_	_	_	0.23±0.05*	0.65±0.06*	0.76±0.12*
SPL 20%	_	_	_	_	_	0.32±0.05*	0.61±0.05*
SPL 25%	_	_	_	_	_	0.06±0.01*	0.56±0.05*

(–) Represent absence of root growth.

* Represent the means statistically differences in relation to the control ($p<0.05$).

5.3. Cytogenetic analysis

With the objective of investigating the possible mechanisms involved in root growth inhibition, cytogenetic analysis was performed. In both plant tests, SPL provoked strong inhibition of the mitotic index, where significant differences in relation to the control can be noticed in SPL 5% (Tables 3 and 4).

Comparing the SPL 25% (high concentration) to the control in *A.cepa* and *Z.mays*, it was observed, respectively, 94% and 84% of reduction in the mitotic index (Tables 3 and 4).

Cytogenetic alterations were also investigated and the results can be seen in Table 3 for *A. cepa* and in Table 4 for *Z. mays*.

The SPL induced chromosome and cytological alterations in both plant tests. Later segregation, anaphase bridges, stickness metaphase, chromosome fragments, c-metaphases, multipolar anaphases and cell death were observed (Figure 1 – not showed in the published article).

In general, it was possible to observe an increase of different abnormalities as the SPL concentration increased (Tables 3 and 4). In both plant tests, a strong SPL toxic effect was observed, supported by great occurrence of stickness metaphase, leading to cell death. The increase in relation to the control is 2.75 and 7.35 times (stickness metaphase) and 10.23 and 4.41 times (cell death), respectively, for *A. cepa* and *Z. mays*. For both plant tests, significant differences for these abnormalities were detected even in SPL 10% concentrations.

In addition to this toxic effect, the observation of anaphase bridges and chromosome fragments showed the clastogenic effect of SPL. Increase in the percentage of anaphase bridges is 3.32 and 6.33 times, respectively, for *A. cepa* and *Z. mays*. For these abnormalities, significant differences were already detected in SPL 5% (*A. cepa*) and SPL 15% (*Z. mays*). In *Z. mays*, the occurrence of chromosome fragments allows observation of significant

differences at SPL 15%, while in *A. cepa*, this parameter did not show any significant difference (Tables 3 and 4).The observation of some cells with later segregation, c-metaphase, and multipolar anaphase were less evident. However significant differences were observed, mainly in *A. cepa* (Table 3 and 4).

Considering the two species and different chromosome and cell alterations analyzed, in general, it was observed that SPL provoked a significant increase in the percentage of abnormalities. The smallest concentration where it had been possible to detect cytotoxicity effect was SPL 5% in *A. cepa* (for anaphase bridges and cell death) and SPL 10% in *Z. mays* (for later segregation, stickness metaphase and cell death). These results, showing that the SPL 5% (low concentration) was able to induce alterations, demonstrate the strong cytotoxic effect of SPL.

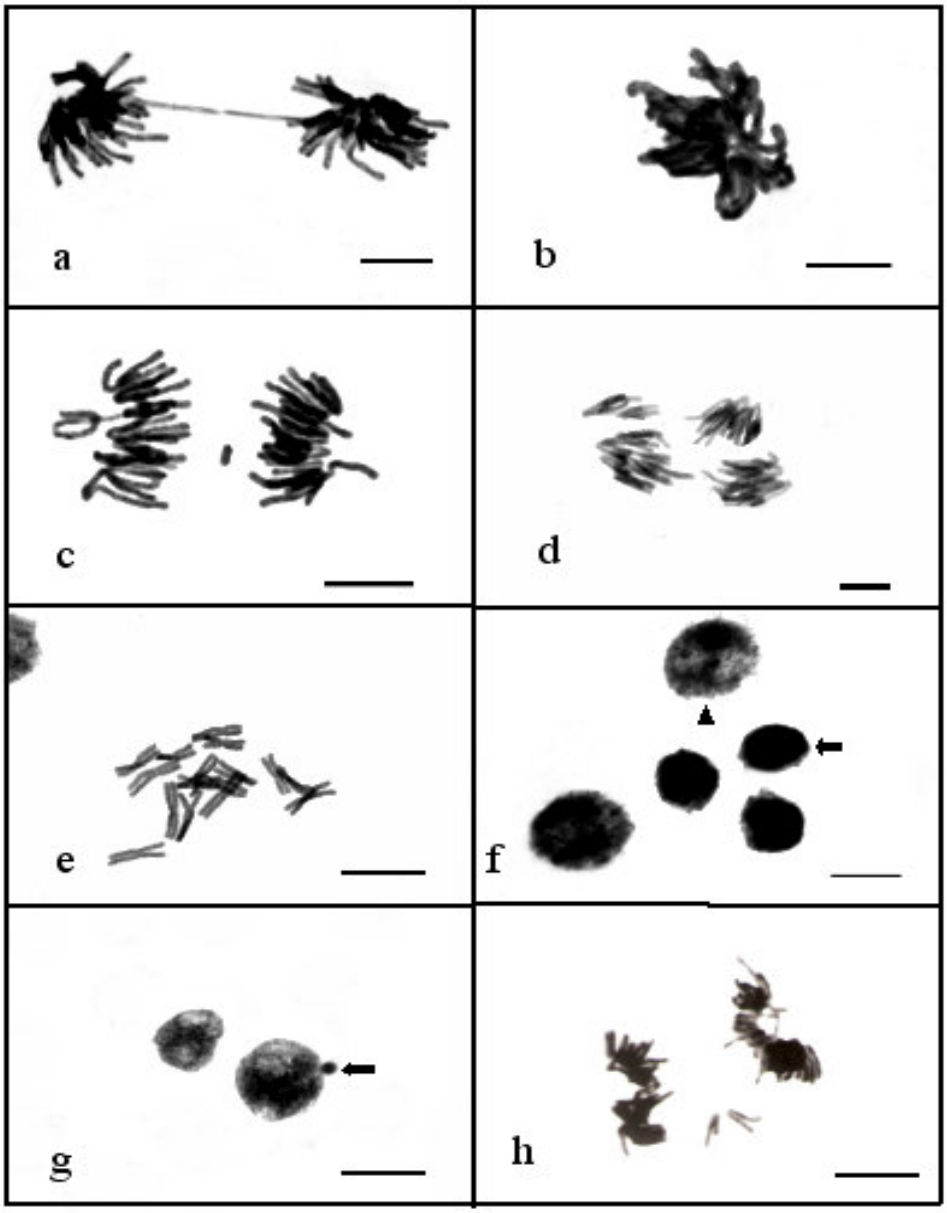

Figure 1 Chromosome alterations in meristematic cells after SPL treatment. (a) Chromosome bridge; (b) Stickness chromosomes; (c) Chromosome fragment; (d) Multipolar anaphase; (e) c-metaphase; (f) Cell death (arrow), note the normal interphase nuclei (head arrow); (g) Micronuclei (arrow); (h) Later segregation. Scale bar = 10μm.

Table 3 Percentage of alterations (mean±SD) in meristematic cells from *A. cepa* after exposure to SPL concentrations.

SPL	Mitotic index	Later segregation	Bridges	Stickness	Fragments	Cell death	C–metaphase	Multipolar anaphase
Control	21.02±2.2	0.21±0.01	1.55±0.24	1.68±0.33	0.11±0.05	4.93±1.0	1.15±0.11	0.00
5%	16.55±1.6*	0.25±0.01	2.07±0.43*	1.77±0.14	0.12±0.03	18.12±3.2*	1.60±0.09	0.00
10%	8.33±1.1*	0.19±0.04	3.12±.21*	3.34±0.36*	0.09±0.05	28.05±4.5*	1.62±0.23	0.00
15%	4.89±0.9*	1.23±0.40*	4.56±0.54*	3.97±0.16*	0.07±0.04	32.45±3.6*	1.65±0.11	0.00
20%	4.33±1.0*	1.31±0.22*	5.12±0.54*	4.26±0.16*	0.04±0.05	45.67±4.5*	1.71±0.23*	0.13±0.01*
25%	1.23±0.11*	1.25±0.19*	5.16±0.33*	4.62±0.34*	0.02±0.03	50.47±4.1*	1.71±0.24*	0.08±0.01*

* Significant differences in relation to the control ($p<0.05$).

Table 4 Percentage of alterations (mean±SD) in meristematic cells from *Z. mays* after exposure to SPL concentrations.

SPL	Mitotic index	Later segregation	Bridges	Stickness	Fragments	Cell death	C–metaphase
Control	8.23±0.54	0.00	0.56±0.08	1.30±0.23	0.41±0.02	5.15±1.23	0.37±0.12
5%	7.1170.23*	0.00	0.80±0.09	2.23±0.11	0.49±0.05	7.08±0.99	0.29±0.13
10%	4.3270.21*	0.59±0.11*	1.12±0.11	6.15±0.34*	0.81±0.11	11.97±1.19*	0.46±0.16
15%	3.8970.11*	1.11±0.12*	2.66±0.12*	7.64±0.67*	1.21±0.22*	12.33±2.33*	0.48±0.12
20%	2.6670.13*	1.36±0.21*	3.66±0.13*	8.48±0.54*	1.91±0.21*	16.49±2.67*	0.47±0.15
25%	1.32±0.09*	1.56±0.15*	3.67±0.13*	9.56±0.23*	1.92±0.18*	22.75±3.23*	0.54±0.17

* Significant differences in relation to the control ($p<0.05$).

6 DISCUSSION

It is obvious from the results of the present investigation that SPL is cytotoxic on meristematic cells of plant tests (*A. cepa and Z. mays*) and that *A.cepa* is more efficient to biomonitore the toxic of pollutants. The cytotoxic effect has been evaluated at micro and macroscopic levels. Macroscopically, we have observed reduction of root growth in both plant tests. Additionally, the treated roots showed a different appearance from that of the normal roots, which was due to the toxic effect. In this respect, inhibition of root growth with significant differences is observed for all concentrations after 48 h of time exposure.

The cytogenetic analysis showed that inhibition of root growth was due to the toxicity of SPL through disturbance of mitotic process and induction of chromosome aberrations and cell death.

The mitotic index inhibition is an evident effect of SPL on both plant tests. Reduction of percentage of cells in division was already observed in the SPL 5% concentration (significant differences in relation to the control). The inhibition of mitotic index can also be attributed to the effect of environment chemicals on DNA/protein synthesis of the biological system (Chauhan et al., 1998). These effects are manifested by induction of interphase arrest or cell death. By analyzing cells of both plants exposed to SPL, the main cause of mitotic inhibition was verified to be the increase of cell death. In this case, the SPL seems to act in the same way, causing programmed cell death (PCD). We observed cells with cytoplasm shrinkage, nuclear condensation, and formation of apoptotic bodies as common morphological characteristics of PCD in plants (Pennell and Lamb, 1997; Solomon et al., 1999). This process is a kind of ‘‘cellular suicide’’, genetically controlled and involved in the specific

elimination of damaged cells (Lamar et al., 1999). The observation of stickness metaphases reinforces the hypothesis of the toxic effect of SPL. Metaphases with sticky chromosomes lose their normal appearance, and they are seen with a sticky ‘‘surface’’, causing chromosome agglomeration (Babich et al., 1997). The presence of this type of aberration reflects the toxic effect on chromatin, which generally leads irreversibly to cell death (El-Ghamery et al., 2003). Stickness has been attributed to the effect of pollutants on the physical–chemical properties of DNA, protein or both, on the formation of complexes with phosphate groups in DNA, on DNA condensation or on formation of inter-and-intra-chromatid crosslinks (El-Ghamery et al., 2003).

Regarding clastogenic alterations, it was possible to observe anaphase/telophase bridges and chromosome fragments in both plant tests. The presence of chromosome fragments is an indication of chromosome breaks, and can be a consequence of anaphase/telophase bridges (Sharma and Sen, 2002; Singh, 2003). Those observations demonstrated that somehow the SPL not only interferes with the cell cycle, but also affects chromatin organization and causes chromosome breaks. Additionally, the observation of later segregation, c-metaphases and multipolar anaphases suggests the effect on microtubule assembly. The microtubules carry out important cell functions during the growth and mitotic cycle, acting in several processes, such as chromosome migration, cell structure, formation of cellular wall, among others (Jordan and Wilson, 1999). The exposure to SPL seems to affect the normal operation of the microtubules. This hypothesis is reinforced by an increase of the c-metaphase number, of chromosomes with late segregation and multipolar anaphases. In general, those alterations are caused by disturbances in the dynamics of the microtubules (Haroun and Shehri, 2001). It is possible that SPL interferes with the polymerization and depolymerization of the microtubules.

The induction of chromosome breaks, disturbances on microtubule assembly and cell death can be related. Micronuclei (MN) often result from the acentric fragments or lagging chromosomes that fail to incorporate into the daughter nuclei during telophase of the mitotic cells and can cause cell death due to the deletion of primary genes (Yi and Meng, 2003). In this present investigation, some cells were observed with MN (data not shown).

