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Influence of humus on chromium absorption by coffee seedlings grown on substrate containing tannery sludge

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ABSTRACT

Chromium is present in the tannery sludges worldwide making it a problem for agriculture. This study aimed to evaluate whether humus functions as a chromium stabilizing agent when using tannery sludge in the substrate of conilon coffee seedlings (*Coffea canephora*) and to determine what effects there are on leaf development and anatomy. Treatments consisted of a fixed dose of tannery sludge (30% of volume) on substrates of conilon coffee seedlings with different proportions of humus and subsoil (T-10, T-20, T-30, T-40% of humus). Information for the evaluation of leaf anatomy and seedling development was collected at 180 days after the planting of cuttings. From the phenological point of view, the treatments that best promoted seedling quality were T-30 and T-40. However, the greater the amount of humus in the substrate the greater the absorption of chromium by plants, which directly affected the organization of epidermal cells and leaf mesophyll. In addition, intense cytoplasmic degradation, ultrastructural changes in chloroplasts and mitochondria, and an increase in autophagic vacuoles were observed. We conclude that increasing the amount of humus in substrate with tannery sludge provides higher quality coffee seedlings, despite promoting greater absorption of chromium by plants and the consequent major intracellular disturbances.

Keywords: leaf anatomy; chromium; oxidative stress; plant nutrition; *Coffea canephora*.

INTRODUCTION

The large volume of agricultural business globally and in Brazil is directly reflected in other sectors, such as the leather market, which generate much foreign exchange for countries; however, they also generate a significant amount of processing waste (Cavallet & Selbach, 2008). Waste from leather processing, called tannery sludge, has been the focus of many studies into its use in agriculture because its composition is rich in nitrogen and organic matter and has high soil reducing power, giving it great potential for agricultural use (Sales *et al.,* 2017; Berilli *et al.,* 2018; Berilli *et al.,* 2020). However, large amounts of sodium and chromium are added during leather processing, which produces a potentially polluting waste for ecosystems with the bioaccumulation of chromium in organisms and soil (Gorni *et al.,* 2014; Berilli *et al.,* 2015).

Many studies have been revealing the effects of the accumulation of heavy metals in tissues and their consequences for plant anatomy and development (Jamal *et al.*, 2013). Depending on the element involved and the tissue concentrations, disorders range from cell disorganization, cell death, compromising physiology, development and productivity of plants (Panda & Choudhury, 2005; Silva *et al.,* 2014).

Studies on conilon coffee seedlings reported that the

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use of tannery sludge in association with pure soil was not efficient at promoting plant development and produced seedlings with quality below that of conventional seedlings (Berilli *et al.,* 2015; Berilli *et al.,* 2018). These authors also observed that when grown on substrates with tannery sludge, conilon coffee seedlings accumulated chromium in root, stem and leaves, which represents the probable cause of low-quality seedlings. Therefore, in order to make use of tannery sludge as an alternative source of fertilization for plants, other components that act in the stabilization, structuring and complexation of chromium need to be added to seedling substrate.

Humus can be a good alternative for stabilizing unwanted elements in tannery sludge, such as chromium, because its particles have great physical-chemical and biological activities (Primo *et al.,* 2011; Souza & Santana, 2014). However, the concentration of humus and the pH of the medium can positively or negatively influence the stabilization of heavy metals. Kerndorff & Schnitze (1980) demonstrated that, in a medium containing 250 mg of humus, the absorption of chromium increases significantly with an increase in pH from 2.4 to 5.8. In this sense, the efficiency of humus as a chromium stabilizing agent depends on its concentration and mainly on the pH of the medium.

In addition, the capacity to change the valence of chromium from trivalent (Cr^{3+}) to hexavalent (Cr^{6+}) is another characteristic that needs to be taken into account during the administration of humus with tannery sludge. The Cr^{6+} form is more mobile and toxic and can accumulate in greater amounts in the aerial part of plants (Panda and Choudhury 2005), generating severe oxidative stress in leaf mesophyll cells (Singh *et al.,* 2015; Berilli *et al.,* 2018). Oxidative stress induced by chromium can cause disorganization and plasmolysis of epidermal cells and leaf mesophyll (Sridhar *et al.,* 2005); reduce the number of cells in the palisade and spongy parenchyma (Panda & Choudhury, 2005); cause severe cytoplasmic degradation and ultrastructural changes in membrane systems of chloroplasts and mitochondria (Berilli *et al.,* 2018); increase the number of vacuoles in cells (Han *et al.,* 2004) and induce cell death (Mahalakshmi *et al.,* 2020).

