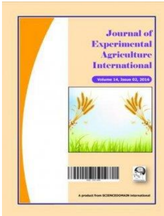

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Effect of Substrate Treated with Tannery Sludge on Growth and Anatomy of Conilon Coffee Cuttings

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Authors' contributions

This work was carried out in collaboration between all authors. The authors AAFZ and RAS were responsible for running the experiment in the field, as well as performing the submission and doing the evaluators' chores. The authors SSB and APCGB were responsible for managing the entire experiment as well as statistical analyzes and writing the paper. The authors SP, FGT and MDC were responsible for the analysis of electron and optical microscopy as well as the interpretation of the results. All authors read and approved the final manuscript.

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ABSTRACT

This work evaluated the development and anatomy of the conilon coffee (*Coffea canephora* Pierre - Rubiaceae) seedlings on substrates with varying doses of dehydrated bovine tannery sludge. The experiment was divided into two stages; The first one was carried out in the field in a nursery of seedlings in the city of Colatina and the second was carried out in a Laboratory of Cell and Tissue Biology of Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes. During the field phase, the experiment was performed in randomly selected sections of a

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propagating nursery area in which each section received five treatments with twelve replicates per treatment. Seventeen replicates of seedlings were included in each treatment for a total of 85 plants per section and 1,020 plants for the whole experiment. Biometric analyzes and gravimetric evaluation of the development of seedlings were carried out at 120 days post - planting at the house of propagation of seedlings. For anatomical and ultrastructural analysis, plant material was processed in accordance with standard techniques for light and electronic microscopy. Despite increasing chromium levels in leaves with increasing doses of sludge, there was no impairment to plant development associated with other components of the tannery sludge treated substrate, such as humus and soil. Structural analysis revealed reduction and disruption of the palisade parenchyma and alteration to the shape and internal structure of chloroplasts. The use of tanning sludge in the proportion of 20% showed positive results, with potential of use in the substrate of *Coffea canephora* seedlings.

Keywords: Sustainability; tannery sludge; chromium; sodium; residue; structure; Coffea canephora.

1. INTRODUCTION

As a country, Brazil harbors one of the largest herds of cattle in the world, with the production of leather being one of the outcomes. In just the first trimester of 2014, 9,164 million units of cattle hides were produced in the country. A great amount of residue is produced on a daily basis as a byproduct of leather processing in tanneries, which has potential agricultural applications. Although there are no federal laws regulating the use of tannery sludge in Brazil, some states have local regulations (e.g., São Paulo) while others, such as Espirito Santo, discard the sludge in leased warehouses at high costs for tanneries. Because the residue is rich in organic matter and essential elements for plants, it has been the subject of numerous investigations into its use and viability, or restriction, for agriculture [1-7].

Tannery sludge may become contaminated with chromium during leather processing, leaving the destination of the residue more problematic. Thus, when used in the substrate of perennial plants, such as conilon coffee, it is diluted and systematically redistributed in the soil in an attempt to prevent major changes to the original basic structure of the soil [8]. Other wastes from human activity, such as sewage sludge, have been considered for the purposes of recovering degraded areas or for the production of biomass for burning. These wastes have desirable characteristics for these purposes, particularly organic matter content and high concentrations of macronutrients needed by plants, such as nitrogen [9,10] similar to the characteristics of tannery sludge.

The chromium contained in tannery sludge can bioaccumulate in plant tissues, which may lead to alterations of cellular structures and compromise the processes involved in cellular division, thus leading to reduced biomass production [11]. Han et al. [12] showed that chromium accumulation in leaves can delay the growth and development of this organ and reduce the number of cells of parenchyma that compose leaf mesophyll.

Other effects attributed to chromium toxicity are directly related to its participation in the formation of reactive oxygen species (ROS) [13,14]. These molecules act as agents that promote lipid peroxidation and cause severe injury to lipid cellular membranes [15]. Damage to the membrane structure of chloroplasts, in the form of perceptible alterations to chloroplast membranes and disorganization of thylakoids membranes, is evident that when plants are treated with high doses of chromium [16].

