Liming applications and the SPAD chlorophyll index and stomatal conductance in cocoa exposed to cadmium in the soil

Aplicaciones encalántes y el índice de clorofilas SPAD y la conductancia estomática de cacao expuesto a cadmio en el suelo

ABSTRACT

Cadmium is a heavy metal that affects cell structures, such as walls and membranes, especially in the photosynthetic apparatus (PSII), chlorophylls, chloroplasts and stomata, producing losses in production quantity and quality. In addition, it is harmful to the health of humans and animals. The objective was to analyze the behavior of the relative chlorophyll index (SPAD units) and stomatal conductance in clone CCN-51 cacao plants every 45 days (45, 90, 135 and 180 days) after liming application. Four doses of a dolomite + agricultural gypsum mixture were applied, increasing Ca\(^{2+}\) saturation in the soil to 7, 8 and 9 cmol kg\(^{-1}\). The control treatment did not have applications. The results indicated a reduction in SPAD units in the plants without liming, with high cadmium levels in the soil (3.3 mg kg\(^{-1}\)), and there were no statistical differences in the other treatments, possibly because of edaphic factors such as pH, organic matter content and Al\(^{3+}\). The best stomatal conductance was observed with 7 cmol kg\(^{-1}\) in the foliar gas exchange. Supersaturated liming applications efficiently reduce the losses in quality and quantity caused by the accumulation of cadmium in cacao plants.

Additional key words: heavy metals; soil amendments; transpiration; pigments; *Theobroma cacao* L.


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Cadmium (Cd) is a heavy element found on the surface of the Earth’s crust at low concentrations in the divalent form (Cd$^{2+}$), with high mobility in the soil and in plants (Santos et al., 2020). Currently, cadmium concentrations in agricultural soils are high and present an environmental problem (Meter et al., 2019). Notably, some crops are bioaccumulators, creating a threat to human and animal health (Li et al., 2022a).

Cadmium accumulates in soil in various ways, most related to anthropogenic activities (Argüello et al., 2018). Per European regulations, the maximum permitted concentration of cadmium in fruits is 0.05 mg kg$^{-1}$ of fresh weight, a factor for the Latin American market given the current concentrations in the soil and cocoa beans (European Commission, 2021). For the United States, the permissible range is 0.8 to 1.2 mg kg$^{-1}$; however, high levels in the cocoa market are concerning (Vanderschueren et al., 2021).

In Latin America, cocoa-producing areas have exceptionally high concentrations that are associated with fertilizer applications with high concentrations of cadmium (0.22-10.8 mg kg$^{-1}$) and suitable geological traits, especially in young soils, such as Entisols and Inceptisols (Ramtahal et al., 2019; Rodríguez et al., 2019; Vanderschueren et al., 2021).

Once cadmium is in the soil solution, it passes through the cells of the cortical root and moves the apoplastic or symplastic xylem route to the upper parts of the plant, with adverse effects on cell stability that result in oxidative burst and altered cellular functions that reduce photosynthesis and alter concentrations of mineral nutrients, energy transduction, protein synthesis and gas exchange (Castro et al., 2015; Barraza et al., 2017; Kapoor et al., 2021).

The accumulation of cadmium (Cd$^{2+}$) alters the structure of the chloroplast, inhibits the electron transport chain, and decreases the chlorophyll content and photosynthetic rate (Huo et al., 2022). According to Bayona-Penagos (2020) and Sánchez-Zepeda et al. (2021), these effects result from the replacement of Mg$^{2+}$ in chlorophylls $a$ and $b$, and competition in the entry of Ca$^{2+}$ in the protective cells, causing poor stomatal closure, limiting the establishment of intercellular spaces in the leaves, inducing fusion of membranes and the cell wall, and increasing the size and quantity of starch grains, plastoglobules and lipids in the chloroplasts (Rabêlo et al., 2022).