The content of SPL suggests the possible involvement of some chemicals in cytotoxicity alterations. SPL includes aluminum, cyanide, fluoride and metals, such as cadmium, copper, iron, lead, manganese, sodium, and zinc. The cytogenotoxic effect of several of these chemicals is known. Cell alterations due to the aluminum exposure have been reported as fragments and bridges at anaphase/telophase (Fiskesjö, 1988; Roy et al., 1989; Campos and Viccini, 2003); decrease in mitotic index (Fiskesjö, 1988; Kaymak, 1996; Campos and Viccini, 2003), binucleate and micronucleate cells at interphase (Fiskesjö , 1988; Roy et al., 1989), sticky chromosomes (Caetano-Pereira et al., 1995; Liu and Jiang, 2001), nucleolar dissolution (Fiskesjö, 1983), cell death (Pan et al., 2001, Campos and Viccini, 2003) and inhibition of DNA synthesis by increasing the rigidity of the DNA double helix (Rout et al., 2001). In our work, to simulate the natural condition, the pH was not adjusted. The high pH in the SPL 15%, 20%, and 25% treatments (>8.0) difficult the bioavailability of the aluminum. However, aluminum is present in toxic concentration in all treatments ($<0.5 mgL^{-1}$). Regarding the cyanides and fluorides, a little is known about the genotoxicity effects. For metal composition, the SPL contains heavy metals that cause chromosome abnormalities and cell death, which may appear as a decline in the mitotic index. For copper the occurrence of stickness, c-mitosis and decrease of the mitotic index are related (Fiskesjö, 1988); for manganese, sticky chromosomes constituted the most typical parameter (Fiskesjö, 1988); for zinc, mitotic inhibition and chromosome abnormalities (sticky chromosomes, c-

metaphases, bridges, and metaphase disturbed by lagging chromosomes) were observed (El-Ghamery et al., 2003); cadmium induces chromatin fragmentation, cell death and causes changes in the nucleolus. (Behboodi and Samadi, 2004); lead reduces root growth and causes mitotic irregularities, including c-mitosis, anaphase bridges, and chromosome stickness. Also, interphase cells with MN, irregularly shaped nuclei and nuclei with decomposed nuclear material were noticed (Liu et al., 1994). Sodium was detected as the most abundant element in SPL leachate. Salinity induces ionic stress driven by the toxic action of sodium (Bernstein et al., 1995). In addition to osmotic and ionic stress, hyperosmolarity also generates secondary stress-like oxidative stress, which is caused by excessive amounts of reactive oxygen species (ROS). ROS can have damaging effect on cellular structures and macromolecules, especially DNA. Radic et al. (2004) showed total absence of mitotic activity in root-tip cells of Centaurea ragusina treated with higher concentrations of salt. Concentrations of NaCl (150mM) induced high frequencies of mitotic abnormalities, such as chromosome breaks, anaphase bridges and stickness, c-metaphase, irregular anaphases, lagging chromosomes, multipolar spindles, aneuploidy and polyploidy.

The presence of these contaminants and other pollutants in agricultural soils has led to bioaccumulation of various toxicants in food crops. Hence the interaction among elements, even at lower levels, present in leachates can have synergistic or additive actions. It is concluded that SPL contains complex mixtures of hazardous chemicals. The treatment of this waste and mitigation tools for its hazardous effects is essential prior to disposal. These are the challenges for further investigations. Work is under way to make these known**.**

7 CONCLUSION

It is obvious from the results of the present investigation that SPL is cytotoxic on meristematic cells of plant tests (*A. cepa* and *Z. mays*), causing alterations such as reduction of mitotic index and increase of different abnormalities as the SPL concentration increased. We observed bridges, chromosome fragments, stickness, multipolar anaphase, later segregation and cell death. The abnormalities increase significantly on the lowest concentration (5% of SPL) in *A.cepa* while for *Z. mays* significant increae was observed in SPL 10%. Thus, it was concluded that *A.cepa* is more indicated in this type of test because it demonstrated more sensibility to SPL than *Z. mays.*

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CHAPTER 3

Changes in mitotic activity and nuclear content in SPL (Spent Pot Liners)-treated root meristem cells of *Allium cepa*

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1 RESUMO

ANDRADE, L.F.; CAMPOS, J.M.S.; DAVIDE, L.C. Alterações na atividade mitotica e conteúdo nuclear de células meristemáticas radiculares de *Allium cepa* tratadas com SPL (Spent Pot Liners). In: ____. **Citogenotoxidade do SPL (Spent Pot Liner) por meio de bioensaios vegetais em ápice radicular.** 2008. 135 p., p. 63-88. Dissertação (Mestrado em Genética e Melhoramento de Plantas) – Universidade Federal de Lavras, Lavras, MG.

O SPL (Spent Pot Liner) é um complexo resíduo sólido gerado na indústria de alumínio. Sua composição inclui compostos orgânicos, metais, fluoreto e cianeto. Considerando a toxicidade destes compostos, este trabalho teve como objetivo estudar o potencial da influência do SPL no ciclo celular e na atividade do DNA em *Allium cepa*, por meio de análises citogenéticas e de citometria de fluxo. A análise citogenética mostrou um grande aumento nos núcleos condesados, além de redução no índice mitótico. Adicionalmente, mudança na razão núcleo-citoplasma foi observada como conseqüência da alteração no tamanho da célula e núcleo. O conteúdo de DNA durante o ciclo celular reduziu com o aumento da concentração de SPL. Não obstante, após a pós-exposição em água destilada, as células meristemáticas recuperaram sua propriedade de divisão.

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2 ABSTRACT

ANDRADE, L.F.; CAMPOS, J.M.S.; DAVIDE, L.C. Changes in mitotic activity and nuclear content in SPL (Spent Pot Liners)-treated root meristem cells of *Allium cepa.* In: ____. **Cytogenotoxicity of SPL (Spent Pot Liner) by plant root tip bioassays.** 2008. 135 p., p 63-88. Dissertation (Mester in Genetics and Plant Breeding) – Federal University of Lavras, Lavras, MG.

SPL (Spent Pot Liner) is a complex solid waste from aluminum industry. Its composition includes organics, metals fluriode and cyanide. Considering the toxicity of these compounds, this study aimed to assess the potential of SPL to influence in the cell cycle and DNA activity in *Allium cepa* by cytogenetics and flow citometry analysis. Cytogenetics analysis showed great increase on condensed nucleus and also a decrease on mitotic index. Change in nucleo-plasmic ratio was also observed as consequence of cell and nucleus size alteration. The content of DNA during cell cycle decreases as SPL concentrations increase. Nevertheless, after post-exposure in water, the meristematic cells recover their property of division.

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3 INTRODUCTION

The industrial waste usually contains complex mixtures of chemicals that substantially contaminate ground and surface water reservoirs used for drinking purposes (Mumtaz, 1995; Dewhurst et al., 2002, Chandra et al., 2005), and might become hazardous to human health. Then, numerous potentially mutagenic chemicals have been studied mainly because they can cause damaging and inheritable changes in the genetic material (Caritá and Marin-Morales, 2008).

SPL (Spent Pot Liner) is a solid waste from aluminum industry. Its composition is highly variable, including organic and inorganic compounds such as cyanide, fluoride salts, sodium and metals (aluminum, cadmium, zinc, lead, manganese and copper), thus, considered as hazardous waste (US EPA, 1991; Silveira et al., 2003). Although it is stored at controlled deposits, there is no effective option for the excess of SPL produced, and it may be leached to the environment.

It is known that toxic heavy metal ions induce several cellular stress responses and cause damage to different cellular components such as membranes, proteins and DNA. They might influence DNA via the production of free radicals or by general interference in the DNA activity (Patra et al., 1998; Kovalchuc et al., 2001; Waisberg et al., 2003; Jimi et al., 2004). At this context, a variety of tests have been employed in order to identify the harmful effect of substances in different concentrations and times of exposure. These tests are commonly used for biomonitoring the extent of pollution and to evaluate the effects of toxic and mutagenic substances in the natural environment (Matsumoto, 2004; Matsumoto et al., 2006).

The species *Allium cepa* has been considered as an efficient test organism to indicate the presence of mutagenic chemicals (Fiskesjö, 1985), due to its kinetic characteristics of proliferation and chromosomes suitable for this type of study (2n = 16 large chromosomes) (Matsumoto, 2004; Matsumoto et al., 2006).

Hence, this study aimed to assess the potential of SPL to influence in the cell cycle and DNA activity in *Allium cepa* by cytogenetics and flow citometry analysis.

4 MATERIAL AND METHODS

4.1 SPL leachates and treatment

SPL leachates were prepared following the absorption methods described in Andrade et al. (2008). In brief, 20g of SPL(mg)/soil (mg) (0, 10 and 25%) was mixed with 200mL of $CaCl_2$ 0.01M solution and then incubated for 12h in a rotary shaker, followed by 12h at rest (repeated twice). The samples were centrifuged and the supernatant was collected. The leachate composition was previously analyzed in Andrade et al. (2008).

The onions root tips from commercial *Allium cepa* were pre-exposed to distilled water, for root emergence, and later treated with SPL leachates in different concentration (0, 10 and 25%) for 4, 8, 12, 24, and 36h of exposure. The SPL 0% is used as negative control. After treatement, a set of bulbs was left in distilled water for 24h of post-exposure to recovery.

4.2 Cytogenetic assays

After treatments, root tips (5mm) were collected and fixed (fresh cold methanol: acetic acid (3:1 v/v) solution. Briefly, four fixed root tips from each treatment were hydrolyzed in 1N HCl at 60ºC for 10 min and stained with Schiff reactive for 1:30h. After that, they were observed under the stereomicroscope; meristem was chopped into several tiny fragments, squashed, covered and frozen in liquid nitrogen. The slides were analyzed under light microscope and about 5000-7000 cells per treatment were scored. The following parameters were analyzed: mitotic index, percentage of micronucleated cells and aberrant cells and frequency of condensed nucleus in interphase.

4.3 Nucleo-plasmic ratio (NPR)

Fixed roots from each treatment were stained and hydrolyzed in 2% aceto-orceine solution with 1N HCL (v/v) (9:1) at room temperature for 20-30min. Then, slides were prepared following the squash microscope preparation as described in the section 4.2. Digital images from 30 cells per treatment were taken. The dimension of the largest and smallest diameter of cytoplasm and nucleus were measured in Sigma ScanPro 5.0. The NPR was calculated with the means of these diameters using the equation:

$$\mathrm{NPR} = \frac{\text{nucleus area}}{\text{total area off cell} - \text{nucleus area}}$$

4.4 Flow cytometry analysis

For cell cycle analysis, the meristematic cells exposed to SPL treatments were analyzed by flow cytometry. The treatments were arranged in a completely random design with 4 replications. Each replication corresponds to three samples analyzed and each sample was obtained by the analysis of three meristems. The fresh plant meristems were chopped with a razor blade in the presence of 1mL LB01 iced buffer for nuclei releasing (Dolezel et al., 1997). The chopped tissue was aspirated through two layers of cheesecloth with a plastic pipette, filtered through a 50µm nylon filter and collected in a polystyrene tube. The nuclei suspension was stained with 25 µL of a 1mg/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 analyzed after 1 to 2h. For each sample, at least 10,000 nuclei were analyzed using a logarithmic scale display. The analysis was performed with a FacsCalibur cytometer (Becton Dickinson). Each flow cytometric histogram was saved using Cell Quest software and analyzed with WinMDI 2.8 software (available at http://facs.scripps.edu/software.html). The frequency of nuclei in G1, S and G2 and particles in sub-G1 were scored. The variations in light scatter by the

forward angle light scatter intensity in G1 cells were also scored in order to identify the condensation level.

4.5 Statistical analysis

Data from all parameters analyzed on the assays (cytogenetic, NPR and flow cytometry) were taken and their significance was determined by the Scott-Knott test ($p<0.05$) (Scott-Knott, 1974). Statistical analysis was done using SISVAR software (Ferreira, 2000).

5 RESULTS

5.1 Cytogenetics analysis

The effects of different concentrations of SPL at five times of exposure on mitotic index (MI) micronuclei formation (MN) and chromosome aberration (CA) are shown in table 1. MI after exposure time decreased. Significant difference to control was observed from treatment of SPL 25% for all exposure time, except for 12h of exposure where the inhibition do not differ from control (Control = 0.59%, SPL 25% = 0.17%, Scott-Knott $p<0.05$). The cells exposed on SPL and left 24h in water from recovery showed increase in MI from all concentrations at different times of exposure. The greatest recovery was noticed at 36h of exposure ranging from 45% in control to 100% in treatment of SPL 25%. Induction of micronucleus (Figure 1) was observed in high concentration of SPL (25%SPL) after exposure time. Statistical difference to control was noticed in 12h and 36h of exposure. However, after recovery time, in post-exposure treatments, an increase on the percentage of micronucleated cells was observed. Statistical analysis revealed differences for the SPL 25% after 24h and 36h of exposure. CA is also counted. Changes in mitotic cells as c-metaphases, stickness, fragments and anaphase-telophase bridges were observed (Figure 1). The percentage of all alterations increased with SPL treatments after exposure time (Table 1). The statistic difference was noticed from SPL 10% after 4h and 24h of exposure. After post-exposure time no recovery was observed.