In addition to chromium, tannery sludge contains high concentrations of sodium, which is used in the cleansing of hides. The resulting high salinity of the residue may cause osmotic disequilibrium in plants when it is used as a component of the substrate, thereby altering plant physiology and anatomy.

Waste generated throughout the world should be appropriately used and allocated as close as possible to the site of generation, since the cost of logistics for transporting these wastes often precludes their use in agriculture. In the state of Espirito Santo, some producers of conilon coffee, the main agricultural activity of the state, could benefit from the use of the tannery residues produced locally. Thus, this work aimed to determine whether structural characteristics of conilon coffee seedlings grown on substrate with increasing doses of dehydrated bovine tannery sludge are altered, and if these alterations interfere with plant development.

2. MATERIALS AND METHODS

The experiment was divided into two stages; the first was carried out in the field in a seedling nursery in the city of Colatina (19º32'22" S, 40º37'50" W; 71 m; Espírito Santo, Brazil), and the second was performed in a Laboratório de Biologia Celular e Tecidual. During the field phase, the experiment was conducted in randomly selected sections of an area of the coffee seedling propagation nursery. Each section received five treatments with twelve repetitions per treatment. Seventeen seedling repetitions were included in each treatment for a total of 85 plants per section and 1,020 plants for the entire experiment.

The four treatments consisted of dehydrated tannery sludge, soil and humus, with the concentration of sludge differing among treatments with 10%, 20%, 30% and 40% of the total volume of the substrate (T-10, T-20, T-30 and T-40). The subsoil – humus – chemical fertilizer mixture traditionally used by producers of conilon coffee seedlings in the northern region of Espírito Santo, was used as a control. The genotype used for this work was the clonal conilon coffee variety Vitoria Incaper 8142. This variety has 13 clones; however, for the purposes of the experiment, only clone V8 was tested since it is considered by the producers of the region to be the most vigorous. The composition of the treatments T-C, T-10, T-20, T-30 and T-40 are detailed in Table 1.

The characteristics of the soil used in the treatment substrates are: pH 5; contents of the P; Cr; K; Fe; Cu; Zn; Mn; S and B was respectively of the 5, 32; 48; 7; 0,6; 0,8; 7,9; 112; 0,4 $\mu g.g^{-1}$DW; contents of the Ca; Mg; and Na was respectively of the 0,8; 1,3; 0,03 $cmol_c.kg^{-1}$ DM; and base saturation of the 74.

Tannery sludge was provided by a company located in Baixo Guandu (Espirito Santo, Brazil). After processing bovine rawhide, the sludge, released in a concentrated liquid form with 97% humidity (dry base), was placed in evaporation tanks until it reached a humidity of approximately 14% (dry base). The characteristics of the dehydrated sludge used in this experiment are: pH 12,3; contents of the N; P; K Ca; and Mg was respectively of the 37; 0,09; 0,8; 27 and 1 $\mu g.g^{-1}$DW; content of organic matter was 29%; electric conductivity was 17,3 $dS.m^{-1}$; contents of the de Cr; Na; Fe; Cu; Zn and Mn was respectively of the 9952; 550; 57; 1; 1; 1 $\mu g.g^{-1}$DW.

The humus used in the treatment substrates was obtained from the warmer at IFES-Itapina and made with bovine manure. The characteristics of the humus are: pH 6,8; contents of the Cr; remaining Phosphorus; K; and Ca was respectively of the 0,01; 90; 201; 93 $\mu g.g^{-1}$DW; contents of the Mg; Fe; Cu; Zn and Mn was respectively of the 146; 57; 1; 1 e 1; and base saturation of 82.

Mineral analyses of the soil and the humus used in the treatment substrates were performed by atomic absorption spectrophotometry [17]. Chromium was analyzed according to EPA-SW-846 Test Method 3051A [18].