Cadmium (Cd$^{2+}$) is usually found with sulfur (S) at high concentrations in cytoplasmic enzymes that maintain a reduced state through a constant supply of metabolic energy, causing oxidative stress,
which damages cells in structures responsible for gas exchange (Herrera, 2000; Qin et al., 2020). There is also an increase in stomatal resistance to CO₂ intake, toxic effects initiated at the root where cadmium interferes with the absorption and translocation of water (Moreno et al., 2013). However, Fernández (2022) noted the lack of knowledge on how cadmium affects gas exchange processes.

Mitigation techniques for cadmium in tropical cacao soils include applications of lime, which involve calcium and magnesium, to improve the soil exchange complex and benefit plant nutrition, where calcium shares many chemical properties with cadmium, meaning they compete for absorption by roots (Meter et al., 2019).

In Latin American soils, the effectiveness of calcium supersaturation has been proven through calcareous amendments such as dolomite and gypsum, generating an oversupply of Ca²⁺ and Mg²⁺ cations that, through a combined effect, neutralize acidity interchangeable and contribute to the soil nutrition, facilitating more efficient absorption of nutritional elements with biochemical functions by plants that improve yield, where cell structures affected by Cd will gradually recover (molecules of chlorophyll and other structures associated with calcium and magnesium) (Castro et al., 2015; Suárez-Salazar et al., 2017; Huang et al., 2020; Zhang et al., 2020; Huo et al., 2022). Successful cases of Cd mitigation were reported by Tantalean and Huauya (2017), Wong (2017), Pérez et al. (2019), Florida et al. (2019) and Huaraca-Fernandez et al. (2020), where Cd decreased in the cacao zone of central Colombia (Rodríguez et al., 2019).

The CCN-51 cacao clone is one of the more commercial, with the highest production and planted area in Latin America (Espinoza, 2019). The objective was to evaluate the application of gypsum and dolomite lime as liming agents to determine the behavior of the relative chlorophyll index (SPAD units) and stomatal conductance in CCN-51 cacao clone exposed to cadmium in western Boyaca (Colombia). Increasing the concentration of calcium in the soil displaces the exchangeable aluminum (5 meq mol kg⁻¹ of soil), where the oversupply of calcium means plants absorb it efficiently, and adsorbed cadmium decreases, along with its negative impacts on cellular tissues.

**MATERIALS AND METHODS**

This experiment was carried out on the Mata de Limón farm in the Palenque village of the municipality of Otanche (5°39’28” N and 74°10’50” W), in the western province of the Department of Boyacá (Colombia), with a 6-year-old cacao crop (clone CCN-51) at 1,100 m a.s.l., monthly average rainfall of 264.55 mm, monthly relative humidity of 80%, and monthly average environmental temperature of 24.03°C, a Tropical Humid Forest life zone according to the Holdridge scale (Motta-Delgado and Ocaña-Martínez, 2018).

A completely randomized design with four treatments was carried out and replicated three times. The treatments corresponded to the mixture of calcareous amendments, dolomite (Do) and gypsum (y) at 7, 8 and 9 cmolc kg⁻¹ of Ca²⁺ in the soil. The control did not have applications of calcareous amendments. The concentrations of the liming material were calculated using equation (1) of Cochrane et al. (1980) and the information of soil analysis (Tab. 1).

\[
\text{Lime required} \quad \text{(CaCO}_3 \text{ equivalent, t ha}^{-1}) = 1.5 \left( \frac{[\text{Al-RAS}] \times \text{ECFC}}{100} \right)
\]

Table 1. Physicochemical analyses of the soil before applications of the amendment.

<table>
<thead>
<tr>
<th>Texture</th>
<th>Sand (%)</th>
<th>Lime (%)</th>
<th>Clay (%)</th>
<th>pH</th>
<th>OM (%)</th>
<th>EA</th>
<th>Al³⁺</th>
<th>H⁺</th>
<th>Cd (cmol kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clayey</td>
<td>29</td>
<td>24</td>
<td>47</td>
<td>4.75</td>
<td>2.49</td>
<td>5.60</td>
<td>5.0</td>
<td>0.60</td>
<td>3.3</td>
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<tr>
<td>Ca²⁺</td>
<td></td>
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<td>Mg²⁺</td>
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<td>10.23</td>
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<td>Fe</td>
<td></td>
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<td>131.86</td>
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<td>Mn</td>
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<td>2.20</td>
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<td>Zn</td>
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<td>1.53</td>
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<td>B</td>
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<td>0.13</td>
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</tbody>
</table>