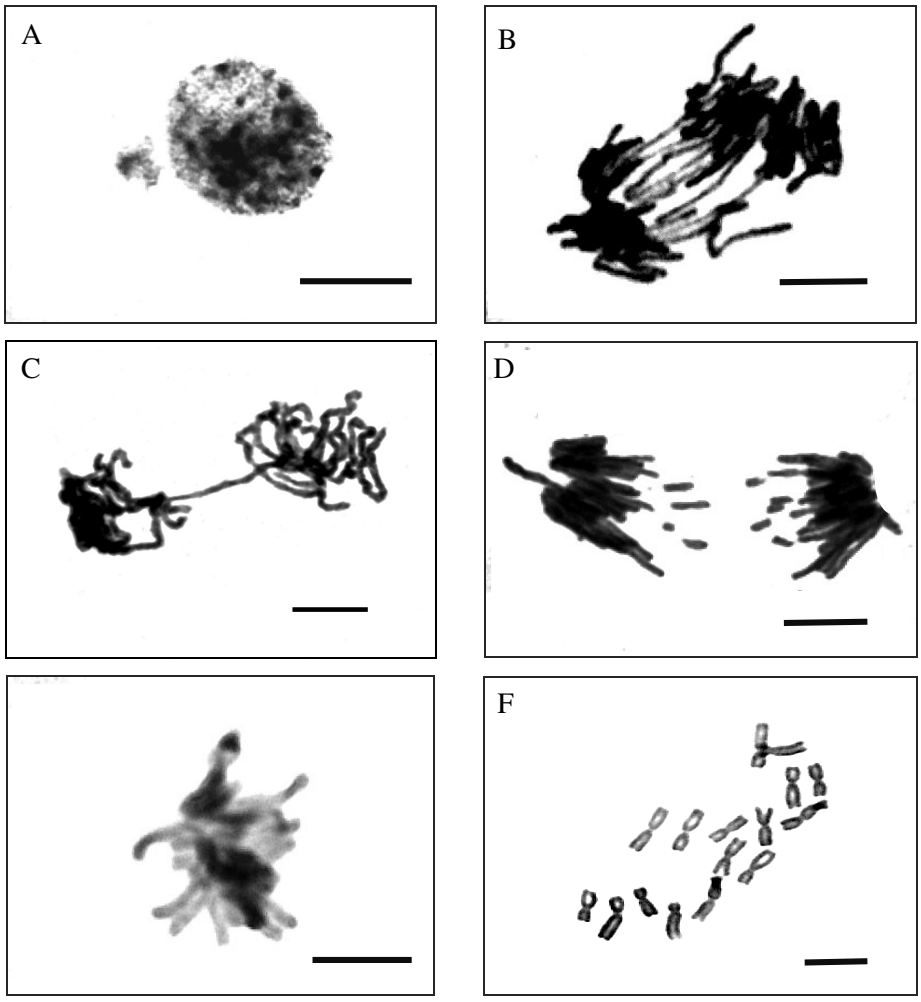

Figure 1 Mitotic alterations after SPL exposure. (A) Micronucleous; (B-C) Anaphase-telophse bridges; (C) Fragments; (E) Stickness; (F) C-metaphasis. Bar = 10µm.

Table 1 Mitotic Index, Micronucleated cells and Chromosome aberrations in root meristem of *Allium cepa* after SPL treatments.

		Mitotic Index		% Micronucleated cells		Chromossome Aberrations	
Exposure Time	**SPL**	**After Exposure**	**After Recovery time (24h)**	**After Exposure**	**After Recovery time (24h)**	**After Exposure**	**After Recovery time (24h)**
4h	0% [a]	2.50 ± 0.72 a	2.44 ± 0.71 a	1.14 ± 0.75 a	1.79 ± 1.45 a	0.15 ± 0.16 a	0.10 ± 0.08 a
	10%	2.69 ± 0.54 a	2.29 ± 0.24 a	1.68 ± 0.43 a	1.61 ± 0.98 a	0.56 ± 0.28 b	0.26 ± 0.18 a
	25%	1.02 ± 0.40 b	1.61 ± 0.58 a	2.22 ± 0.27 a	1.35 ± 0.47 a	0.31 ± 0.20 a	0.62 ± 0.18 b
8h	0%	0.94 ± 0.49 c	1.13 ± 0.60 b	1.42 ± 0.89 b	0.96 ± 0.81 b	0.00 ± 0.00 c	0.19 ± 0.18 c
	10%	1.48 ± 0.66 c	1.12 ± 0.93 b	1.58 ± 0.67 b	1.57 ± 0.77 b	0.16 ± 0.18 c	0.16 ± 0.14 c
	25%	0.00 ± 0.00 d	3.00 ± 0.99 c	2.16 ±1.19 b	1.68 ± 0.81 b	0.00 ± 0.00 c	0.06 ± 0.12 c
12h	0%	0.59 ± 0.56 e	1.88 ± 1.34 d	1.35 ± 0.37 c	1.53 ± 0.61 c	0.00 ± 0.00 d	0.31 ± 0.25 d
	10%	2.27 ± 1.52 f	3.17 ± 1.13 d	0.62 ± 0.21 c	2.19 ± 0.88 c	0.41 ± 0.43 d	0.60 ± 0.15 d
	25%	0.17 ± 0.14 e	2.72 ± 0.77 d	2.53 ± 0.66 d	1.73 ± 0.44 c	0.06 ± 0.04 d	0.28 ± 0.27 d
24h	0%	1.82 ± 0.58 g	2.57 ± 0.78 e	1.11 ± 0.79 e	0.49 ± 0.66 d	0.02 ± 0.03 e	0.41 ± 0.29 e
	10%	4.36 ± 1.16 h	2.06 ± 1.04 e	0.89 ± 0.48 e	0.15 ± 0.29 d	0.67 ± 0.45 f	0.26 ± 0.31 e
	25%	0.04 ± 0.08 i	2.50 ± 1.44 e	1.55 ± 0.19 e	2.10 ± 0.49 e	0.02 ± 0.05 e	0.23 ± 0.12 e
36h	0%	2.40 ± 1.10 j	3.49 ± 1.48 f	0.74 ± 0.31 f	0.42 ± 0.50 f	0.13 ± 0.19 g	0.46 ± 0.29 f
	10%	3.31 ± 0.68 j	3.60 ± 0.38 f	1.09 ± 0.44 f	0.99 ± 0.71 f	0.51 ± 0.43 g	0.94 ± 0.47 g
	25%	0.00 ± 0.00 l	3.00 ± 1.10 f	1.88 ± 0.85 g	4.82 ± 0.92 g	0.00 ± 0.00 g	0.59 ± 0.44 f

Data presented as means ± SD from four replicates. [a] Negative Control.

*There is no statistical difference (P<0.05) between means followed by the same letter from the same exposure time.

Mitotic Index = percentage of cells in mitosis (prophase. metaphase. anaphase and telophase) / total evaluated cells Micronucleated cells = percentage of micronucleus / total evaluated cells; Chromosome Aberrations = percentage of aberrations (bridges, sticknes, c-metaphases, fragments) / total evaluated cells.

Condensed nucleus cells were analyzed separately. Table 2 shows the frequencies of interphasic and condensed nucleus cells observed after exposure of SPL and after recovery. Root meristem treated with SPL exhibited high percentage of condensed nucleus (Figure 2). For each time of exposure (4, 8, 12, 24 and 36h) SPL concentrations (10 and 25%) were significantly different ($p<0.05$) when compared to control (SPL 0%). The difference could be already noticed on the lower time of exposure (4h) where the increase for the concentration of 10% SPL was two-fold than control (Control = 12.75%, SPL 10% = 25.85% and SPL 25% = 44.49%, $p<0.05$). Considering the time of 36h after exposure the increase on percentage of condensed nucleus was 2.8 and 5.19-fold, respectively for 10-25% SPL compared to control (SPL 0% = 14.18%, SPL 10% = 39.83%, SPL 25% = 73.64%).

In addition, considering the exposure time, the greatest increase on frequency of condensed nucleus was noticed at the concentration of 25%SPL. Statistical difference could be observed after 8h of exposure, comparing to 4h after exposure (lower exposure time).This increased 65% at the higher (36h) exposure time (SPL 25%: 4h = 44.49%, 8h = 53.11%, 36h = 73.64%, $p<0.05$). It can also be observed that frequency of interphase cells decrease significantly (<0.05), depending on concentration and exposure time. For all these parameters, recovery post-exposure time (24h) was observed. The recovery about 50% was observed in the treatment of 10% SPL after 4h of exposure (After exposure = 25.85%, Post-exposure = 11.98%, $p<0.05$), while the major recovery observed was for the treatment of 25%SPL after 36h of exposure (After exposure = 73.64%, Post-exposure = 20.31%, $p<0.05$).

Table 2 Percentage of interphases cells with normal and condensed nucleus in the root meristem of *Allium cepa* after SPL treatments.

		Interphase Cells		Condensed Nucleus		
Treatment	**Exposure Time**	**After Exposure**	**After Recovery time (24h)**	**After Exposure**	**After Recovery time (24h)**	**% Recovery at 24h post-exposure**
0% SPL [a]	4h	83.46 ± 5.03 a*	85.07 ± 3.23 a*	12.75 ± 4.48 a*	10.07 ± 0.75 a*	21.02
	8h	82.58 ± 4.63 a	86.19 ± 1.64 a	15.25 ± 5.98 a	10.62 ± 0.81 a	30.36
	12h	85.35 ± 2.43 a	86.24 ± 1.50 a	12.90 ± 2.46 a	10.03 ± 0.12 a	22.25
	24h	81.85 ± 1.82 a	84.16 ± 7.11 a	15.78 ± 3.78 a	10.44 ± 1.16 a	33.84
	36h	82.55 ± 1.32 a	85.35 ± 1.73 a	14.18 ± 1.43 a	10.27 ± 1.25 a	27.57
10% SPL	4h	69. 21 ± 2.90 b	83.14 ± 2.25 b	25.85 ± 3.48 b	11.98 ± 3.46 b	53.66
	8h	62.73 ± 0.94 c	83.26 ± 1.11 b	34.53 ± 2.31 c	14.02 ± 1.33 b	59.40
	12h	59.91 ± 3.63 d	81.13 ± 2.84 b	36.97 ± 2.30 c	13.18 ± 0.90 b	64.35
	24h	58.17 ± 2.90 e	84.17 ± 0.80 b	35.91± 3.78 c	13.36 ± 0.41 b	62.80
	36h	55.29 ± 1.37 e	78.77 ± 2.61 b	39.83 ± 0.79 c	15.68 ± 2.15 c	60.63
25% SPL	4h	51.95 ± 1.71 f	82.28 ± 3.52 c	44.49 ± 2.24 d	12.34 ± 2.60 d	72.26
	8h	44.72 ± 4.38 f	76.77 ± 3.27 d	53.11± 3.83 e	17.39 ± 1.82 e	67.26
	12h	42.89 ± 2.31 f	71.24 ± 1.81 e	54.35 ± 2.50 e	23.77 ± 0.70 g	56.26
	24h	34.26 ± 2.93 g	62.15 ± 2.96 e	64.13 ± 2.98 f	27.71 ± 0.88 h	56.79
	36h	24.48 ± 2.50 h	70.75 ± 4.86 e	73.64± 2.88 g	20.31 ± 0.78 f	72.42

Data presented as means ± SD from four replicates obtained from 6000 to 8000 cells. [a] Negative Control.
*There is no statistical difference (P<0,05) between means followed by the same letter from the same SPL treatment.
[a]Interphase Cells = percentage of interphase (G1, S e G2) / total evaluated cells; Condensed nucleus = percentage of condensed nucleus /total evaluated cells.

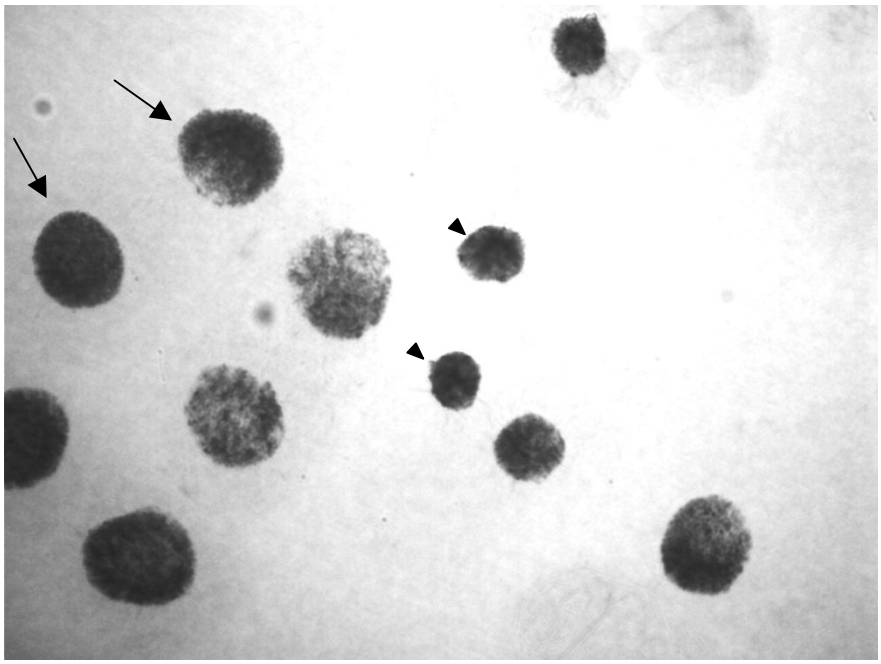

Figure 2 Condensed Nucleus formated after SPL treatment. Note normal nucleus (arrows) and a condensed nucleus (head arrows).