Seedlings of the species *Coffea canephora*, or conilon coffee, were chose as subjects to better understand the effects of chromium in association with humus due to the great importance of this species in some countries with large tanneries, such as Brazil. Although humus is widely used in plant propagation, there have been no studies on its efficiency as a stabilizing agent of chromium in tannery sludge. Therefore, the present study aimed to evaluate whether the association of humus and tannery sludge in the substrate is beneficial for the cultivation of conilon coffee seedlings. However, due to the complexity involving humus ionization potential and chromium valence instability, some questions were raised: (1) Does humus really have the capacity to make chromium in tannery sludge unavailable? (2) Does the use of humus in association with tannery sludge positively affect the development of conilon coffee seedlings compared to conventional substrate? (3) If humus does make chromium unavailable, will the plants show signs of oxidative stress induced by chromium?

MATERIAL AND METHODS

Experiment implantation

This study was conducted at the Federal Institute of Education, Science and Technology of Espírito Santo – Itapina campus, located in the municipality of Colatina, northwestern Espírito Santo State, within the geographic coordinates 19° 32’22” south latitude; 40° 37’50” west longitude and altitude of 71 meters. The climate in the region is Tropical Aw, according to the Köppen’s climate classification, with average minimum and maximum temperatures in the region of 19 and 31 ºC and with a well-defined rainy season from October to January and an average climatological rainfall of 1029.9 mm (Alvares *et al.*, 2013; Sales *et al.*, 2018a).

The experiment was carried out in a propagation nursery for irrigated conilon coffee seedlings, in a randomized block design, containing 6 treatments with different concentrations and mixtures of substrates, 12 blocks, with the portion of each treatment in the block consisting of 17 plants, accounting for 102 seedlings per block and a total of 1224 seedlings in the experiment.

The treatments consisted of four different concentrations of the mixture of humus, dehydrated tannery sludge and subsoil; subsoil alone; and subsoil with conventional fertilization, according to Table 1. The chemical characteristics of the subsoil used for the substrate mixtures with tannery sludge treatments and conventional treatment are described in Table 2.

The conventional substrate used in the composition of the treatments was a mixture recommended by the Capixaba Institute of Research, Technical Assistance and Rural Extension (INCAPER), for the production of quality Conilon coffee seedlings: for each cubic meter prepared (m^3), the substrate showed in its composition 75% of sieved subsoil earth; 25% bovine manure; 1.5 kg of dolomitic limestone; 5.0 kg of simple superphosphate and 0.5 kg of potassium chloride (Ferrão *et al.*, 2012).

The dehydrated tannery sludge was supplied by the company Capixaba Couros LTDA ME, located in Baixo Guandu, Espírito Santo, with a moisture content of 13.8% (dry basis). The humus used in this study was produced from manure from cattle raised in a confinement system. The characteristics of the dehydrated sludge and humus used in the experiment are shown in Tables 3 and 4.

Table 1: Description of the evaluated treatments and their respective components

Treatment	Substrate component
T-C	100% conventional substrate
T-10	10% humus + 30% dehydrated tannery sludge + 60% subsoil
T-20	20% humus + 30% dehydrated tannery sludge + 50% subsoil
T-30	30% humus + 30% dehydrated tannery sludge + 40% subsoil
T-40	40% humus + 30% dehydrated tannery sludge + 30% subsoil
T-S	100% subsoil

Table 2: Chemical characteristics of the subsoil used as a component of seedling substrate

pH	O.M.	P	K	Ca	Mg	Al	Na	S.B.	T	t	m	V	Fe	Cu	Zn	Mn
	-------g/dm^{-3}-------			------------cmol$_c$/dm^{-3}-----------							---%---		------mg/dm^{-3}------			
5	8.1	0.005	0.048	0.8	1.3	0	0.03	2.3	3.1	2.3	0	74	7	0.6	0.8	7.9

Note: potential of hydrogen (pH); organic matter (O.M.); phosphorus (P); potassium (K); calcium (Ca); magnesium (Mg); aluminum (Al); sodium (Na); S.B.: sum of bases; T: cation exchange capacity; t: cation exchange capacity effective; m: aluminum saturation; V: base saturation; Iron (Fe); copper (Cu); zinc (Zn); manganese (Mn).

Table 3: Characteristics of dehydrated tannery sludge used in seedling substrate

pH	O.M.	N	P	K	Ca	Mg	E.C.	Fe	Cu	Zn	Mn
	---------- g/dm^{-3}---------				-cmol$_c$/dm^{-3}-		dS/m^{-1}	----------- mg/dm^{-3}-----------			
12.30	305	3.7	2	0.8	2.70	0.1	17.30	57	1	1	1

Note: potential of hydrogen (pH); organic matter (O.M.); nitrogen (N); phosphorus (P); potassium (K); calcium (Ca); magnesium (Mg); E.C.: electrical conductivity; iron (Fe); copper (Cu); zinc (Zn); manganese (Mn).