Seedlings were produced from cuttings obtained from adult tissue of orthotropic branches collected from plants with good phytosanitary and nutritional conditions. After removal, the branches were sent to a greenhouse where 30 cm of the extremities of the branches were eliminated (branches containing several cuttings). Standardization of cuttings was then carried out by setting them at a height of 6 cm and leaves with 1/3 of the leaf blades. The cuttings were planted in 11x20 cm polyethylene bags, which had been filled with treatment substrate 30 days prior to the implementation of the experiment. Irrigation by micro sprinklers was done on a daily basis throughout the experiment, always maintaining the field capacity of the substrates.

Table 1. Description of the treatments containing the conventional treatment (control T-C), and the different proportions of the tannery sludge in the soil (T-10, T-20, T-30 and T-40)

Treatment	Components of substrate
T-C	136 dm^{-3} DM soil (even soil used in treatments T-10 and T40) + 625 g simple superphosphate + 200 g limestone + 200 g KCl and 3 dm^{-3} DM + Humus.
T-10	10% tannery sludge + 30% humus + 60% soil
T-20	20% tannery sludge + 30% humus + 50% soil
T-30	30% tannery sludge + 30% humus + 40% soil
T-40	40% tannery sludge + 30% humus + 30% soil

The experimental field stage lasted for 120 days beginning on the day when the cuttings were planted. The number of leaves, seedling height and stem diameter were evaluated and gravimetric analyses were performed with fresh and dry mass of the shoot, root and entire plant (circulation furnace at 70ºC until reaching a constant weight for dry masses). Through obtaining of the dry mass of the root and aerial part, the root dry mass / shoot dry mass ratio was calculated. For weighing shoots (stem and leaves), only leaves that had emerged from the planted cuttings were considered, excluding the original mass of the cutting. Only the new leaves were taken into account when determining the number of leaves. After being dried in the furnace, tissue samples were ground in a Willey knife grinder equipped with two opening sieves with 2 mm mesh. The analysis of leaf chromium was performed by means of wet digestion with HNO_3 + $HClO_4$.

Quantitative data were subjected to analysis of variance with the F test, and means compared by the Duncan's test ($p<0.05$). The statistical analyses were performed using the Assistat program version 7.7 [19].

For anatomical and ultrastructural analyses, fragments of the middle third of the leaf blade were removed and placed in Karnovsky solution [20]. The samples were then washed in sodium cacodylate buffer at 0.05 M and post-fixed in 1% osmium tetroxide in the same buffer for hours at room temperature. The samples were then dehydrated in increasing amounts of acetone, infiltrated and embedded in epoxy resin (Polybed), and polymerized in a furnace at 60ºC. Semi-thin sections of approximately 0.7 µm were then obtained with the aid of an ultra-microtome (Reichert Ultracuts Leica Instruments®) using a diamond knife (Diatome®). The sections were then stained with 1% toluidine blue and 1% borax buffer, and observed under a light microscope (Axioplan, ZEISS), using a Hamamatsu C3077 digitizing camera or a Cannon Power Shot A640 camera and Analysis® software - LINK/ISIS/ZEISS (Oxford, UK). For ultrastructural analyses of the chloroplasts, ultrafine sections were collected in copper grids (300 mesh), which were then contrasted with 5% lead citrate and 1% uranyl acetate, and observed under a transmission electron microscope (TEM 900, ZEISS) at a voltage of 80 Kv.

The anatomical and ultrastructural analyses were performed at the Laboratório de Biologia Celular e Tecidual da Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, Rio de Janeiro, Brazil.

3. RESULTS

There were significant differences among treatments for practically all characteristics evaluated, with the only exception being stem diameter and root dry mass/shoot dry mass ratio, which exhibited no statistical differences.