5.2 Nucleo-plasmic ratio (NPR)

Figure 3 shows the changes in NPR after SPL treatment. Treatments were compared within the same exposure time. After exposure time of 4h and 8h no statistically changes were detected (Scott Knott, $p<0.05$) (4h after exposure, SPL 0% = 44.62%, SPL 10% = 34.56%, SPL 25% = 38%; 8h after exposure, SPL 0% = 36.73%, SPL 10% = 31.89%, SPL 25% = 30.41%). Whereas, for 12, 24 and 36h of exposure NPR decreased significantly in SPL 25% comparing to control ($p<0.05$) (12h after exposure, SPL 0% = 48.36%, SPL 25% = 30.08%; 24h after exposure, SPL 0% = 45.47%, SPL 25% = 29.17%; 36h after exposure, SPL 0% = 39.84%, SPL 25% 21.19%). Considering the post-exposure time (24h) the reduction of NPR was less prominent. However, recovery was noticed for all treatment, except for SPL 25% after 4h and 12h of exposure ($p<0.05$).

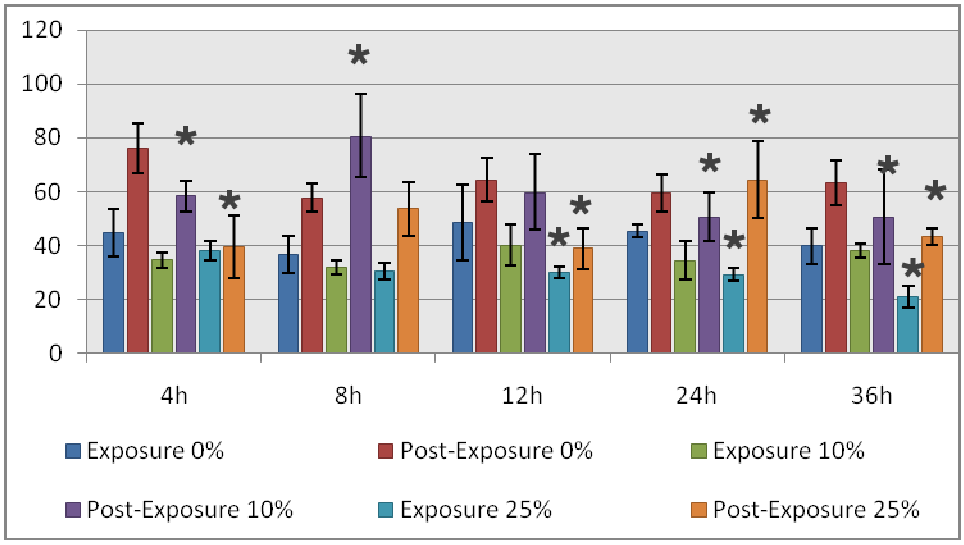

Figure 3 Nucleo-plasmic ratio (NPR) in root meristem of *Allium cepa* after SPL treatments. Vertical axis means NPR (%) and horizontal axis means time exposure (h). Bars followed by (*) represent statistic difference to control (SPL 0%) considering the same exposure and post-exposure time.

5.3 Flow cytometry analysis

The alteration in DNA content at cell cycle is shown in table 3. It was observed a concentration and exposure-dependent manner in the changes for all parameters analyzed. Exposure to SPL treatments increases the frequency of sub-G1 cells. Considering the times of exposure, significant increase was observed after 12h of exposure (SPL 0% = 5.68%, SPL 10% = 9.54%* and SPL 25% = 14.11%*, $p<0.05$). After 36h of exposure this increase was 6.4-fold and 10.3-fold, respectively to SPL 10% and SPL 25% (SPL 0% = 6.14%, SPL 10% = 39.33%, SPL 25% = 63.53%). In addition, for the concentration of SPL the increase in relation to the lower exposure time (4h) was significant after 12h to

SPL 10% (after 4h = 5.45% and after 12h = 9.54, $p<0.05$) and to SPL 25% (after 4h = 5.98% and after 12h = 14.11%, $p<0.05$). For post-exposure (24h) treatments, in general, a concentration-dependent increase was observed comparing with the exposure treatments. Significant difference between SPL concentrations were already noticed after 8h of exposure (SPL 0% = 6.00%, SPL 10% = 9.48% and SPL 25% = 15.78%, $p<0.05$).

The other parameter analyzed was G2 and M cells (Table 3). After exposure treatment a great decrease on the frequency was observed for SPL concentrations (10 and 25%). A reduction of 45% was observed after 24h of exposure to SPL 10% (SPL 0% = 9.99%, SPL 10% = 5.55). However, the major decrease was for 36h after exposure following 86% and 91% for SPL 10% and 25% respectively (SPL 0% = 9.82%, SPL 10% = 1.35%, SPL = 0.87%). Comparing treatments after exposure and post-exposure time it was observed an increase after recovery time mainly for 24h after exposure at SPL 10% and SPL 25% and also for 36h at SPL 10%. Furthermore, SPL treatments reduce S cells in interphase for treated meristem after exposure and post-exposure time (Table 3). The prominent reduction was observed for treatment after higher exposure times ranging, respectively, for exposure and post-exposure time from 85% and 56% for SPL25% after 24h (after exposure, SPL 0% = 14.88%, SPL 25% = 2.18%; after post-exposure, SPL 0% = 15.22%, SPL 25% = 6.63%); 84% and 65% for SPL 10% after 36h (after exposure, SPL 0% = 14.81%, SPL 10% = 2.32%; after post-exposure, SPL 0% = 16.00%, SPL 10% = 5.47%) and also 85,61% and 85,75% for SPL 25% after 36h (after exposure, SPL 0% = 14.81%, SPL 25% = 2.13%; after post-exposure, SPL 0% =16.00 %, SPL 25% = 2.28%). Apart of this, a decrease on percentage of cells in G1 was also observed (Table 4). The greatest reduction was after 36h of exposure in SPL 25% which ranges from 51% (SPL0% = 69.23%, SPL 25% = 33.48%, $p<0.05$). At post-exposure time a reduction of 54.4% in G1 cells was already noticed at treatment of 10%

after 24h of SPL exposure (SPL = 72.96%, SPL 10% = 33.23%, $p<0.05$). The induction of condensed nucleus was concentration and exposure-dependent manner (Table 4). Though, significant frequencies were observed at 10% and 25%SPL from all exposure time. Thus, the significant increase on percentage of condensed nucleus was first observed for SPL10% after 4h of exposure (SPL 0% = 11.87%, SPL 10% = 16.71%, $p<0.05$). Similar results were observed for treatments after exposure time. The exceptions were to 4h of exposure when a decrease was noticed for SPL 10% and 25% comparing to exposure treatments (see table 4).

Table 3 DNA content of cell cycle in flow cytometry analisys on root tips of *Allium cepa* after SPL treatments.

		% Sub-G1		% Cells in S		% Cells in G2 and M	
Treatment	**Exposure Time**	After Exposure	After Recovery time (24h)	After Exposure	After Recovery time (24h)	After Exposure	After Recovery Time (24h)
0% SPL [a]	4h	5.32 ± 0.15a*	4.55 ± 0.23a	14.64 ± 2.06a	10.34 ± 0.95a	9.69 ± 4.25a	9.26 ± 1.00a
	8h	5.04 ± 0.17a	6.00 ± 0.33a	14.84 ± 0.27a	15.47 ± 0.16b	9.50 ± 0.67a	9.22 ± 0.95a
	12h	5.68 ± 0.19a	5.77 ± 0.51a	14.84 ± 0.19a	14.85 ± 2.06b	9.89 ± 0.79a	9.81 ± 0.38a
	24h	5.92 ± 0.21a	4.88 ± 0.39a	14.88 ± 0.46a	15.22 ± 1.89b	9.99 ± 1.51a	7.33 ± 0.40a
	36h	6.14 ± 0.22a	5.23 ± 2.00a	14.81 ± 0.36a	16.00 ± 2.22b	9.82 ± 3.32a	9.62 ± 0.86a
10% SPL	4h	5.45 ± 0.89a	5.44 ± 1.02a	13.89 ± 0.59a	11.15 ± 0.14a	10.44 ± 1.51a	11.43 ± 0.76a
	8h	5.13 ± 0.14a	9.48 ± 0.83b	10.57 ± 0.57b	11.30 ± 1.01a	7.37 ± 1.84b	10.99 ± 0.33a
	12h	9.54 ± 1.76b	20.89 ± 1.28c	17.28 ± 0.89c	12.53 ± 2.74a	9.59 ± 3.80a	9.92 ± 0.18a
	24h	20.85 ± 2.79c	40.84 ± 1.30d	11.23 ± 1.40b	11.45 ± 1.11a	5.55 ± 1.00c	13.60 ± 1.89b
	36h	39.33 ± 2.43d	61.29 ± 2.81e	2.32 ± 1.01d	5.47 ± 1.12a	1.35 ± 0.31d	11.15 ± 1.03a
25% SPL	4h	5.98 ± 0.23a	6.36 ± 0.40a	15.29 ± 3.27a	15.65 ± 4.16a	7.85 ± 1.24a	6.56 ± 0.68b
	8h	5.28 ± 0.61a	15.78 ± 1.06b	9.48 ± 0.86b	9.24 ± 1.01b	6.68 ± 1.31a	7.02 ± 0.29b
	12h	14.11 ± 0.58b	34.54 ± 2.98c	16.33 ± 0.68a	10.44 ± 1.69b	2.57 ± 0.72b	0.39 ± 0.06a
	24h	33.04 ± 1.31c	54.33 ± 3.50d	2.18 ± 1.38c	6.63 ± 0.59c	2.22 ± 0.89b	11.62 ± 0.72c
	36h	63.52 ± 1.57d	75.26 ± 2.56e	2.13 ± 1.90c	2.28 ± 0.45d	0.87 ± 0.46b	1.83 ± 0.23a

Data presented as means ± SD from four replicates. [a] Negative Control.
*There is no statistical difference ($P<0.05$) between means followed by the same letter from the same SPL treatment.

Table 4 Flow cytometry analisys of G1 cells by FSC light scatter on root tips of *Allium cepa* after SPL treatments.

		% Cells in G1					
		% Cells in G1		Normal nucleus		Condensed nucleus	
Exposure Time	**SPL**	**After Exposure**	**After Recovery time (24h)**	**After Exposure**	**After Recovery time (24h)**	**After Exposure**	**After Recovery time (24h)**
4h	0% [a]	70.36 ± 2.27a	72.34 ± 1.11a	88,13 ± 0.31a	87,13 ± 0.78a	11,87 ± 0.31a	12,87 ± 0.78a
	10%	70.21 ± 1.09a	70.48 ± 1.72a	83,29 ± 1.39b	88,48 ± 1.0 a	16,71 ± 1.39b	11,52 ± 1.01a
	25%	70.87 ± 4.57a	70.88 ± 1.28a	77,87 ± 0.64b	87,22 ± 1.46a	22,13 ± 0.64b	12,78 ± 1.46a
8h	0%	70.62 ± 0.58b	69.82 ± 2.51b	87,89 ± 0.67c	88,10 ± 1.37b	12,11 ± 0.67c	11,90 ± 1.37b
	10%	76.92 ± 1.27c	69.81 ± 1.73b	84,33 ± 0.89d	83,59 ± 1.88c	15,67 ± 0.89d	16,41 ± 1.88c
	25%	78.56 ± 1.52c	68.78 ± 2.63b	77,33 ± 1.09e	76,06 ± 2.48d	22,67 ± 1.09e	23,94 ± 2.48d
12h	0%	69.59 ± 0.62d	71.07 ± 6.21c	87,44 ± 0.64f	88,69 ± 0.78e	12,56 ± 0.64f	11,31 ± 0.78e
	10%	63.92 ± 1.53e	57.91 ± 2.28d	74,33 ± 1.52g	71,97 ± 3,59f	25,67 ± 1.52g	28,09 ± 3,59f
	25%	66.95 ± 1.58d	58.66 ± 1.25d	70,13 ± 0.83g	63,28 ± 2.29g	29,87 ± 0.83g	36,72 ± 2.29g
24h	0%	69.22 ± 0.85f	72.96 ± 2.56e	89,68 ± 0.42h	87,34 ± 0.68h	10,32 ± 0.42h	12,66 ± 0.68h
	10%	62.37 ± 2.51g	33.23 ± 2.22f	63,22 ±2.01i	58,18 ± 2.97i	36,78 ± 2.01i	41,82 ± 2.97i
	25%	62.55 ± 0.78g	28.51 ± 4.36g	58,77 ± 1.01j	53,34 ± 1.11i	41,23 ± 1.01j	46,66 ± 1.11i
36h	0%	69.23 ± 3.49h	65.89 ± 2.39h	86,79 ±0.64l	84,87 ± 1.39j	13,21 ± 0.64l	15,13 ± 1.39j
	10%	57.00 ± 1.97i	21.78 ± 1.50 i	53,22 ±0.78m	41,07 ± 1.58l	46,78 ± 0.78m	58,93 ± 1.58l
	25%	33.48 ± 2.38j	21.03 ± 1.25 i	41,10 ±1.51n	32,76 ± 1.86m	58,90 ± 1.51n	67,24 ± 1.86m

Data presented as means ± SD from four replicates. [a] Negative Control.
*There is no statistical difference (P<0.05) between means followed by the same letter from the same exposure time.

6 DISCUSSION

The results of our studies showed that SPL diminished MI and induced numerous disturbances in cell cycle in root tips of *Allium cepa*.