Table 4: Chemical characteristics of humus used in seedling substrate

pH	O.M	P	K	Ca	Mg	Fe	Cu	Zn	Mn	m	V
	---------g/dm^{-3}--------			--cmol$_c$/dm^{-3}--		------------mg/dm^{-3}-----------				-----%----	
6.8	202	0.09	0.21	93.1	146	57	1	1	1	1.9	82

Note: potential of hydrogen (pH); organic matter (O.M.); phosphorus (P); potassium (K); calcium (Ca); magnesium (Mg); iron (Fe); copper (Cu); zinc (Zn); manganese (Mn). m: aluminum saturation; V: base saturation.

The species used in this experiment was *Coffea canephora*, also known as conilon coffee. Seedlings were produced using cloning techniques with cuttings obtained from adult tissue of orthotropic branches, which were removed from crops with good phytosanitary and nutritional aspects (Clone 8 of the Vitória cultivar). After removing the branches from the mother plants, they were sent to the greenhouse and 30 cm were removed from the ends of the orthotropic branches. The cuttings were then standardized to 6 to 8 inches in height with leaves with 1/3 of the leaf blade, plagiotropic branches and above the insertion of the pair of leaves with 1 cm. The cuttings were planted in 11 x 20-cm polyethylene bags, with capacity of 500 ml, filled 30 days previous with substrate. Irrigations were performed daily throughout the experiment by micro-sprinkler such that the field capacity of the substrates was maintained.

Evaluation of seedling development and chromium quantification

At 180 days after the planting of the cuttings, the number of leaves, seedling height and stem and canopy diameters (digital caliper) were measured and gravimetric analyses, including fresh and dry mass of the aerial part and roots, were performed with the aid of a forced circulation oven at 70 ˚C until reaching constant weight. Only new shoots, and excluding original cuttings, were considered for determining fresh and dry mass of the aerial part. After drying in an oven, tissue samples were ground in a Willey knife mill equipped with a 2 mm diameter mesh opening sieve. Chromium analysis was performed following the method proposed by Nogueira *et al.* (2008), where the tannery sludge samples were submitted to wet digestion with $HNO_3 + HClO_4 + H_2O_2$ in a water bath at 150 °C. Then, the material was filtered through quantitative filter paper (Inlab-type 30), and the extract resulting from the digestion was collected for subsequent chromium quantification. The determination of chromium in the samples was performed using an atomic absorption spectrophotometer (Avanta Sigma, GBC Scientific Equipment), with a wavelength = 357.9 and acetylene-nitrous oxide flame. The Dickson seed quality index (DQI) proposed by Dickson *et al.* (1960), which takes into account gravimetric and developmental characteristics, was also calculated. All analyzes were performed in triplicate.

Anatomical and ultrastructural analyzes

Anatomical analyses were performed using fragments of the middle third of the leaf blade, which were fixed in 2.5% aqueous glutaraldehyde and 0.05 M sodium cacodylate buffer (Karnovsky, 1965 modified by Da Cunha *et al.,* 2000), and then post-fixed in 1% osmium tetroxide and 0.05 M sodium cacodylate buffer for 2h at room temperature. The samples were dehydrated in an increasing acetone series and infiltrated and soaked in epoxy resin (Epon®) at 60 ºC in an oven. Semi-thin sections of approximately 70 µm were obtained with the aid of an ultramicrotome (Reichert Ultracuts Leica Instruments®) using a diamond knife (Diatome®). The sections were then stained with 1% toluidine and 1% Borax buffer (Johansen, 1940), and observed under a light microscope (Axioplan, ZEISS, Germany) coupled to an image capture system (Moticam Pro 282B, Hong Kong). Ultrastructural analyses used the same blocks as used for the anatomical analyses, however, ultra-thin sections of approximately 0.7 nm were obtained and collected in 300-mesh copper grids. The samples were then contrasted with 5% lead citrate and 1% uranyl acetate and subsequently observed with a transmission electron microscope (JEM 1400 Plus, JEOL, Japan) at a voltage of 80 Kv.

Statistical analysis

All quantitative analyses were submitted to the F test and applied to Dunnett's means test ($p < 0.05$); for chromium level in tissues, the degrees of freedom for treatments were deployed via regression analysis. The regression was selected according to the level of significance (R^2). Statistical analyses were performed using the program Assistat (version 7.7), and the graph was generated using the program Origin (version 9.0 Professional – Academic).

RESULTS AND DISCUSSION

Seedling development

All developmental characteristics of the plants differed between treatments with tannery sludge or pure soil and the conventional treatment. In general, plants developed in pure soil, that is without the addition of fertilizers, humus or tannery sludge, had inferior development of most of the evaluated characteristics (Table 5 and 6).