When evaluating the plants height, it was observed that the treatment with the dosage of 20% dehydrated sludge had the highest average plant height, differing from practically all the other treatments. This proportion of sludge/soil/humus was the only treatment that had a positive significant difference ($p<0.05$); in other words, the increase in height of the coffee seedlings in this treatment was greater than that for the control substrate with conventional chemical fertilizer (Table 2).

The number of leaves per plant at 120 days did not differ among tannery sludge treatments, and only the 10% treatment had a greater number of leaves than conventional substrate (Table 2). There was no significant increase in chromium level in leaves of the 10% sludge treatment (Table 2), with the level being equivalent to that

Table 2. Mean plant height, number of leaves, stalk diameter and chromium level in leaves of conilon coffee seedlings grown with different concentrations of dehydrated tannery sludge at 120 days

Treatment	Plant height (cm)	Number of leaves	Stalk diameter (cm)	Chromium level in leaves ($\mu g.g^{-1}$DW)
T-10	8.75 b	4.45 a	2.82 a	1.65 c
T-20	9.76 a	4.16 ab	2.82 a	3.75 b
T-30	8.29 b	4.14 ab	2.64 a	4.05 b
T-40	8.87 ab	4.07 ab	2.79 a	5.21 a
T-C	8.23 b	3.92 b	2.67 a	1.20 c
Average	8.78	14.15	2.75	3.03

Means followed by different letters in a column differ statistically according to Duncan's test at the 5% level found for plants developed on conventional substrate (without sludge). However, higher proportions of sludge produced increased levels of chromium in leaves, with the 40% treatment having statistically higher levels than the others. Chromium levels in treatments T-10; T-20; T-30 and T40 were 37, 212, 237 and 334%, respectively, which were all higher than the chromium content of leaves grown on conventional substrate (T-C, without sludge).

Practically all characteristics evaluated for the conilon coffee seedlings exhibited higher gain for the 20% sludge proportions of sludge when compared to the treatment with conventional fertilization (T-C, Table 3).

Anatomical examination showed a decrease in palisade parenchyma and a consequent increase in spongy parenchyma, with the changes being most evident in the 30% and 40% sludge treatments. In addition to these changes, disorganization of the treatment (T-20), exceeding not only the treatments with other palisade cells resulting from the loss of cellular volume was observed mainly in the 10%, 30% and 40% sludge treatments (Fig. 1).

The chloroplasts of the conilon coffee plants grown on substrates enriched with tannery sludge exhibited significant structural differences from those of the control plants. The control plants (T-C) possessed chloroplasts with an ellipsoidal shape, internal membranes organized in thylakoids, and stroma with starch grains and oil droplets (Fig. 2. A and B). In the 10% and 20% tannery sludge treatments there was an increase in the grana and in the stroma, and a decrease in the quantity of starch grains (Fig. 2. C – F). The chloroplasts of plants of the 30% and 40% treatments exhibited internal membranes organized in thylakoids and grana similar to those of the control plants. However, there was also disorganization of the membranes and an absence of starch grains in the stroma of the chloroplasts, with only oil droplets being present (Fig. 2. H – J). The chloroplasts of plants from all the treatments exhibited changes in shape, compared to those of the control plants (Fig. 2).

4. DISCUSSION

Seedling height is one of the main indicators of quality, and has been the subject of several studies involving conilon coffee seedlings. It is also one of the main developmental parameters taken into account by nurseries [21]. In this sense, the average height of plants developed on conventional substrate and substrates containing tannery sludge are within acceptable limits of previous studies.

Berilli et al. [2] showed that the height of conilon coffee seedlings grown on a substrate containing soil from the subsoil, tannery sludge in different proportions and without humus reached heights of 5 cm at 120 days. For seedlings on conventional substrate, the heights were approximately 8 cm, similar to those observed in the present study. However, in contrast to what was observed by Berilli et al. [2] the height of the seedlings developed on substrates with tannery sludge with humus and soil exhibited heights that were similar to or higher than that of seedlings developed on conventional substrate. These results reveal that the presence of humus in the substrate, with sludge and soil, produced significant increases in seedling height, most notably in the proportion of 20% sludge, 30% humus and 50% soil.