Data suggested that the decrease on MI is related to the reduction in frequency of S and G2/M cells observed in cytometry analysis. The S cells are cells that replicate DNA in interphasis, preceding the division, while G2 and M cells have the double DNA content though they undergo to division. The MI was 100% inhibited in 25% SPL treatment after 36h of exposure while the percentage of G2/M cells in interphasis decrease 91%. Thus, it was also observed a reduction in frequency of S cells around 85% (SPL 25% after 36h of exposure) which corroborates with the changes in cell cycle and mitotic index. Glinska et al. (2007) suggested that reduction of MI value in metal treated *A. cepa* roots is probably from disturbances in the cell cycle as well as from chromatin dysfunction induced by metal-DNA interactions.

The increase in sub-G1 cells is due to an alteration on DNA content. Altough the frequency of alterations was low, the high percentage of sub-G1 cells suggested that SPL provoked chromatin damage leading to chromosome abnormalities. These changes are associated with loss of DNA content due to the cytotoxicity of SPL. Andrade et al. (2008) demonstrated that SPL lead to chromosome fragments, stickness, multipolar anaphase, bridges and to later segregation. The presence of abnormalities in treated roots reinforces the clastogenic effects of SPL that somehow not only interferes with cell cycle but also affects chromatin organization and causes chromosome breaks.

Therefore, strong increases on condensed nucleus were observed. The nuclear condensation is a common feature of apoptosis-like cell death (or programmed cell death) (Pennel and Lamb, 1997; Bebhoodi and Samnti, 2004).

It was observed a decrease on percentage of cells in G1, what reflects the increase on frequency of sub-G1 cells. In addition, the frequency of condensed nucleus cells in G1 enhanced dependently with SPL concentration and exposure time. This observation is reinforced by the changes on nucleo-plasmic ratio (NPR) once that nucleus condensation decreases this relation.

On the other hand, after post-exposure on distilled water for 24h it was observed a recovery in some parameters analyzed such as condensed nucleus, mitotic index and NPR. The recovery in frequency of condensed nucleus ranged 21% to 72% while for NPR it was from 22% to 60%, whereas this was not observed in cytometry analysis for frequency of condensed nucleus in G1 cells. These observations probably are related to the population of cells that was damaged in time exposure and left to elongation zone during the post-exposure time. Then it was counted in cytometry analysis, but not in cytogenetic analysis where only the meristematic cells were analyzed. In addition, the recovery of MI may reflect the role of quiescent center (QC) under stress conditions. The QC is essential for normal root growth, since the exposure of roots to environmental stress causes alteration of the QC size and meristem architecture. These QC are activated indirectly in response to environmental damage to the adjacent cells. The activation of the QC leads to the replacement of the damaged meristematic cells and consequently restores the balanced between the meristematic dividing cells and QC stem cells (Jiang and Feldman, 2005). Thus, SPL treatment probably leads to a change in QC size and induces some QC cells to divide recovering the root-damaged cells. Therefore, a recovery in condensed cells is also observed but only in lower exposure time (4h and 8h), suggesting that this step of nucleus condensation is reversible for mild treatments.

Thus, these finds confirm the sinergistc cytotoxicity of SPL components (Cd, Cu, Al, and Pb) in meristematic cells of *Allium cepa* what corroborate with literature where the individual toxicity of some heavy metals is shown.

7 CONCLUSION

Our findings showed that SPL affects cell cycle, DNA content and nucleus size. Nevertheless, after post-exposure in water, the meristematic cells recover their property of division.

8 ACKNOWLEDGEMENTS

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CHAPTER 4

Cell death feutures in root tip cells of *Allium cepa* induced by SPL (Spent Pot Liners)

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1 RESUMO

ANDRADE, L.F.; DAVIDE, L.C.; GEDRAITE, L.S.; TORRES, G. A.; ALVES, E. Características de morte celular induzidas pelo SPL (Spent Pot Liners) em células radiculares de *Allium cepa.* In: ____. **Citogenotoxidade do SPL (Spent Pot Liner) por meio de bioensaios vegetais em ápice radicular.** 2008. 135 p., p. 89-111. Dissertação (Mestrado em Genética e Melhoramento de Plantas) – Universidade Federal de Lavras, Lavras, MG.

Vários efeitos tóxicos têm sido atribuídos a poluentes ambientais. O SPL (Spent Pot Liner) é um resíduo sólido gerado na indústria de alumínio durante que apresenta composição variável, incluindo cianeto, fluoreto, composto orgânicos e metais. Dessa forma, o objetivo do estudo foi determinar o efeito do SPL em ápice radicular de *Allium cepa.* Foi observado estresse oxidativo, fragmentação de DNA, condensação de cromatina, e núcleos esféricos nas raízes tratadas com SPL 25%. Essas características estão associadas a morte celular programada causada por fatores abióticos. Também foram observadas alterações nas células superficiais da coifa, como conseqüências de danos às células. Portanto, os dados sugerem que o SPL induz morte celular programada, semelhante à apoptose, nas células radiculares de *Allium cepa.*

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2 ABSTRACT

ANDRADE, L.F.; DAVIDE, L.C.; GEDRAITE, L.S; TORRES, G. A.; ALVES, E. Cell death features in root tip cells of *Allium cepa* induced by SPL (Spent Pot Liners). In: ____. **Citogenotoxidade do SPL (Spent Pot Liner) por meio de bioensaios vegetais em ápice radicular.** 2008. 135 p., p. 89-111. Dissertação (Mestrado em Genética e Melhoramento de Plantas) – Universidade Federal de Lavras, Lavras, MG.

There is a variety of toxic effects of environmental pollutants including apoptosis and carcinogenesis. SPL (Spent Pot Liner) is a solid waste from aluminum industry which composition is highly variable including cyanide, fluoride, organics and metals. Thus, the aim of this study was to determine the features in root tips of *Allium cepa* induced by SPL. Oxidative stress, DNA fragmentation, condensed chromatin and spheres nuclei were observed in root tips treated with SPL 25%. These features are associated with programmed cell death under abiotic stress. Root tips disturbance on cap cells were also observed, as consequence of cell damage. Therefore, this data suggests that SPL induces cell death like apoptosis in root tips of *Allium cepa.*

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3 INTRODUCTION

Environmental pollutants have long-term effects on cellular development. There is a variety of toxic effects of these pollutants, including apoptosis and carcinogenesis (Mountassif et al., 2007).

SPL (Spent Pot Liner) is a solid waste from aluminum industry. It is generated in electrolytic cells during the reduction of alumina to aluminum metal. The SPL composition is highly variable, but the components of major environmental concern are cyanide and soluble fluoride salts. Therefore, it has been classified as a hazardous waste by US EPA (Silveira et al., 2002, 2003).

Additionally, the leachates of SPL showed contain compounds as cadmium, copper, iron, lead, manganese, sodium zinc and also aluminum (Andrade et al., 2008). It is well known that these heavy metals can induce toxic effects in plants such as inhibition of root growth, reduction of mitotic index (Mohanty et al., 2004; Liu et al., 2005; Seth et al, 2008), induction of micronuclei (Qian et al., 2006; Feng et al., 2007), c-metaphasis (Samardakiewicz and Wozny, 2005), chromosome bridges (Jiang et al., 2000; Chandra et al., 2005), chromossome stickness (Fsikejö, 1988; Samardakiewicz and Wozny, 2005), also lagged chromosomes (El-Ghamery et al., 2003) and nuclei with more condensed chromatin (Behboodi and Samadi, 2004). Moreover, those metals can disturb RNA and DNA synthesis (Glinska et al., 2007) and cause changes in the root cell ultraestructure (Kopittke et al., 2007). In addition, they induced DNA damage (Seth et al., 2008), oxidative stress (Pan et al., 2001) and cell death (Boscolo et al., 2003). These alterations were also observed after SPL exposure (see Chapter 2 and 3).

Cell death is a basic biological process of living organisms. Two kinds of cell death are well characterized: apoptosis also called by programmed cell

death (PCD) and necrosis. PCD occurs during development and in response to environmental stress. It is a genetically defined process associated to morphological and biochemical characteristic changes including condensation of cytoplasm, fragmentation of nucleosomal DNA, condensation of nuclear chromatin, membrane blabbing, and formations of apoptotic bodies (Kerr and Wyllie, 1972; Pennel and Lamb, 1997). On the other hand, necrosis is an accidental death associated to a passive process involving disruption of membrane integrity and cellular lysis (Xiong et al., 2006). Nevertheless, evidences suggest that apoptosis and necrosis are just endpoints of a range of morphologic and biochemical possibilities of death (Subbaiah & Sachs, 2003). At this point numerous assays are used in order to detect cell death features.

Changes in nucleus provide the most useful markers to detect apoptosis, which can be induced by environmental stress in plants (Fesus et al., 1991; Heath and Ryerson, 1996; Levine et al., 1996; Mittler and Lam, 1996; Pan et al., 2001). Another feature of programmed cell death (PCD) is the formation of reactive oxygen species (ROS). Thus, PCD in plants is likely to be influenced by cellular redox status. (Jabs, 1999; Bethke and Jones, 2001; Boscolo et al., 2003; Lin et al., 2006).

In this way, in a previous work it was demonstrated that SPL leachates induced cytogenetic alterations and mainly cell death in root tips of *Allium cepa* and *Zea mays* (Andrade et al., 2008). *Allium cepa* demonstrated to be more sensitive as expected. Considering this, the aim of this study is to determine the features of cell death in root tips of *Allium cepa* induced by SPL.

4 MATERIAL AND METHODS

4.1 SPL leachates and treatment

SPL leachates were prepared following the absorption methods described in Andrade et al (2008). In brief, 20g of SPL (mg)+soil (mg) (0, 10 and 25%) was mixed with 200mL of $CaCl_2$ 0.01M solution and then incubated for 12h in a rotary shaker, followed by 12 h at rest (3 times). The samples were centrifuged and the supernatant was collected. The composition of these leachates was previously analyzed in Andrade et al. (2008).

The onion root tips from a commercial cultivar of *Allium cepa* were pre-exposed to distilled water, for root emergence, and later treated with SPL leachates in different concentration (0, 10 and 25%) for 4, 8, 12, 24, ad 36h of exposure. SPL 0% was used as negative control

4.2 Enzyme Assays

The measurement of enzymatic activity to detect the formation of reactive oxygen species (ROS) was carried out with spectrophotometric enzyme assays. Briefly, fresh roots (200mg) from each treatment (0, 10 and 25% SPL for 24h of exposure) were homogenized in 1.5mL of 100mM sodium phosphate buffer (pH 7.8), containing 0.1mM ethylenediamine-tetra acetic acid (EDTA) and 10mM ascorbate, in liquid nitrogen with 50% (w/v) polyvinyl-polypyrrolidone (PVPP). The homogenate was centrifuged at 13000g for 10 min at 4ºC and the resultant crude supernatant was collected and desalinized in Sephadex G-25 (PD-10) column. Soluble protein content was determined according to the method of Bradford (1976) with bovine serum albumin as the standard. The antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione redutase (GR) were

analysed specrophotometrically. CAT activity was determined by measuring the decrease in absorbance at 240nm for 2 minutes in a 28ºC reaction mixture containing 100mM sodium phosphate buffer at pH 7.0 and 10mM of H_2O_2 (Azevedo et al., 1998). SOD activity was determined by measuring the inhibition of the photochemical reduction of NBT, by the enzyme in the reaction mixture containing 100mM sodium phosphate buffer (pH 7.8), 0.1µM of EDTA, 75µM of NBT, 14mM methionine and 0.2µM riboflavin. The reaction was initiated at room temperature by switching on the light (20W) for 7 min, and stopped by switching off the light. For controlling, the same reaction without sample was also carried out. The absorbance of formazone then formed was recorded at 560 nm. One unit of SOD activity was defined as the amount of the enzyme that inhibited NBT reduction by 50%. APX activity (Nakano and Asada, 1981) was measured in fresh enzyme extract by using reaction mixture containing 100mM sodium phosphate buffer (pH 7.0), 0.5mM ascorbic acid and 0.1mM H_2O_2. The hydrogen peroxide-dependent oxidation of ascorbate was followed monitoring the decrease in absorbance at 290nm for 2 minutes. GR activity (Cakmak et al., 1993) was measured by monitoring the NADPH oxidation followed by the decrease in absorbance at 340nm during 2 minutes. The reaction mixture contained 50mM potassium phosphate buffer (pH 7.0), 1mM glutathione and 0.075 mM NADPH.

Data from enzymes activites were taken and their significance was determined by the Scott-Knott test ($p<0.05$) (Scott-Knott, 1974). Statistical analysis was done using SISVAR software (Ferreira, 2000).

4.3 DNA extraction and electrophoresis assay

Total DNA was extracted from 1g of fresh weight treated roots (25%SPL for 36h) according to Katsuhara and Kawsaki (1996). Briefly, the roots were homogenized in liquid nitrogen, incubated in buffer containing 2%

hexadecyltrimethylammonium bromide and DNA was extracted with chloroform-isoamyl alcohol and precipitated with cold isopropanol. Isolated DNA was kept in TE buffer (10mM Tis-HCl, pH 7.4 nd 1mM EDTA). Samples were subjected to electrophoresis on 1.8% agarose gel in Tris-boric EDTA buffer. DNA-laddering was visualized by staining with 0.5 μg/mL ethidium bromide (EB) and UVB illumination.