Conventional substrate (T-C) had the highest average values for seedling height, although treatments T-20, T-30 and T-40 did not differ significantly from T-C. On the other hand, treatments T-10 and T-S had significantly lower values than T-C (Table 5), indicating that this would be the minimum limit for achieving seedlings with height standards similar to conventional treatment. Leaf number and canopy diameter did not differ significantly between treatments with different proportions of humus and T-C. However, leaf number and canopy diameter were significantly lower for T-S than for T-C (Table 5). Treatments that had lower average values for stem diameter compared to T-C were those with the highest (T-40) and lowest (T-10) proportions of humus in the substrate, in addition to seedlings developed on pure soil (T-S) (Table 5).

Table 5: Mean ± standard deviation of height, leaf number, canopy diameter and stem diameter of conilon coffee seedlings grown on conventional substrate with different concentrations of humus associated with dehydrated tannery sludge at 180 days after the planting of cuttings

Treatment	Seedling height (cm)	Leaf number	Canopy diameter (cm)	Stem diameter (mm)
T-C	114.49 ± 34.59	6.9 ± 1.84	196.2 ± 25.08	3.01 ± 0.27
T-10	86.15 ± 31.18*	6.0 ± 1.15	168.9 ± 23.27	2.66 ± 0.23*
T-20	101.4 ± 24.47	6.7 ± 1.03	191.5 ± 11.52	2.89 ± 0.17
T-30	111.6 ± 41.97	6.7 ± 1.31	192.0 ± 27.77	2.88 ± 0.26
T-40	93.08 ± 42.38	6.3 ± 1.70	189.1 ± 27.69	2.78 ± 0.30*
T-S	45.5 ± 14.10*	4.5 ± 0.94*	142.4 ± 50.79*	2.42 ± 0.14*

*differs significantly from conventional substrate (T-C) according to Dunnett's test at 5% probability.

Table 6: Mean ± standard deviation of total fresh mass (TFM), total dry mass (TDM), Dickson quality index (DQI) of conilon coffee seedlings grown on conventional substrate and with different concentrations of humus in association with dehydrated tannery sludge at 180 days after the planting of cuttings

Treatment	TFM (g)	TDM (g)	DQI
T-C	6.85 ± 2.59	2.18 ± 0.89	0.055 ± 0.03
T-10	8.11 ± 2.19	2.48 ± 0.58	0.072 ± 0.01
T-20	7.18 ± 0.92	2.07 ± 0.25	0.057 ± 0.01
T-30	9.76 ± 1.27*	3.48 ± 1.13*	0.094 ± 0.05*
T-40	9.60 ± 3.76*	2.99 ± 1.25	0.090 ± 0.04*
T-S	2.96 ± 1.61*	0.98 ± 0.55*	0.048 ± 0.03

*differs significantly from conventional substrate (T-C) according to Dunnett's test at 5% probability.

The results presented in Table 5 reveal that humus, between the proportions of 20 to 30%, associated with tannery sludge enriches the substrate and favors the development of seedlings. The effect of these mixtures of humus and tannery sludge did not affect seedling development. This fact corroborates other authors who used tannery sludge in association with humus for the propagation of other species, such as *Passiflora edulis*, where proportions of 5 to 50% of humus did not affect the quality of passion fruit seedlings (Sales *et al.,* 2018b).

For total fresh mass (TFM) and total dry mass (TDM), treatments T-30 and T-40 had higher values than did T-C (Table 6). This was directly reflected in the quality of the seedlings since T-30 and T-40 had higher DQI values than did T-C (Table 6). The better seedling quality in T-30 and T-40 is directly related to the concomitant increase in total dry mass, stem diameter and canopy diameter, which are variables that are considered in the calculation of DQI (Dickson *et al.,* 1960). Some authors have demonstrated that increases in dry mass, canopy area and stem diameter are allometric characteristics of plant development that are dependent on the allocation of nitrogen, phosphorus and potassium (Cai *et al.,* 2017; Dubey *et al.,* 2017; Pireda *et al.,* 2019). Therefore, it is possible to infer that the combination of 30% or 40% humus with tannery sludge provides the necessary nutrients for the development of conilon coffee seedlings with better quality.

The quality gain of seedlings in T-30 and T-40 demonstrates that the composition of the substrates enriched with 30% tannery sludge and 30% or 40% humus are superior to conventional treatments and/or pure soil. Berilli *et al.* (2014) found that dehydrated tannery sludge added to pure soil did not result in good development of conilon coffee seedlings, with characteristics of mass, number of leaves, stem diameter, canopy diameter and height always lower than seedlings grown in conventional substrate. This finding reveals that the addition of humus to the substrate, in association with the tannery sludge and the soil, is a viable option for stabilizing the substrate and promoting seedlings with similar or superior development to seedlings grown on conventional substrates, without the addition of chemical fertilizers to the substrate.