Mean heights of the conilon coffee seedlings observed in this experiment were below those of coffee seedlings of other studies [21,22]. An

Table 3. Mean fresh mass of shoot (MFPA), root (MF-Root), and plant (MFP); and mean dry mass of shoot (MSPA), root (MS-Root), plant (MSP) and root dry mass/shoot dry mass ratio (RDM/SDM) for conilon coffee seedlings grown on conventional substrate and substrate with different concentrations of dehydrated tannery sludge at 120 days

Treatment	MFPA	MFR	MFP	MSPA	MSR	MSP	RDM/SDM
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T-10	3.26 ab	1.84 b	5.10 b	0.68 b	0.26 b	0.94 b	0.38 a
T-20	4.08 a	2.79 a	6.87 a	0.90 a	0.48 a	1.38 a	0.53 a
T-30	2.40 b	1.89 b	4.29 b	0.53 b	0.23 b	0.76 b	0.43 a
T-40	3.07 b	1.96 b	5.04 b	0.69 b	0.34 b	1.03 b	0.49 a
T-C	2.62 b	1.85 b	4.47 b	0.56 b	0.29 b	0.84 b	0.52 a
Average	3.09	2.06	5.15	0.67	0.32	0.99	0,47

Means followed by different letters in a column differ statistically according to Duncan's test at the 5% level explanation is that the seedlings of the present study had different ages and were not provided supplemental fertilization, because the purpose of this study was to assess only the influence of the application of tannery sludge as a source of initial organic fertilization, in contrast to the conventional chemical fertilizer used by Berilli et al. [2].

Apparently, the presence of high doses of tannery sludge in the substrate tends to decrease the number of leaves per coffee seedling. This is probably due to the greater amount of factors inhibiting the development of plants, such as the greater presence of chromium and the salinization of the substrate caused by greater doses of sludge. Excessive