4.4 Scannig Electron Microscopy

Three treated roots from each treatment (25% SPL for 4, 8, 12, 24 and 36h of exposure) and untreated roots (control – distillated water) were collected and fixed in modified Karnorvsky solution (2.5% Gluteraldehyde, 2% Paraformaldehyde in a 0.05M sodium cacodilate buffer at pH7.2 containing 0.001M $CaCl_2$) for at least 48 hours. Then, they were dehydrated in a gradient series of acetone solutions (30, 50, 70, 90 and 100), dried with carbon dioxide in a critical-point dryer (Bal-tec CPD 30), mounted at stubs with double sided tape, coated by vacuum evaporation with a gold layer of 20nm (Bal-tec SDC 50), and observed in an Evo40 Leo scanning electron microscope.

4.5 TUNEL assay

The TUNEL reaction (TdT-mediated deoxy-uracil nick end labeling) is used for analyzing DNA fragmentation by labeling the 3-OH ends of the DNA strand breaks. This method is based on the ability of terminal deoxynucleotidyl transferase (TdT) to attach a fluorescein-conjugated deoxy-uracil to the 3'-OH end of cut DNA (Behboodi and Samadi, 2004). The TUNEL reaction was made using an APO-BrdU^{TM}TUNEL assay kit (Invitrogen, A35125) with Alexa fluor. Three treated roots (25% SPL for 4, 8, 12, 24 and 36h of exposure) and untreated roots (control – distillated water) were fixed in 1% paraformaldehyde

during 12h. Then, dehydrated in a gradient series of ethanol solutions (30, 50, 70, 90 and 100%, each step 1h), embedded in Steedman's wax at 37ºC using a series of wax:ethanol (v:v) (50:50%, 70:30%, 90:10% and 100% wax, each step 1h) and sectioned longitudinally on a microtome at a thickness of 10µm.The slides with sections were prepared with TUNEL reactions as described by the manufacturer. The slides were observed at epifluorescent microscope by using 500nm wave length (Olympus BX60).

5 RESULTS

The activities of antioxidant enzymes catalase (CAT), superoxidase dismutase (SOD), ascorbate peroxidase (APX) and glutathione redutase (GR) after 24h of SPL exposure is shown in Figure 1. The enzymatic activity increased on SPL 10% and decreased on SPL 25% for all enzymes. However, the significant ($p<0.05$) increase was observed only for GR (SPL 0% = 1.56, SPL 10% = 3.05 µmol NADPH $min^{-1}gMF^{-1}$).

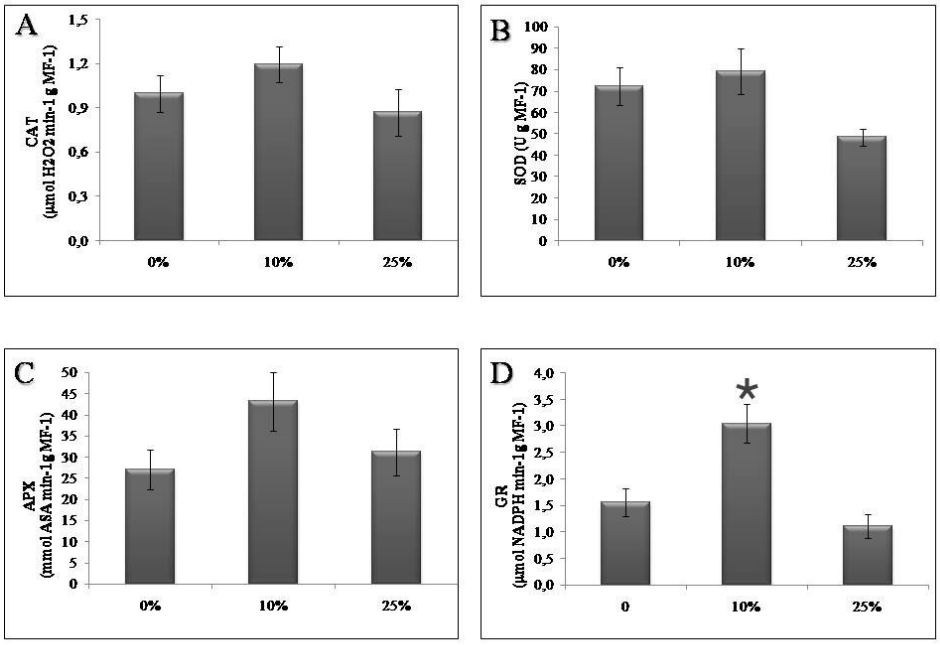

Figure 1 Antioxidants enzymes activity in *Allium cepa* root tips after 24h of SPL exposure. (A) catalase (CAT), (B) superoxidase dismutase (SOD), (C) ascorbate peroxidase (APX) and (D) glutathione redutase (GR). Bars followed by (*) presents significant difference to control.

Figure 2 shows the analysis for DNA fragmentation by electrophoresis on 1.8% agarose gel. A ladder of fragmented DNA was observed. Additionally, the analysis of root tip surfaces on a Scanning Electron Microscopy (SEM) showed a disturbance at SPL 25% treated root tips after 36h of exposure comparing to control (distillated water). After SPL treatment the root cap presents injuried and pointless.

The TUNEL assay showed in Figure 3 was carried out for 25% SPL after 4, 8, 12, 24 and 36h. The root tip morphogenic regions (Figure 3a) were observed on DAPI-stained roots. Epidermal and cap root cells were TUNEL-positive for all treatments. In addition, TUNEL-positive nuclei were also observed for meristem cells after all SPL exposure time. The nuclei showed condensed chromatin, appearance of spheres and formation of buds.

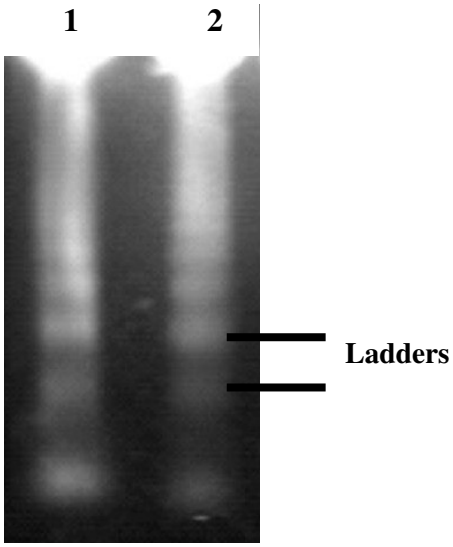

Figure 2 DNA fragmentation in root tips of *Allium cepa*. Nuclear DNA was isolated from treated roots cells after 24h of exposure. Line 1: positive control with aluminum; Line 2: SPL 25%.

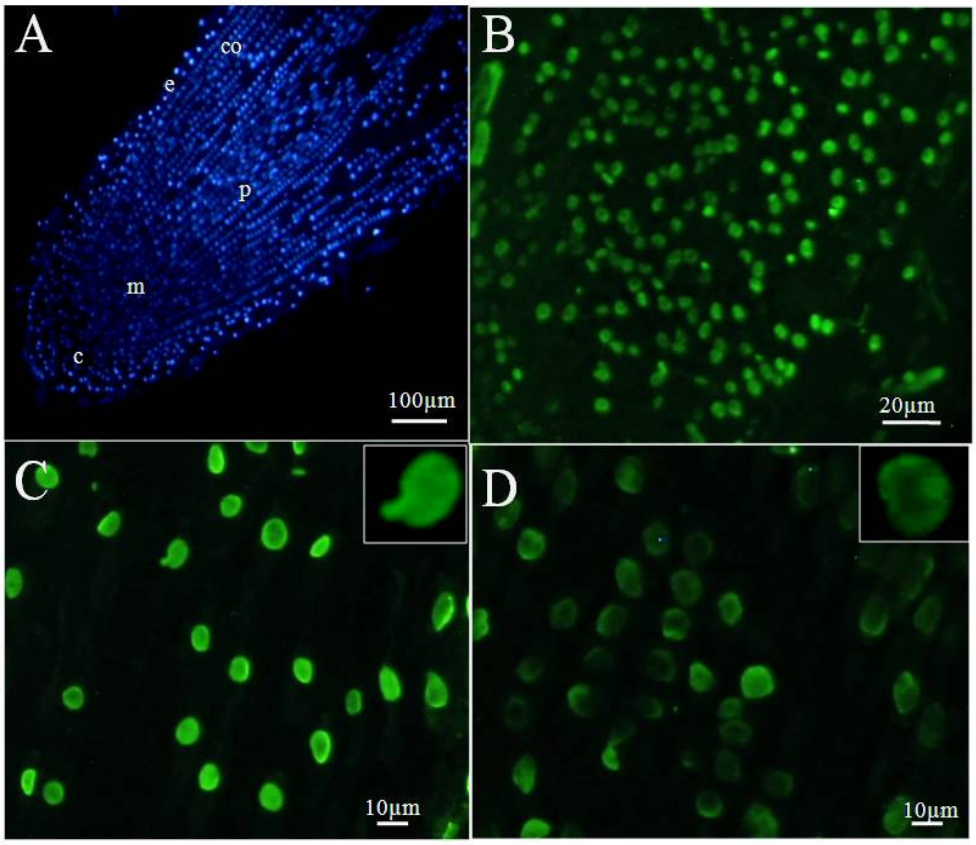

Figure 3 TUNEL assay after treatment with SPL 25%. (A) Control DAPI-stained root. The morphogenic regions in the proliferation zone: meristem (m), cortex (co), epidermis (e), pericycle (p) and root cap (c). (B-D) Meristem cell TUNEL-positive: 36h after SPL exposure (B), 8h after exposure (C), notes the budding nuclei (detail), and 12h after exposure (D), note the condensed chromatin (detail).

6 DISCUSSION

In the present study we observed changes in nucleus in TUNEL-positive treatments after SPL exposure times. TUNEL-positive reaction indicates an increase in the 3'-OH of DNA strands, which is recognized as one feature of apoptosis (Katsuhara, 1997). However, apoptosis, a PCD, is also characterized by other features, such as ladders of DNA on agarose gel, cell shrinkage and formation of apoptotic bodies. The DNA fragmentation with ladder pattern was observed in this study, suggesting the production of oligonucleossomal fragments of DNA derived from endonucleases breakage (Katsuhara, 1997; Behboodi and Samadi, 2004). Nuclei with condensed chromatin and budding nucleus were other features of PCD observed in TUNEL-positive cells. The buddies were noticed 8h after exposure. Behboodi and Samadi (2004) suggested that this "budding" nucleus is apoptotic bodies formed by degradation of plant cell nuclei, facilitating their division and digestion in plant vacuole. In addition, condensed chromatin is also observed after 12h of SPL exposition.

Additionally, it was related that SPL treatment increases the activity of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), peroxidase and others. These enzymes participate in cell process and reduce ROS under normal condition. If complete reduction of ROS does not occur, the result may be a state of oxidative stress, leading to oxidation of biomolecules or even cell death (Richter and Schwcitzer, 1997; Buckner et al., 2000; Jones, 2000). It is well known that heavy metals, including those presents in SPL composition, induce oxidative stress, generating ROS (Rai et al, 2004) and modifying the activity of antioxidant enzymes (Hegedüs et al., 2001; Bagchi et al., 2002; Rai et al., 2004). Thus, a significant increase of GR in SPL 10% is probably due to the increase of ROS in cell compartments (cytosol,

chloroplast, endoplasmic reticulum, vacuoles, and mitochondria). This enzyme protects plants against stress including oxidative stress, heavy metals and certain exogenous and endogenous chemicals (Hong-bo et al., 2008). In the treatment of 25% of SPL, this increase was not observed suggesting that cells started an advanced cell death process (Lin et al., 2007).

Considering the metal composition of SPL 25% leachate (Andrade et al., 2008) these results are related to heavy metal effects on cell cytoplasm, proteins and DNA. Boscolo et al. (2003) demonstrated that Al^{3+} induces dose-dependent formation of reactive oxygen species (ROS) and protein oxidation in maize, indicating that oxidative caused by Al harm several components of the cell. Moreover, Al treatment and oxidative stress induced cell death in root tip cells revealed by high chromatin fragmentation detected by TUNEL analysis. These findings are also observed by other authors who suggested that oxidative stress may induce plant cell death by generation of ROS (Buckner et al., 2000; Pan et al., 2001; Breusegem et al., 2001, Hong-bo et al., 2008). ROS also cause deterioration of membrane lipids, proteins and nuclei acids. DNA is particularly sensitive to OH-induced damage (Sarma and Sharma, 1999). Nevertheless, there are evidences that Al^{3+} binds to DNA, causing condensation and stabilization of chromatin (Matsumoto, 1988, 2000). A condensation of chromatin was observed in SPL-treated roots after TUNEL assay.