These results indicate that the combination of tannery sludge and humus provided an increase in the growth and quality of conilon coffee seedlings when compared to pure soil. This fact becomes predictable due to the greater availability of essential nutrients in the plants present in the tannery sludge and in the humus, while also having the benefit of a better structuring of the substrate with the presence of the humus. In addition, some authors report that humus can act as a structuring and stabilizing agent for some unwanted components present in the tannery sludge, such as chromium present in tannery sludge (Primo *et al.,* 2011; Tavares *et al.,* 2013; Souza & Santana, 2014).

Chromium absorption by plants

In order to evaluate whether humus can act as a stabilizing agent of the chromium present in tannery sludge, the chromium concentration in tissues of the aerial part of the conilon coffee seedlings was analyzed. The regression analysis of the results revealed a strong trend ($R^2 = 0.92$) between the increase in the humus concentration in the substrate and the chromium concentration in the aerial part of the seedlings (Figure 1). These results reveal that the humus, despite promoting better development of seedlings, also promotes an increase in the accumulation of chromium in the tissues of their aerial part. Therefore, it is possible to affirm that the humus had an effect opposite to what was expected — instead of acting on the stabilization of chromium, it acted in favoring the absorption of chromium by the plant. This may have occurred due to the mixture of the basic (pH = 12.30) tannery sludge with the acidic (pH = 6.8) humus. This mixture may have changed the chromium valence from trivalent (Cr^{3+}), mainly present in the tannery sludge, to hexavalent (Cr^{6+}). The Cr^{6+} can take the form of chromate or dichromate, making it more soluble in water (Medda & Mondal, 2017), which favors its mobility throughout the plant body via xylem (Panda & Choudhury, 2005). This process can help us understand the synchronous increase in the concentration of humus in the substrate and the concentration of chromium in the aerial part of the conilon coffee seedlings.

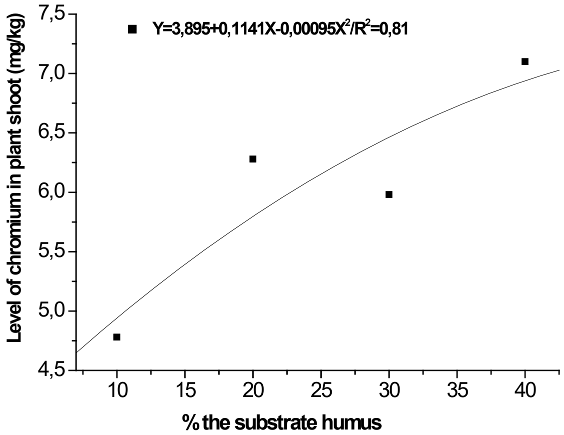

Figure 1: Regression analysis of the concentration of chromium accumulated in the tissues of the aerial part of conilon coffee seedlings as a function of the percentage of humus present in the substrate.

Anatomical and ultrastructural aspects of leaves

Due to the increased concentration of chromium in the aerial part of the seedlings, anatomical and ultrastructural analyses of the leaves were carried out to identify possible signs indicative of oxidative stress mediated by chromium.

The anatomical analyses of the leaves found that treatments T-10 and T-20 had smaller epidermal cells and with greater sinuosity of the anticlinal walls in relation to T-C (Figure 2 A, B and C). According to Sridhar *et al.* (2011), chromium can induce the shrinkage of epidermal cells, elucidating the reduction in size and the greater sinuosity of the epidermal cell wall in T-10 and T-20. However, shrinkage of epidermal cells was not seen in T-30 and T-40, which had larger epidermal cells with slightly thicker cuticles compared to T-C (Figure 2 A, D and E). These changes in anatomy found for leaves of T-30 and T-40 may reflect an alternative response of the plants to water stress conditions induced by the high concentrations of chromium and sodium present in the tannery sludge. Gomes *et al.* (2011) demonstrated that the greater accumulation of metal in leaves of *Brachiaria decumbens* can induce thickening of the epidermis and minimize water loss through evapotranspiration. Additionally, the greater thickening of the cuticle in T-40 may be related to the excessive accumulation of chromium in the aerial part of these plants. The cuticle consists of negatively charge molecules, such as pectin and cutin, which can act as cationic exchangers and establish covalent bonds with chromium (Greger, 1999). Therefore, chromium may be unavailable through association with the cuticle, leading to cuticle hardening and thickening (Krzesłowska, 2011; Sujkowska-Rybkowska *et al.*, 2020).