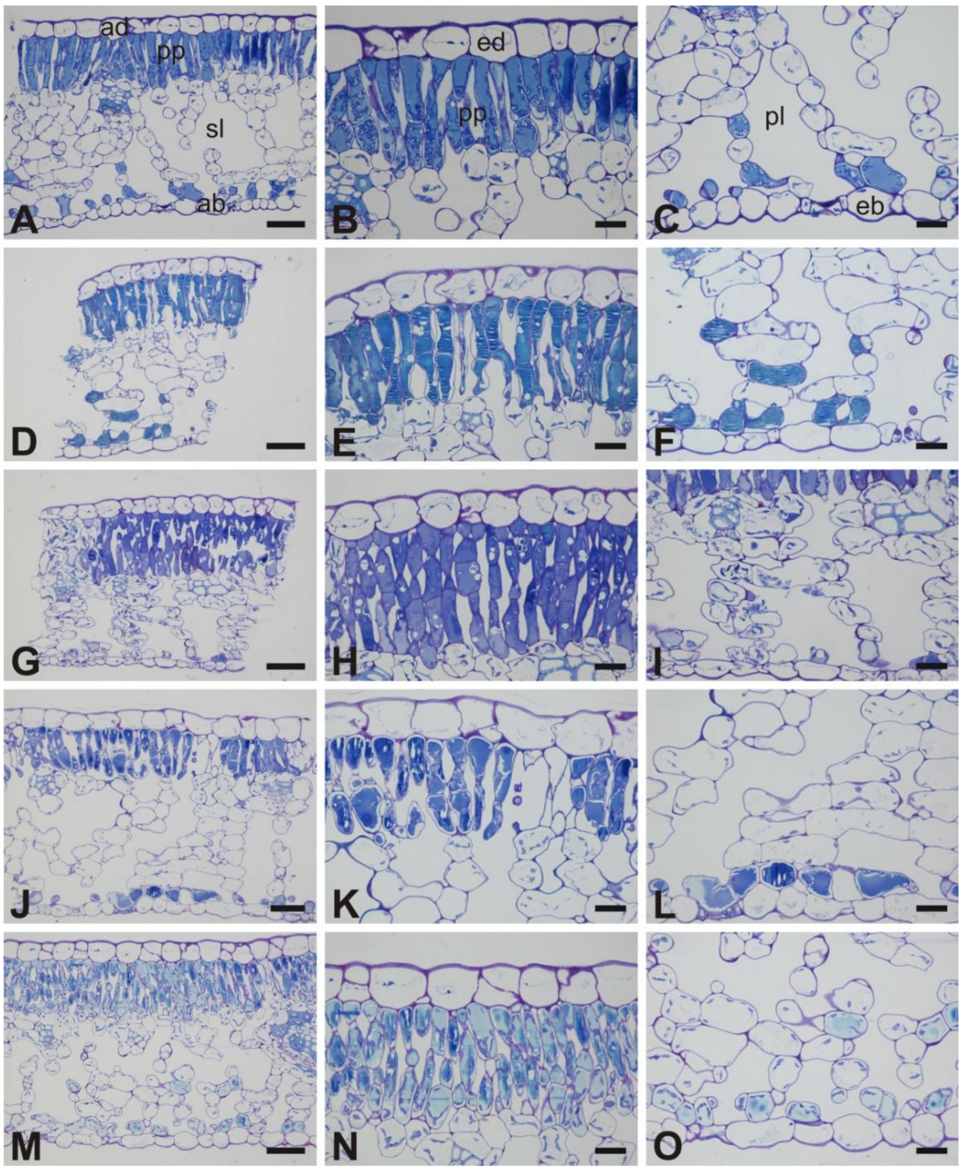

Fig. 1. Anatomical characteristics of the leaf blade of conilon coffee plants grown in substrates enriched with different quantities of tannery sludge; A, B and C – control plant; D, E and F – plants grown with 10% tannery sludge; G, H and I – plants grown with 20% tannery sludge; J, K and L – plants grown with 30% tannery sludge; and M, N and O – plants grown with 40% tannery sludge. Adaxial epidermis (ed); palisade parenchyma (pp); Spongy parenchyma (pl); abaxial and abaxial epidermis (eb). Bars: A, D, G, J and K = 50μm; B, C, E, F, H, I, K, L, N and O = 20 μm