Furtheremore, Ikegawa et al. (2000) suggested that the Al-enhanced peroxidation of lipid is a direct cause of cell death in tobacco. The lipid peroxidation is associated to SOD activity. The increase in SOD acivity after SPL treatment was observed, despite being not significant. Sodium is other SPL component that constitutes the major part of SPL leachate (Andrade et al., 2008). Some studies from Katsuahara and co-workers demonstrated the apoptosis-like DNA degradation after salt stress. The cleavage of DNA was observed 1h after salt stress while oligonucleosomal fragments were detected

electrophoretically after 8h of salt stress. They also observed the nuclear deformation and degradation. Thus, they suggested that the DNA degradation leads to sequential nuclear degradation, cell death and inhibition of root growth (Katsuhara and Kawasaki, 1996; Katsuhara, 1997; Katsuhara and Shibasaka, 2000). Andrade et al. (2008) observed strong root inhibition after SPL treatment. Then, SPL TUNEL-positive root cells and DNA ladder pattern observed on the present study could be due to sodium leading to stress and cell death.

On the other hand, in mammalian cells, fluoride increased intracellular Ca^{2+} concentration and the population of shrunken cells, both associated with early stages of apoptosis (Matsui et al., 2007). Since fluoride is a compound of SPL leachate, the apoptotic features of SPL-treated roots cells could be attributed to fluoride too.

To sum it up, SPL is a waste with a complex mixture of metals, cyanide, fluoride and sodium. Then, the results of this work and literature data suggested that SPL causes root cell damage mainly in nucleus, leading to cell death.

7 CONCLUSION

SPL affects root tips of *Allium cepa* by oxidative stress, DNA fragmentation, condensed chromatin and spheres nuclei. These features are associated with apoptosis-like cell death (programmed cell death) under abiotic stress.

8 ACKNOWLEDGMENTS

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CHAPTER 5

Biomonitoring the cytotoxicity of SPL (Spent Pot Liner): changes in root tips of *Lactuca sativa*

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1 RESUMO

ANDRADE, L.F.; DAVIDE, L.C.; GEDRAITE, L.S. Biomonitoramento da citotoxidade do SPL (Spent Pot Liner*)*: alterações em raízes de *Lactuca sativa*. In: ____. **Citogenotoxidade do SPL (Spent Pot Liner) por meio de bioensaios vegetais em ápice radicular.** 2008. 135 p., p. 112-135. Dissertação (Mestrado em Genética e Melhoramento de Plantas) – Universidade Federal de Lavras, Lavras, MG.

O SPL (Spent Pot Liner) é um resíduo sólido gerado pela indústria de alumínio. Sua composição é altamente variável (cianeto, fluoreto, compostos orgânicos e metais). Algumas plantas têm sido utilizadas em testes para biomonitoamento da poluição de contaminantes ambientais. Logo, o objetivo do presente trabalho foi determinar os efeitos do SPL em raízes de *Lactuca sativa* através de bioensaios vegetais. Foi observado um decréscimo nos testes de germinação de sementes (razão de germinação e percentual de geminação) à medida que aumentou a concentração de SPL. Adicionalmente, ocorreu redução no crescimento radicular em 44% após 72h de exposição ao SPL. Essa redução foi relacionada ao decréscimo no índice mitótico em 58% após 24h de exposição no SPL 25%. Não obstante, um forte aumento na percentagem de cromossomos pegajosos de células em divisão foi notado. Além disso, foi observado um aumento em células com núcleos condensados. O SPL provocou, ainda, alteração na superfície radicular, reduzindo os pelos radiculares absorventes. Esses resultados demonstraram que o SPL é um agente tóxico, que causa danos e distúrbios celulares.

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2 ABSTRACT

ANDRADE, L.F.; GEDRAITE, L.S.; DAVIDE, L.C. Biomonitoring the cytotoxicity of SPL (Spent Pot Liners*)*: changes in root tips of *Lactuca sativa*. In: ____. **Citogenotoxidade do SPL (Spent Pot Liner) por meio de bioensaios vegetais em ápice radicular.** 2008. 135 p., p. 112-135. Dissertação (Mestrado em Genética e Melhoramento de Plantas) – Universidade Federal de Lavras, Lavras, MG.

SPL (Spen Pot Liner) is a solid waste from aluminum industry. This waste has a composition high variable (e.g. cyanides, fluorides, organics and metals). Several plants tests have been used for biomonitoring the pollution of environmental contaminants. Thus, the aim of this work was study the SPL effects on root tips of *Lactuca sativa* with some current plant bioassays. It was observed a decrease on germination rate (GR) and seed germination (SD) as increased SPL concentration. In addition, SPL reduced the root growth in 44% after 72h of exposure. This reduction was related to a decrease on mitotic index in 58% after 24h of exposure in SPL 25%. Nevertheless, a great enhanced on percentage of stickness in dividing cells was noticed. It also was observed an increase on condensed nucleus cells. Moreover, SPL provoked an alteration on root tip surface reducing the root hair. These results demonstrated that SPL is a toxic agent which leads to cell damage and disturbance.

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3 INTRODUCTION

A wide variety of organic and inorganic compounds contribute to chemical pollution. Anthropogenic contamination by these chemicals is an especially dangerous side effect of civilization (Kovalchuk et al., 2001).

SPL (Spent pot liner) is a solid waste generated by the aluminum industry during the reduction of alumina to aluminum metal in electrolytic cells. This waste has a composition highly variable (e.g. cyanides, fluorides, organics and metals), but the components of the greatest environmental concern are cyanides and soluble fluoride salts (Andrade et al., 2008). These elements had the tendency to leach in contact with water (US EPA, 1991; Silveira et al., 2002). In Brazil, aluminum industry generates about 35,000 t of SPL a year. The industry has stored the SPL in controlled deposits, but it is known that in a long term, solutions environmentally more viable will need (Silveira et al., 2003).

The study of chemical pollution in complex organic–inorganic mixtures and assessment of their genotoxicity is not an easy task (Minissi, 1998). However, several plant species have been used as bioindicators, and a variety of tests have been employed, such as cytogenetic tests. These tests are commonly used for biomonitoring the extent of pollution and to evaluate the toxicity and mutagenicity of environmental contaminants on vegetal organisms (Liu et al, 2005; Matsumoto et al., 2006). In addition, under pollution stress, roots are the primary point of contact with contaminants (Fiskesjö, 1988; Sandalio et al., 2001; Liu et al, 2005). Tests on germination rate and seed germination in lettuce are indicated to determine phytotoxicity caused by potentially pollutant elements in soils (Enríquez-Peña et al., 2004; Vadillo et al., 2004; Valerio et al., 2007)

In this manner, is necessary to evaluate the cytoxicity of SPL on ultra-structural, microscopically and macroscopically levels in root tips to detect the

physio-biochemical mechanisms responses of cells and tissues. Thus, the aim of this work is biomonitore the SPL effects in *Lactuca sativa* by evaluating changes on root tips with some current plant bioassays.

4 MATERIAL AND METHODS

4.1 Plant Material

Seeds of *Lactuca sativa* L. (2n = 2x = 18) of commercial cultivar Regina were used as material for plant bioassays.

4.2 Preparation of SPL/soil leachates

The preparation of SPL solutions for simulating the adsorption of the SPL in soil and leachability of this pollutant was performed as described Andrade et al. (2008). Six concentrations of SPL were prepared (0%, 5%, 10%, 15%, 20% and 25%) of SPL(mg)/soil(mg). The composition of these leachates was previously analyzed in Andrade et al. (2008).

4.3 Germination test

Seeds were placed on germination paper with 7 ml of SPL solution in a Petri dish (90mm) at room temperature. The treatments were arranged in a completely random design with four replications for each concentration. Each replication was obtained from one Petri dish contained fifty seeds. The following parameters were analyzed: (1) germination rate (GR), calculated by de percentage of germinated seeds per day, measured for three days (24, 48 and 72h after exposure); (2) the seed germination (SG), corresponding to the percentage of germinated seeds after the experiment, calculated after 120h of exposure. For both parameters the number of germinated seeds was counted and compared to the control (0% SPL).

4.4 Root Growth analysis

Seeds were pre-exposed directly to distilled water for root emergence for 24h. Then, germinated seeds were submitted to SPL solutions at room temperature. The root lengths were evaluated after distilled water exposure and 12, 24, 36, 48, 60, and 72 h after the exposure to SPL treatments. The treatments were arranged in a completely random design with four replications for each concentration. Each replication was obtained from the average of six measurements of root growth, i.e. there were 24 root growth measurements for each concentration. The same root was measured for each evaluation time. The final root growth for each concentration/exposure time was obtained by the difference from root length after SPL exposure to root length after distilled water exposure, i.e. the root length after 36h on SPL treatment = root length after 36h on SPL – root length after distilled water.

4.5 Cytogenetic analysis

For cytogenetic analysis the seeds were pre-exposed to distilled water, for root emergence, and later submitted to SPL concnetrations for 24 h. After exposure, root tips (ranging from 0.5 to 1.0cm) were collected from each treatment, washed in distilled water and fixed in fresh cold methanol: acetic acid (3:1 v/v) solution. Fixed root tips were hydrolyzed in 1N HCl at 60ºC for 10 min, and then submitted to enzymatic maceration in Pectinase-Cellulase (40-4%) at 37ºC for 1:30h. After that, observed under the stereomicroscope, the root tips were chopped into several tiny fragments, covered, squashed and frozen in liquid nitrogen. Slides were quickly dried and stained with 5% Giemsa in phosphate buffer (pH 6.8), followed by washing with distilled water, and dried on a heating plate at 50ºC. Four slides were prepared from each treatment and about 5000-7000 cells were scored. The mitotic index was calculated as the number of dividing cells per total of observed cells (Fiskesjö, 1985). The

frequency of aberrant cells (%) was calculated based on the number of aberrant cells per total cells scored at each concentration of SPL.

4.6 Ultra-structural analysis

The seeds were pre-exposed to distilled water, for root emergence, and later submitted to SPL treatments (only control – 0% - and 25%) for 24 h**.** After exposure three root tips from each treatment were collected and fixed in modified Karnorvsky solution (2.5% Gluteraldehyde, 2% Paraformaldehyde in a 0.05M sodium cacodilate buffer at pH7.2 containing 0.001M $CaCl_2$) for at least 48 hours. Then, they were dehydrated in a gradient series of acetone solutions (30, 50, 70, 90 and 100), dried with carbon dioxide in a critical-point dryer (Bal-tec CPD 30), mounted at stubs with double sided tape, coated by vacuum evaporation with a gold layer of 20nm (Bal-tec SDC 50), and observed in an Evo40 Leo scanning electron microscope.

4.7 Statistical analysis

The means of root growth, mitotic index and chromosomal/mitotic alteration data were taken and their significance was determined by the Scott-Knott test ($p<0.05$) (Scott and Knott, 1974). Statistical analysis were done using SISVAR software (Ferreira, 2000).

5 RESULTS

5.1 Germination test

Table 1 shows that the germination rate (GR) tend to decrease as increase the SPL concentration. Significant differences were detected in the SPL 10% sample for the first day (24h) and in the lowest SPL concentration of 5% for the second (48h) and third (72h) days. However, the reduction on GR was greater in the first 24h when in the SPL 25% (high concentration) was 61% lower in relation to the control (Control = 37.3%, SPL 25% = 14,51%, Scott-Knott $p<0,05$). For the following 48h and 72h the reduction was 55% and 54% respectively, in relation to the control (in 48h, Control = 54.86%, SPL 25% = 24,59%; and in 72h, Control = 66,27%, SPL 25% = 30,02%, Scott-Knott $p<0,05$). The reduction of 50% of this parameter was already noticed in the concentration of SPL 10% after 24h of exposure (Control = 37.30%, SPL 10% = 17.28%, Scott-Knott $p<0.05$). Moreover, seed germination (SG) was similar to GR (Table 1). The significant differences comparing to the control were also observed in the SPL 5% sample. The inhibition of SG was increased 1.56 times for the concentration of SPL 5% and 2.31 times for SPL 25% (Control = 69.27%, SPL 5% = 44.4%, SPL 25% = 30.02%, Scott-Knott $p<0.05$).

Table 1 Germination Rate (GR) and Seed Germination (SG) in *Lactuca sativa* after SPL exposure.

Treatment	GR 24h (%)		GR 48h (%)		GR 72h (%)		SG (%)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	37.30	8.49	54.86	13.51	66.27	11.75	69.27	13.71
SPL 5%	27.40	5.05	33.77 *	5.12	44.40 *	2.23	44.40 *	2.23
SPL 10%	17.28 *	6.72	27.37 *	9.17	35.84 *	14.74	35.84 *	14.74
SPL 15%	20.79 *	3.92	29.12 *	5.52	37.01 *	11.61	37.83 *	11.48
SPL 20%	18.86 *	4.62	25.15 *	9.63	37.61 *	13.43	37.61 *	13.43
SPL 25%	14.51 *	3.40	24.59 *	3.71	30.02 *	4.69	30.02 *	4.69

SD = Standard deviation

*Represent statistical difference ($P<0.05$) in relation to the control.

5.2 Root Growth analysis

The results presented in Table 2 show that root growth was reduced by all the concentrations applied as compared with the control values. The inhibition of root growth was greater with increasing concentrations of SPL and treatment duration. Significant differences were first detected at SPL concentration of 15% in 36 h of time exposure (control = 0.80cm, SPL 15% = 0.57cm; Scott Knott $p<0.05$). Significant differences were not observed for 24 h of time exposure. In 48 h and 60 h of time exposure the behavior of root growth was similar to 36 h. Whereas, in 72 h of time exposure the difference can be noticed even in the lowest SPL concentration of 5% (control = 1.66cm, SPL 5% = 1.51cm; Scott Knott $p<0.05$). Additionally, comparing SPL treatments for 72 h of exposure time the inhibition of root growth in the SPL 25% (high concentration) was 44% (control = 1.66cm, SPL 25% = 0.81cm; Scott Knott $p<0,05$).