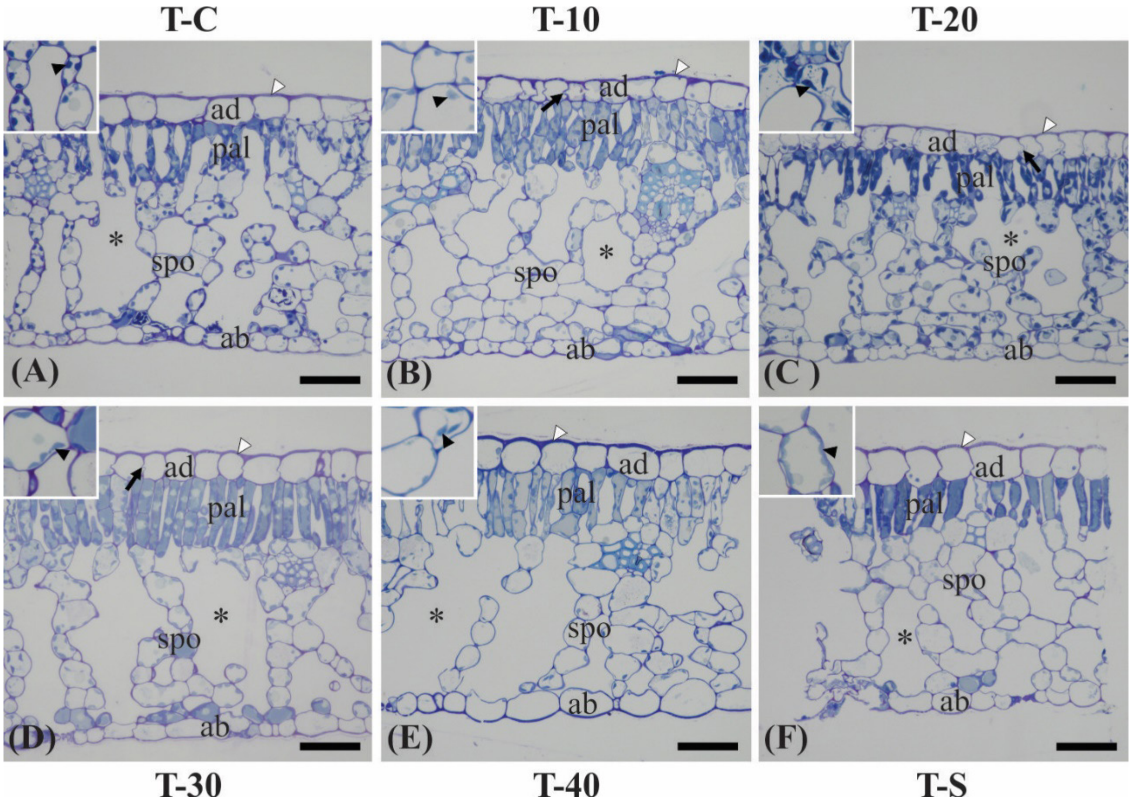

Figure 2: Leaf anatomy of conilon coffee seedlings grown on conventional substrate and with different concentrations of humus in association with dehydrated tannery sludge. A – F: Cross section of leaves observed through optical microscopy. Inserts highlight the distribution of chloroplasts in the cells of the spongy parenchyma. ad – adaxial epidermis; ab – abaxial epidermis; pal – palisade parenchyma; spo – spongy parenchyma; arrow – indicating sinuosity of anticlinal wall of epidermal cell; black arrowhead – indicating chloroplasts; white arrowhead – indicating cuticle; asterisk – indicating the intercellular spaces of the spongy parenchyma. Bars: A – F: 50 µm.

The leaves of the coffee seedlings showed disorganized palisade parenchyma with signs of plasmolysis in all treatments except for T-S (Figure 2 A – F). The spongy parenchyma had wider intercellular spaces in T-30 and T-40 when compared to the other treatments (Figure 2 D and E). Berilli *et al.* (2018) demonstrated similar changes in the palisade and spongy parenchyma in conilon coffee plants treated with tannery sludge and attributed these changes to high concentrations of chromium in the tannery sludge. Additionally, the organization and shape of the chloroplasts underwent severe changes in the humus treatments compared to the control treatments (T-C and T-S). The chloroplasts in treatments T-C and T-S were arranged along the cell wall and were oval to elliptical in shape (Figure 2 A and F), whereas in the humus treatments they were located away from the cell wall and were amorphous in shape (Figure 2 B – E). These results indicate that treatment with humus may be inducing autophagy of chloroplasts, since they mobilize more chromium for the leaves. Excess chromium can cause irreversible damage to a chloroplast and induce chlorophagia so that the selective degradation and nitrogen remobilization of damaged chloroplasts occurs (Liu *et al.,* 2018; Signorelli *et al.,* 2019), which helps to explain the amorphous shape and the displacement of chloroplasts to the central region of cells in plants treated with humus.