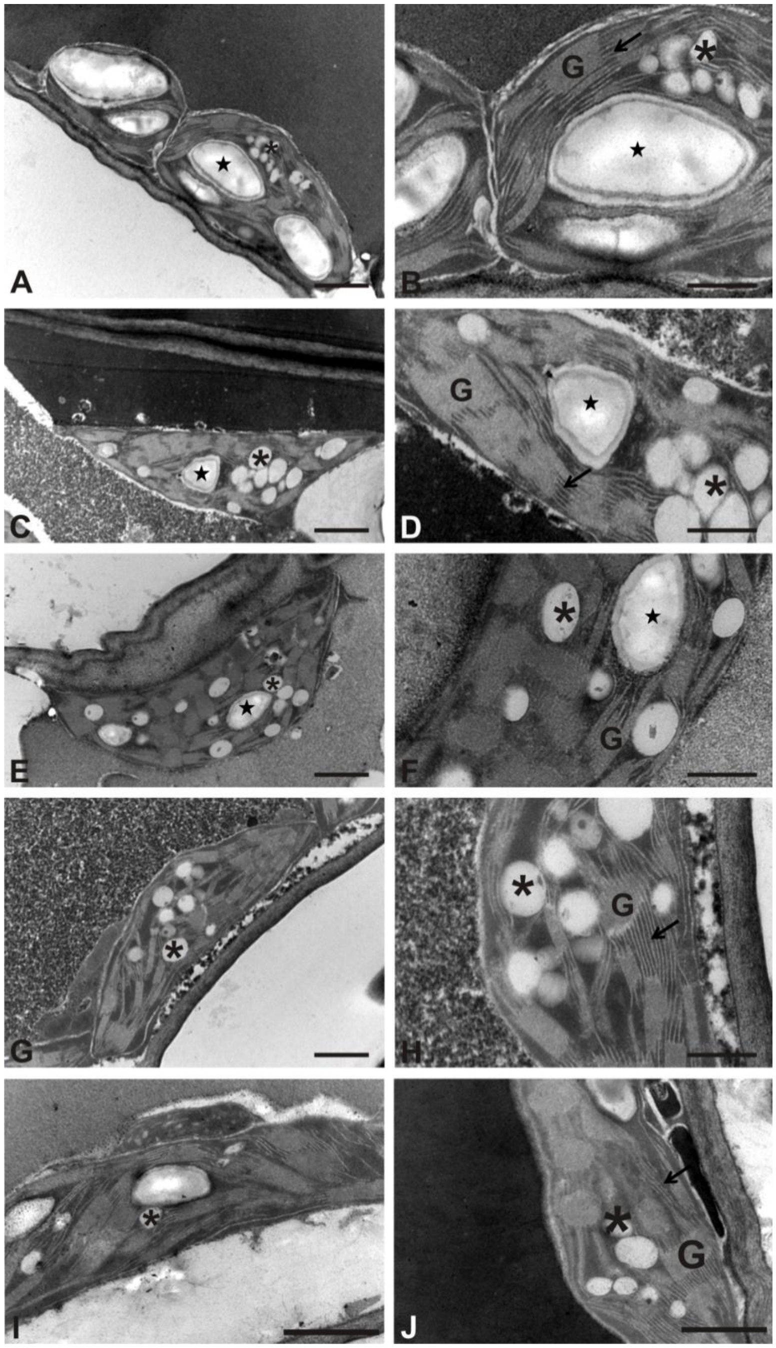

Fig. 2. Chloroplasts of conilon coffee plants grown on substrates enriched with different quantities of tannery sludge. A and B – control plant; C and D – plants grown with 10% tannery sludge; E and F – plants grown with 20% tannery sludge; G and H – plants grown with 30% tannery sludge; I and J – plants grown with 40% tannery sludge; granum (G); thylakoid (arrow); starch grains (star); oil droplets (asterisk). Bars: A, C, E, G and I = 2 μm; B, D, F, H, J = 500 μm

application of tannery sludge has the potential to be inhibitory for several crops, mainly due to factors such as increased salinity and chromium concentration [1,3,4,23].

The root dry mass/shoot dry mass ratio showed a vegetative growth pattern among the treatments. The shoot dry mass presented higher values in relation to root dry mass revealing a larger development of the shoot part in comparison to the roots. This pattern of development was also observed by Sales et al. [24] in seedlings of the *Schinus terebinthifolius* Raddi. (Anacardiaceae) treat with tannery sludge. Thus, seedlings with this high ratio may present problems at transplanting moment, because the root system may present difficulties in water supply to the shoot part under conditions of water stress, if the development of the shoot part is much above the development of the roots.

However, even with increased chromium accumulation in leaf tissues of plants with the increased doses of sludge, there was no apparent interference in mass gain or in seedling development, which is similar to the findings of Potasso et al. [1] who assessed the influence of adding tannery sludge to soil on eucalyptus harvest and the assimilation of chromium by this plant (Tables 2 and 3).

The presence of chromium in leaf tissue may alter the natural biochemistry of cells by causing oxidative stress and disturbances in the ultrastructure of the chloroplasts, compromising the photosynthetic process. Thus, the activity of the chromium in plants depends on sensitivity of the particular crop and/or its concentration in the soil or plant tissue [25]. Thus, despite there being an increased presence of chromium in leaf tissue, it seems that conilon coffee experienced no great compromise to plant development.