Texture (Bouyucos), pH (1:1 ratio), OM-organic material (Walkey-Black), EA-exchangeable acidity [Al³⁺ + H⁺] (KCl 1N), Cd (P-PAS-21 procedure for the determination of cadmium in soils [NTC 3888 – Incontec, 2014]), Ca-Mg-k-Na (atomic absorption), P (Bray II - colorimetry), Fe-Mn-Zn (DTPA extract – atomic absorption), and boron (hot water).
Where, \( Al \) is the current aluminum percentage (cmol, kg\(^{-1} \)), \( RAS \) is required percentage \( Al \) saturation, and \( ECEC \) is the effective cation exchange capacity.

The calcareous materials were selected by taking into account the percentage of participation of the elements calcium and magnesium, the relative power of total neutralization of acidity of the amendment, and references in the literature that an amendment reduces cadmium concentrations in tropical cacao soils.

Healthy 6-year-old plants of a similar size were selected. The amendments were applied in a crescent on the upper part of the trunk. The selected plants had floral differentiation and fruit set, some in the gherkin state (according to the BBCH scale), with a length not exceeding 5 cm.

After the application of the treatments, samples of the chlorophyll index variables (SPAD units) and stomatal conductance were carried out at four intervals of 45 days after application (daa). The samplings were carried out at the same hour. Four leaves that were exposed directly to the sun were selected, from productive branches of the middle-third of 10 plants. Three samples per leaf were evaluated to calculate the average for the data per experiment unit. A SPAD-502Plus chlorophyll meter (Konica Minolta, Osaka, Japan) and SC-1 Leaf porometer (Meter, Decagon Devices Inc., Pullman, WA) were used. The samples for the soil Cd analysis were taken at 0-10 and 10-20 cm depths and were determined with the P-PAS-21 procedure following the NTC 3888 standard (Incontec, 2014).

Statistical analysis

The data were subjected to an analysis of variance after verifying the assumptions of normal and homogeneity with the Shapiro-Wills and Bartlett tests (\( P<0.05 \)), respectively. Subsequently, Tukey’s multiple comparison test of means (\( P<0.05 \)) was performed for each measurement interval. The statistical software Infostat was used.

RESULTS AND DISCUSSION

Relative chlorophyll index

During the four evaluation periods, there were no statistically significant differences between the treatments (Tab. 2).

For the treatment without amendment applications (control), the SPAD units were reduced throughout the four evaluation intervals. The first interval (45 daa) had the highest value with 65.15 SPAD, and 35.73 SPAD units were recorded at 180 daa. The behavior of the chlorophyll index in the control treatment confirmed the reports by Jácome (2017) and Fernández (2022), who stated that SPAD units decrease as plants adsorb more cadmium. Hakeem et al. (2022) reported that the degradation of photosynthetic pigments in plants can decrease by up to 80% under cadmium stress, which can be corrected with calcium applications that reduce the degradation of the chlorophyll molecule.

At 90 daa, the lowest SPAD chlorophyll index was observed, which was related to high concentrations of cadmium, especially in the control treatment with an average value of 3.3 mg kg\(^{-1} \) in the soil, accompanied by low pH values (4.2 - 4.7), coinciding with the highest rainfall (552 mm) and concentrations of Al\(^{3+} \) (7 meq mol kg\(^{-1} \)). These conditions created a greater expression of the stressful action of cadmium, affecting the chlorophyll synthesis, hindering photosynthetic electron transfer, and resulting in excess electrons in the photosynthetic electron transfer chain with a rapid increase in ROS (reactive oxygen species) and feedback inhibition of plant photosynthesis, which

| Table 2. SPAD units in T. cacao clone CCN-51 subjected to calcium supersaturation under cadmium stress. |
| Treatment | Days after the application | 45 | 90 | 135 | 180 |
| Control | 65.15±1.59 | 48.05±1.24 | 43.85±1.99 | 35.73±1.99 |
| Do+y7 | 55.2±1.36 | 41.4±7.44 | 62.55±9.90 | 42.93±6.98 |
| Do+y8 | 55.87±1.65 | 52.4±1.38 | 43.5±4.7 | 62.3±17.1 |
| Do+y9 | 56.3±4.01 | 46±1.67 | 48.4±1.84 | 45.33±13.01 |