Transmission electron microscopy analyses were performed to identify ultrastructural changes in cells that characterize oxidative stress and chromium-induced autophagy. Treatments T-C and T-S showed intact cells with a central vacuole occupying most of the cell, cytoplasm in the peripheral region of the cell, and associations between distributed chloroplasts and mitochondria distributed along the extension of the cell wall (Figure 3 A and F). However, cells in treatments with humus and tannery sludge showed a process of progressive degradation with increasing chromium concentration in the substrate. In treatments T-10 and T-20, it was possible to identify cytoplasm with a granular aspect and the presence of microvacuoles and drops of oil (Figure 3 B and C). Cellular degradation was intensified in treatments T-30 and T-40, with an advanced stage of cytoplasmic degradation with the formation of autophagic vacuoles and cytoplasmic remains containing damaged chloroplasts inside vacuoles (Figure 3 D and E). The presence of granular cytoplasm, autophagic vacuoles and cytoplasmic remains inside the vacuole are indicative of severe oxidative stress (Bassham *et al.,* 2007; Liu *et al.,* 2020). Thus, these results demonstrate that the increase in chromium in the aerial part of the coffee seedlings may have induced oxidative stress and autophagy in leaf mesophyll cells.

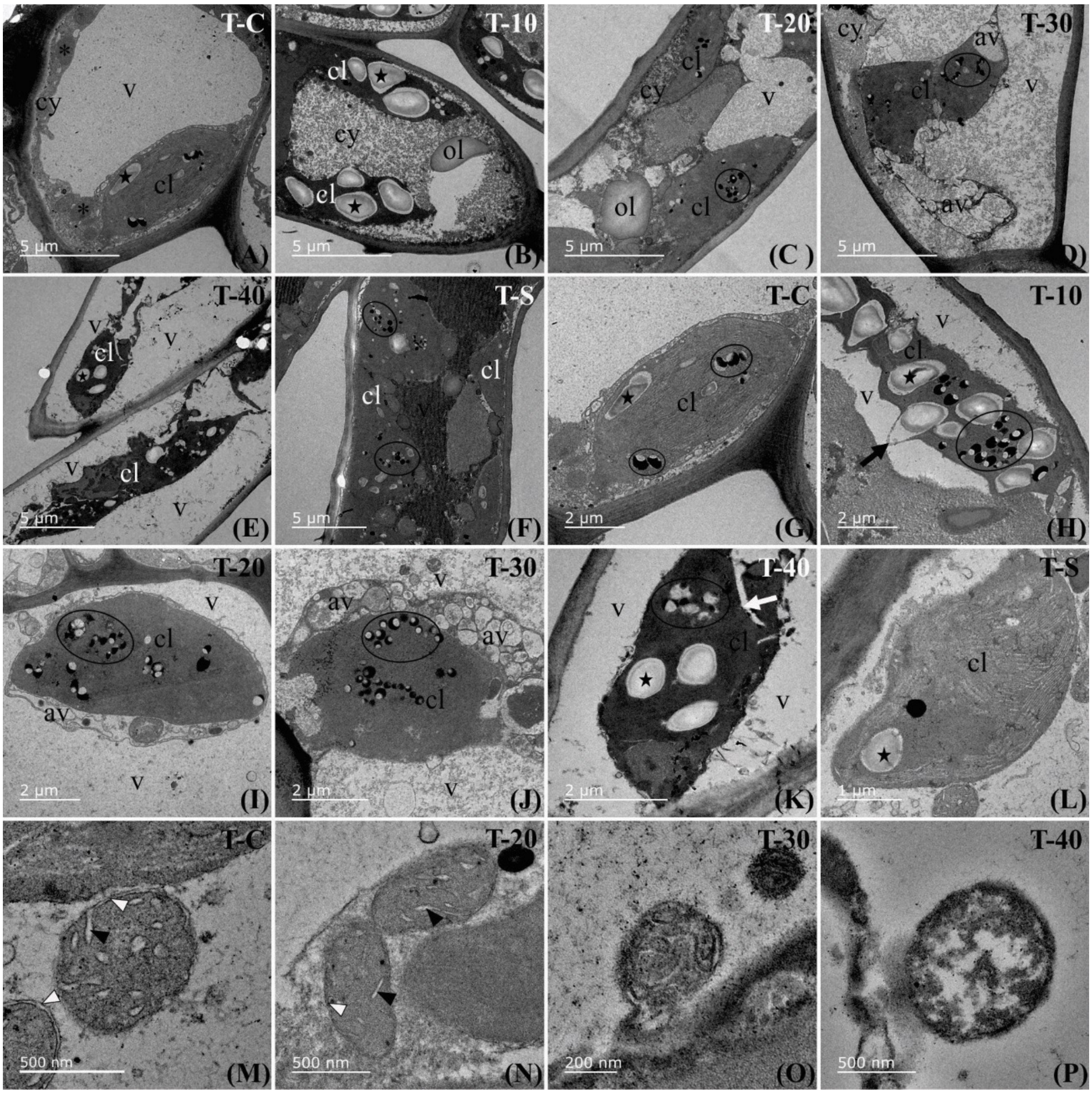

Figure 3: Ultrastructural aspects of leaves of conilon coffee seedlings grown on conventional substate and substrate with different concentrations of humus in association with dehydrated tannery sludge. A – F: Overview of leaf mesophyll cells. G – L: Detail of chloroplasts in leaf mesophyll cells. M – P: Detail of mitochondria in leaf mesophyll cells foliar. cl – chloroplast; v- vacuole; cy – cytoplasm; ol – drops of oil; av – autophagic vacuole; asterisk – mitochondria; star – starch grain in the chloroplast; ellipse – highlighting plastoglobules in chloroplasts; arrow – indicating rupture of the outer membrane of the chloroplasts; black arrowhead – indicating mitochondrial cristae; white arrowhead – indicating double membrane of mitochondria.

The chloroplasts in treatments T-C and T-S presented an elliptical shape, with intact thylakoid membranes and few plastoglobules, ultrastructural characteristics considered normal for chloroplasts (Figure 3 G and L). However, in treatments with humus and tannery sludge, it was possible to observe amorphous-shaped chloroplasts with ruptured external membranes, electrodense stroma, increased amounts of starch grains and plastoglobules, and the presence of autophagic vacuoles on the periphery of chloroplasts (Figure 3 H – K). In addition, in T-40, the damaged chloroplasts were translocated into the vacuoles (Figure 3 E and K). Mitochondria also showed signs of oxidative stress. Mitochondria of treatment T-C presented double intact membranes and prominent mitochondrial cristae (Figure 3M). A slight disorganization of the mitochondrial cristae was observed in T-20 (Figure 3N), however, the mitochondria for T-30 and T-40 exhibited an advanced stage of degradation of their membrane systems (Figure 3 O and P).