The significant mass gain for plants in the 20% tannery sludge treatment reveals that the specific mixtures (T-20) can provide a relative balance among the components of the substrate (soil, humus and dehydrated tannery sludge). In this way, the influence of problematic elements, such as sodium or chromium, are minimized by the activity of the humus, while at the same time the nutrients made available by the tannery sludge aid seedling development better than other treatments, being recommended for coffee seedlings, since it did not negatively affect the quality of seedlings.

Despite the fact that the dehydrated tannery sludge consisted of 29% dry mass organic matter and that its composition is rich in essential nutrients for plants, the elevated pH and the presence of sodium and chromium in high concentrations may preclude the direct use of tannery sludge as source of organic matter or alternative fertilizer [2,8]. However, by associating the sludge with humus in the substrate, there was a positive interaction between the balance of the toxic elements and the availability of the essential nutrients present in the sludge.

Anatomical changes observed in the leaves, such as disorganization of the palisade parenchyma (Figure 1), may be directly related to the quantity of heavy metal, mainly chromium, present in the tannery sludge. Han et al. [12] showed that in the presence of trivalent chromium (Cr^{3+}), white mustard plants (*Sinapis alba* L. - Brassicaceae) exhibit a decrease in both the palisade and the spongy parenchyma, which differs from the results obtained for the present study, in which only a decrease in the palisade parenchyma was observed. Results of studies by Sridha et al. [26] with Zinc (Zn) and Cadmium (Cd), also with mustard plants, were equivalent to those obtained in the present study with respect to the palisade parenchyma, and in which cell disorganization was also evident.

The fact that tannery sludge presents a great amount of chromium may be directly related to the alterations observed in the chloroplasts of the studied plants. Studies with chromium have shown this metal to have phytotoxic effects, mainly by affecting the ultrastructural of chloroplasts and thus altering the organization of the chloroplast membranes and influencing thylakoid membrane arrangements [16,25].

The loss of the standard shape of chloroplasts (ellipsoidal) in plants treated with tannery sludge may also be related to the effect of heavy metals, since radish plants (*Raphanus sativus* L. - Brassicaceae) treated with cadmium also exhibited alterations to chloroplast shape [27]. The same author related these changes to increased stroma volume and the disorganization of thylakoid membranes, characteristics found in the coffee plant chloroplasts analyzed in the present study.

A decrease in the number of starch grains and an increase in the number of oil droplets, mainly in the 30% and 40% treatments, may be related to stress and chromium toxicity. Increased numbers of oil droplets in plants grown on substrates treated with sludge can be explained by the fact that these structures act in response to different types of stress [28]. Oil droplets are lipid reserves that are linked to thylakoid membranes and can act as regions of synthesis and recycling of proteins, and thus have an important role in the response of plants to oxidative stress to the photosynthetic apparatus [29]. The increase in the number of oil droplets in plants treated with tannery sludge can be directly related to this role of protecting these structures against the oxidative stress caused by the excess sodium and chromium present in the substrate.

5. CONCLUSION

The results indicate alterations to the structural characteristics of leaves of conilon coffee seedlings grown on substrates with varying doses of dehydrated bovine tannery sludge, such as disorganization of the mesophyll and alterations to the chloroplasts, suggesting that these plants were experiencing stress. However, these alterations did not interfere with seedling development, of the coffee seedlings and they present an exhibited optimal performance on substrate enriched with tannery sludge, suggesting that this is an adaptive strategy of these plants.

The treatment that obtained the highest plant dry mass was the one with 20% dehydrated sludge in the substrate, which showed potential for use as a substrate for coffee plants. Such use favors mutually close coffee producers and tanneries, by promoting proper disposal of this residue in the environment and facilitating coffee plant production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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