Control: no application of amendment; Do+y7: dolomite + gypsum at 7 cmol, kg\(^{-1} \) of Ca; Do+y8: dolomite + gypsum at 8 cmol, kg\(^{-1} \) of Ca; Do+y9: dolomite + gypsum at 9 cmol, kg\(^{-1} \) of Ca. Means±standard error.
impairs the maintenance of the redox balance in the chloroplast (Huang et al., 2020; Zhang et al., 2020; He et al., 2021; Huo et al., 2022).

Cadmium inhibits root growth, decreases chlorophyll content, and suppresses photoactivation of plant photosystem II (PSII) by preventing electron transport. In addition, it interferes with carbon metabolism and contributes to water stress in plants and the absorption of nutrients, affecting the respiration process and accumulation of cadmium in organs, such as leaves in cacao plants (Huang et al., 2017; Kapoor et al., 2021).

Reducing the quantity and quality of chlorophylls in leaf blades in turn decreases photosynthetic activity, considerably limiting plant growth and development, leading to greater susceptibility to diseases and low-quality production, and resulting in definitive loss of the plant because of a deficient conversion of photoassimilates and nutritional elements (Choudhury et al., 2022).

Among the treatments of calcareous amendments, Do+y8 stood out (Tab. 3) with fewer variations in the relative chlorophyll index, where, 45 daa, values of 55.87 SPAD units were recorded that slowly decreased to 52.4 SPAD on day 90. The lowest value occurred at 135 d (43.5 SPAD), until reaching 62.3 SPAD units at 180 daa of the liming agents.

The trend in treatments Do+y7, Do+y8 and Do+y9 for the relative chlorophyll index (SPAD) may have been related to the action of the applied amendment as a result of a significant increase in soil pH, which started at 4.75 and reached 5.2 on average in all treatments, possibly decreasing the mobility and concentration of cadmium (average of 0.3 mg kg⁻¹ in soil for all treatments, except the sampling at 45 daa with 0.1 mg kg⁻¹ of Cd). Hu et al. (2016), Sun et al. (2016), Huang et al. (2017), Huaraca-Fernandez et al. (2020) and Hakeem et al. (2022) stated that Cd is found in complexes that can precipitate more easily, such as the formation of cadmium sulfate (CdSO₄) because the reaction with calcium sulfate (CaSO₄·2H₂O) or agricultural gypsum in the redox process can lead to dissolution or precipitation of cadmium. The interaction of S within plants promotes the precipitation or immobilization of cadmium in the cells and facilitates contractions of calcium and magnesium, reducing the absorption of cadmium and generating less sensitive cell walls (Cao et al., 2018; Guan et al., 2018; Yao et al., 2021).

By fixing calcium ions on the negative charges of the colloidal fraction in the soil, calcium enters the plant more efficiently and facilitates a recovery of metabolic activity, especially in the reconstruction of chlorophyll molecules where significant concentrations of calcium and magnesium are required, improving photosynthetic efficiency (Choudhury et al., 2022; Li et al., 2022b). Likewise, soil moisture plays an essential role in the release of calcium in the soil solution, as well as the precipitation of cadmium in the form of cadmium sulfate (CdSO₄), because the correct activation of amendments requires a sufficient amount of water; the average rainfall in the area is sufficient (262.53 mm) and does not require additional water applications, which is why, at 45 daa, the cadmium concentrations were 0.1 mg kg⁻¹.