Because they are energetic organelles, chloroplasts and mitochondria are naturally sites of reactive oxygen species (ROS) production. Therefore, under conditions of amplified ROS production, such as heavy metal stress, these organelles are expected to suffer the most from oxidative stress (Choudhury & Panda, 2005; Keunen *et al.,* 2011). Panda (2007) demonstrated that high concentrations of chromium increase the production of hydrogen peroxide (H_2O_2) and superoxide radicals (O^-_2), inducing lipid peroxidation in rice root cells. This helps to explain the intense degradation of the membranes of chloroplasts and mitochondria in T-30 and T-40. In addition to structural changes, it is possible to observe metabolic changes in chloroplasts of humus treatments compare to control treatments. The increase in the amount of starch grains in chloroplasts indicates that there may have been a decoupling between the synthesis of carbohydrate and triose-phosphate (Sharkey, 2019), and the greater presence of plastoglobules represents a targeting of resources for the synthesis of secondary metabolites with antioxidant properties (Bréhélin & Kessler, 2008; Piller *et al.,* 2014).

CONCLUSION

Although the use of humus in association with tannery sludge contributed to the development and quality of conilon coffee seedlings, humus did not have a positive effect on the stabilization and neutralization of chromium, as was initially expected. Humus presented an inverse effect, favoring greater absorption and accumulation of chromium in the aerial part of the plants. The results showed that chromium in the aerial part of seedlings increased synchronously with the increased humus in the substrate. At the cellular level, the greater accumulation of chromium in the aerial part of seedlings treated with humus induced severe signs of oxidative stress and autophagy in leaf mesophyll cells, which was not observed for the control treatments.

In this sense, the use of humus in association with tannery sludge in the substrate of coffee seedlings brings an intriguing discussion about the use of this waste in the propagation of plants, since the association is efficient for the growth and quality of seedlings, while at the same time causes great anatomical disturbance and cellular stress due to increased chromium in tissues. However, it is premature to state that the combination of humus and tannery sludge is inefficient for the development and productivity of conilon coffee. One way to reveal whether the damage at the cellular level induced by chromium could compromise the adult plant would be to carry out planting in the field and assess the phenological and anatomical status of adult plants, which remains a topic for future studies.

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