Table 2 Root growth (cm) in *Lactuca sativa* after SPL exposure in different times (h).

Treatments	24h	36h	48h	60h	72h
Control	0,36 ± 0,10 a*	0,80 ± 0,14 a	1,26 ± 0,17 a	1,44 ± 0,22 a	1,66 ± 0,12 a
SPL 5%	0,27 ± 0,07 a	0,76 ± 0,09 a	1,14 ± 0,07 a	1,45 ± 0,15 a	1,51 ± 0,20 b
SPL 10%	0,31 ± 0,05 a	0,72 ± 0,11 a	1,15 ± 0,15 a	1,40 ± 0,20 a	1,45 ± 0,18 b
SPL 15%	0,29 ± 0,10 a	0,57 ± 0,07 b	0,84 ± 0,11 b	1,10 ± 0,03 b	1,25 ± 0,10 c
SPL 20%	0,20 ± 0,04 a	0,47 ± 0,06 b	0,79 ± 0,08 b	1,02 ± 0,08 b	1,11 ± 0,12 c
SPL 25%	0,12 ± 0,02 a	0,29 ± 0,02 c	0,45 ± 0,05 c	0,71 ± 0,14 c	0,81 ± 0,08 d

Data represent means±SD.
*Means with the same letter do not represent statistically differences ($p<0.05$).

5.3 Cytogenetic analysis

The effect of SPL in cell division is shown in Table 3. SPL reduces significantly the percentage of prophase and metaphase dividing cells. The statistical difference ($p<0.05$) is noticed at SPL 10%. Nevertheless, it was observed an increase in telophases cells after treatment with lower SPL concentration (5% and 10%). In addition, SPL enhanced the frequency of abnormal dividing cells (Figure 1). Statistical difference ($p<0.05$) was observed in the SPL 10% for stickness metaphases. Although c-metaphases and bridges were observed, there was not a significant ($p<0.05$) increase compared to control (SPL 0%). On the other hand, considering the frequency of abnormalities per number of cell analyzed it was not enhanced significantly (Table 4). However, a decrease on mitotic index (MI) was observed, ranging from 22% (5% SPL) to 58% (25% SPL) (Table 4). Moreover, the percentage of condensed nucleus increases significantly ($p<0.05$) still on the lowest SPL concentration (Table 4). After treatment of 25% SPL it was 50% higher than control (0% SPL).

5.4 Ultra-structural analysis

The scanning electron microscopy images of root tips demonstrated a decrease of root hair on root elongate zone as increased SPL concnetrations. After 24h in SPL 25% (high concentration) it was noted a diminished on root hair size comparing to control roots (Figure 2).

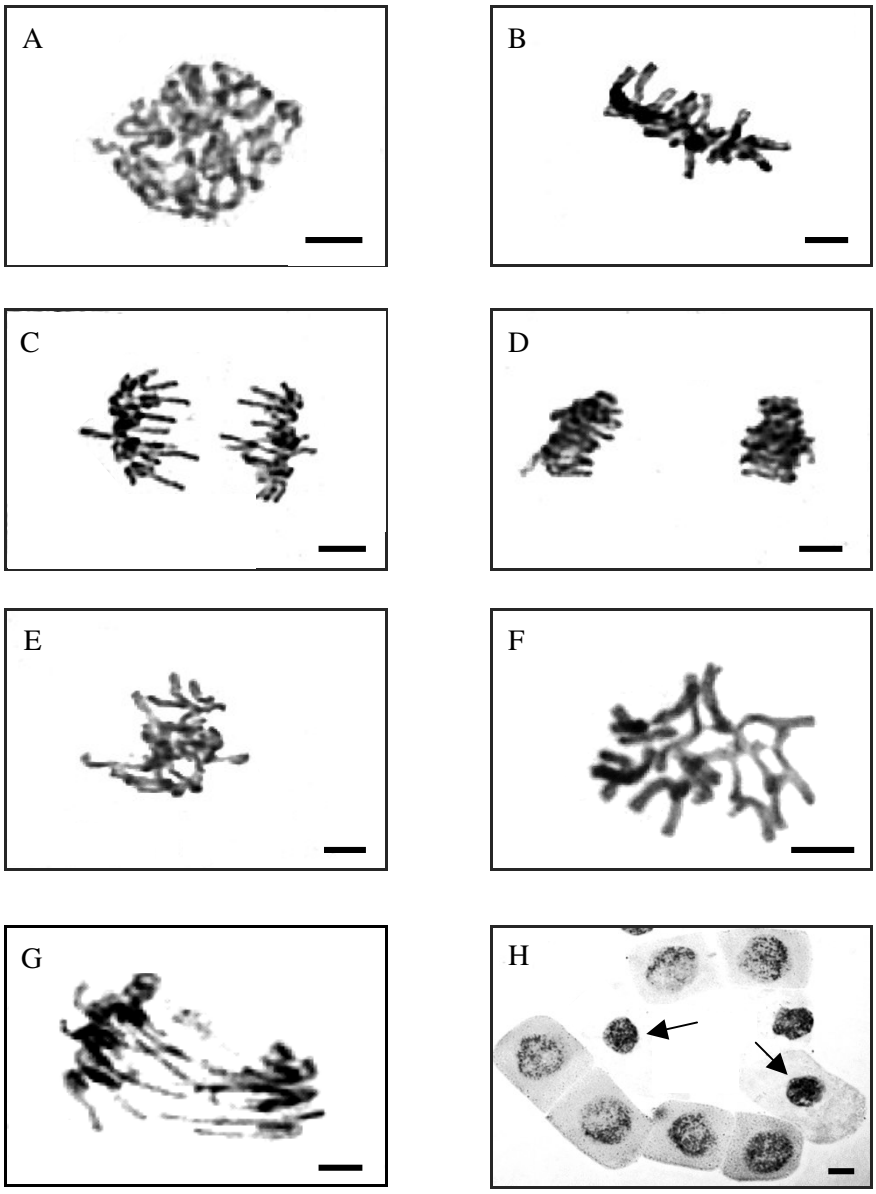

Figure 1 Normal and Abnormal dividing cells of *Lactuca sativa* after SPL exposure**.** (A-D) Satges of mitosis: prophase (A), metaphase (B), anaphase (C), telophase (D); (E–G) chromosome disturbances: stickness (E), c-metaphase (F) and anaphase bridges (H); (H) condensed nucleus (arrows); Bar = 10 µm.

Table 3 Effects of SPL on cell division in root tips of *Lactuca sativa.*

		Normal dividing cells (%)				Anormal dividing cells (%)		
	Number of cells	**Prophase**	**Methaphase**	**Anaphase**	**Telophase**	**C-Metaphasis**	**Stickness**	**Bridges**
SPL 0%	5155	16,28	25,58	3,49	9,30	1,16	43,02	1,16
SPL 5%	5357	10,26	23,08	7,69	17,95*	7,69	33,33	0,00
SPL 10%	5400	0,00*	11,11*	2,78	16,67*	5,56	63,89*	0,00
SPL 15%	4285	3,70*	14,81*	5,56	5,56	5,56	62,96*	1,85
SPL 20%	7058	3,51*	12,28*	1,75	5,26	3,51	70,18*	1,75
SPL 25%	4999	8,70*	8,70*	4,35	8,70	4,35	65,22*	0,00

Data present the percentage of total dividing cells. Means followed by (*) presents no statistical difference ($p<0,05$) comparing to control ($p<0.05$)

Table 4 Mitototic Index, Abnormlities and condensed nucleus cells after 24h of SPL treatment in root tips of *Lactuca sativa*

	Treatments					
	0% SPL	**5% SPL**	**10% SPL**	**15% SPL**	**20% SPL**	**25% SPL**
Mitotic Index (%)	2.54 ± 0.71	1.97 ± 0.21	1.63 ± 0.71*	1.47 ± 0.71*	1.19 ± 0.43*	1.06 ± 0.52*
Abnormalities (%)	0.68 ± 0.24	0.95 ± 0.32	1.03 ± 0.25	0.99 ± 0.34	1.04 ± 0.10	1.28 ± 0.46
Condensed Nucleus	17.62 ± 3.11	25.40 ± 3.35*	29.41 ± 3.95*	32.04 ± 2.14*	33.58 ± 6.89*	35.29 ± 3.58*

Data present the percentage of total number of cells. Means followed by (*) presents no statistical difference comparing to control ($p<0.05$)

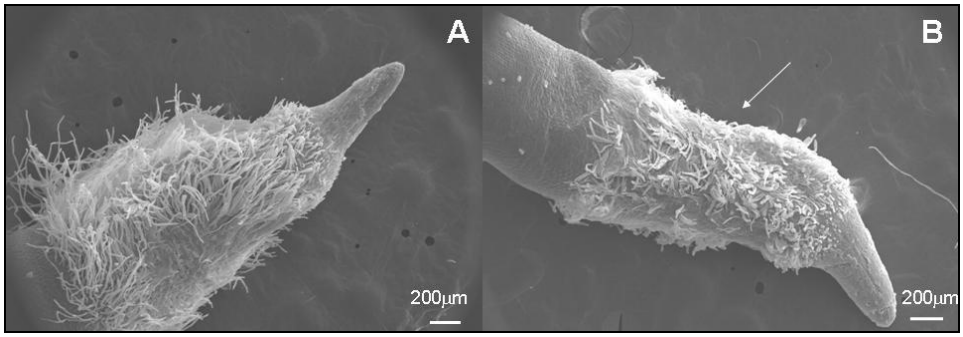

Figure 2 Scanning Electron Microscopy of root tips of *Lactucca sativa*. (A) Control and (B) SPL 25%. Note the reduction of root hair size (arrow).

6 DISCUSSION

The results of the present work demonstrated that SPL have toxic effects in root tips of *Lactuca sativa*.

The SPL leachates exert a toxic effect on GR, SG and root growth. Valerio et al. (2007) proposed the methodology of GR and SG in lettuce to measure the phytotoxicity of elements coming from soil-water extracts as alternative to determine soil toxicity. Our observations showed that SPL reduces all these parameters in about 50% in relation to the control (SPL 0%). In their findings, reduction in GR was the most sensitive parameter observed in determine of the pytotoxicity of soil-water samples with heavy metals (As, Pb, Zn, Mn, Co, Ni).

In addition, the bioassay with lettuce seeds is defined as static and of acute toxicity, where the effects of pure compound or a complex mixture in the germination of seeds and in the development of the seedlings was evaluated during the first days of growth (120h of exposure) (Torres, 2003). Thus, the germination rate and seed germination are variables that are measured in germination tests (Enriquez-Peña et al., 2004; Vadillo et al., 2004). Moreover, these could be associated with cytogenetics assays. Here, we observed that mitotic index has the same comportment of the germination tests and root growth analysis. SPL reduces the percentage of prophasic and mainly metaphasic cells, reflecting in the reduction of 58% on mitotic index (MI). The MI is other parameter considered reliable to determine the presence of cytotoxic compounds in the environment and suitable test for biomonitoring pollution levels (Smaka-Kincl et al., 1997; Fernandes et al., 2007). Then, the reduction of MI value in treated roots is probably due to disturbances in cell cycle as well as from chromatin disfunction induced by metals SPL components-DNA

interactions (Glinska et al., 2007). Apart of this, on ultraestructural analysis a reduction on root hair size were observed what is related with the decrease on root growth and consequently on the MI.

Cytogenetic analysis also revealed the presence of abnormalities in dividing cells. The most frequent aberration observed in SPL treatments was stickness, but c-metaphase and bridges were also present. Pobably the high frequence of stickness metaphases contributed for the no significant frequency of c-metaphase. Sticky chromosomes observed indicate an effect of SPL on the organization of chromatin which is related to a disturbed balance of the quantity of histones or other proteins responsible for controlling the proper structure of nuclear chromatin (Kurás et al., 2006). In spite of being low, the c-metaphases and bridges observed suggested that SPL acts on mitotic spindle apparatus (Seth et al., 2008). These results is according to previous findings reporting the SPL effects on root tips of *Allium cepa* and *Zea mays* where stickness, bridges, lagged chromosomes, fragments and c-metaphases were observed (Andrade et al., 2008).

Additionally the enhanced frequency of condensed nucleus suggested that SPL leads to DNA damage. The condensation of nuclei is one of the first steps on cell death process (Katsuhara, 1997; Vermes et al., 2002). Cell death by exposure to toxic chemicals has been studied in various plant systems (Pan et al., 2001; Boscolo et al., 2003; Behboodi and Samadi, 2004; Seth et al., 2008). This type of cell death in response to abiotic stress, named programmed cell death. It is characterized by DNA fragmentation, chromatin condensation, cytoplasm shrinkage, nuclear and cytoskeleton alterations (Savill and Fadok, 2000; Ueda and Shah, 2000; Ameisen, 2002; Behboodi and Samadi, 2004).

7 CONCLUSION

In our study we observed that SPL affect germination, root growth and mitotic index. Taking into account, it was demonstrated that the use of germination tests with MI is a feasible combination of methods to identify toxic synergistic effect of chemicals compounds on the environment. Moreover, alterations on root tip surface and in cell cycle were observed, showing that SPL is a toxic agent which leads to cell damage and disturbance.

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