### Stomatal conductance

The stomatal conductance presented statistically significant differences (P<0.05) between treatments at

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Days after the application</th>
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<tbody>
<tr>
<td></td>
<td>45</td>
</tr>
<tr>
<td>Control</td>
<td>267±6.95 ab</td>
</tr>
<tr>
<td>Do+y7</td>
<td>184.5±0.57 b</td>
</tr>
<tr>
<td>Do+y8</td>
<td>329±1.70 a</td>
</tr>
<tr>
<td>Do+y9</td>
<td>321±3.51 a</td>
</tr>
</tbody>
</table>

Control: no application of amendment; Do+y7: dolomite + gypsum at 7 cmolc kg⁻¹ of Ca; Do+y8: dolomite + gypsum at 8 cmolc kg⁻¹ of Ca; Do+y9: dolomite + gypsum at 9 cmolc kg⁻¹ of Ca.

Means with different letters in the column show significant differences according to the Tukey test (P<0.05). Means±Standard error.
45, 135 and 180 DAA, with the Do+y7 and Do+y8 treatments showing the best responses (Tab. 4).

The lowest stomatal conductance (Tab. 3) was observed at 135 DAA in the control treatment (62.13 mmol m$^{-2}$ s$^{-1}$), where a greater deterioration of cellular structures was suggested where cadmium replaced calcium, as reported by Kapoor et al. (2021), entering plant as a result of the effect of physiological sensitivity.

Treatments Do+y7 and Do+y8 had higher measurements (greater than 500 mmol m$^{-2}$ s$^{-1}$, Tab. 3). The control treatment had values between 267 mmol m$^{-2}$ s$^{-1}$ (45 dda) and 418.53 mmol m$^{-2}$ s$^{-1}$ (180 dda), and the Do+y7 treatment sustained an increase throughout the experiment, from 184.5 mmol m$^{-2}$ s$^{-1}$ (45 dda) to 481 mmol m$^{-2}$ s$^{-1}$ (180 dda), considered the best treatment, improving the opening and closing capacity of the stomata, providing a better respiratory process, and achieving better efficiency in gas exchange (Suárez-Salazar et al., 2017). Considerable concentrations of cadmium in cellular structures increase susceptibility to water stress because, while calcium has the biological function of opening and closing stomata and allowing adequate gas exchange, stomata with cadmium cannot close normally, affecting the photosynthetic process through an inefficient CO$_2$ exchange, increasing damage to the photosystem by stress (Dell’Amico and Morales-Guevara, 2017; Ramthahal et al., 2019; Choudhury et al., 2022; Li et al., 2022a).

Although little is known about the dynamics of cadmium in affecting stomatal conductance, it can be assumed that the insufficiency in the respiratory process caused by cadmium is related to a calcium deficiency in the system, which can be corrected with calcium applications. Li et al. (2022b) stated that increasing extracellular calcium and cytosolic calcium leads to efficient stomatal closure, allowing better performance of stomatal conductance, giving better conditions for their closing and opening, and increasing stomatal density. In addition, there is a better and greater production of dibromothymoquinone (DBMIB), an enzyme that allows stomatal closure, where H$_2$O$_2$ generated by chloroplast in the mesophyll is necessary to avoid losses from stress. This explains the behavior of the variable in the Do+y7 treatment, which had an increasing trend.

Although no consistent data were found, multiple factors affected the stomatal opening of the plants that were related to stomatal conductance, CO$_2$ assimilation, air humidity and leaf temperature, along with soil moisture and efficiency of plants to transport water under stress from biotic or abiotic factors, which must be taken into account when evaluating the variable (Pino et al., 2019).

**CONCLUSIONS**

The relative chlorophyll index in the treatments with calcareous amendments did not have significant statistical differences; however, it increased throughout the experiment. The Do+y8 treatment presented the best response with 62.3 SPAD units at 180 days after the application of the calcareous amendment.

The stomatal conductance presented significant statistical differences between treatments, where treatment Do+y7 had an increase at each evaluation interval, which could indicate a recovery of stomatal activity, improving the physiological process of gas exchange and decreasing cadmium affection.

Applications of amendments or supersaturated applications of calcium in the soil can be a useful tool for reducing losses caused by the accumulation of cadmium in plants; however, these applications must be made consecutively and must complement each other to achieve long-term results.

Conflict of interests: This manuscript was prepared and reviewed with the participation of the authors, who declare that there exists no conflict of interest that puts at risk the validity of the presented results.

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