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Calcium and Silicon Precipitation in *Pinus taeda* Needles in Response to Soil Application of Cellulosic Residue

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Abstract

The use of biosolids as a sustainable alternative in the management of planted *Pinus taeda* forests affects availability of nutrients and beneficial elements. Calcium (Ca) and Silicon (Si) play important roles in plant regulatory and protective systems; therefore, our objective was to use scanning electron microscopy combined with energy-dispersive spectroscopy (SEM–EDS) to analyze the accumulation of elements in *P. taeda* needles grown in soil that received 0 to 60 Mg ha^{-1} applications of cellulosic waste. Microanalyses of needle sections were performed using a SEM with elemental detection by EDS. Mapping mode allowed for detection and analysis of Ca, Si, and C distributions in needle sections. Calcium and Si precipitation occurred in needles, with Ca accumulating in the phloem and Si in the epidermal cells. Application of 60 Mg ha^{-1} changed the availability and accumulation of elements, which resulted in more Ca crystals and fewer Si crystals.

Keywords: Calcium oxalate, phytoliths, electron microscopy.

Key message: Different patterns of Ca and Si accumulation (with possible physiological and ecological roles) as function of cellulosic waste application were detected in *P. taeda* by using SEM – EDS microanalysis.

1. INTRODUCTION

World wood demand from planted forests is steadily increasing. In Brazil, planted forests of *Pinus* and *Eucalyptus* have very high productivity levels, with *Pinus taeda* being the most important species in terms of planted forest area in southern Brazil (Oliveira et al., 2018; Dobner and Campoe, 2019). In addition to economic relevance, social and sustainable management practices utilizing organic fertilizers (e.g., biosolid residues from pulp and paper industries) are being implemented as alternatives to promote sustainability of planted forests (Ortega Rodriguez et al., 2018).

Biosolids use can change nutrient dynamics in forest systems and enhanced tree growth (Rodrigues et al., 2023). McLaren et al. (2007) found that biosolids applied to forest soils resulted in substantial build-up of macronutrients and metals in forest litter layers, which changes their availability to plants. For example, the application of residues from recycled paper production increased Ca concentration in *P. taeda* needles, trunks, and roots (Rabel et al., 2020).

Pinus species are highly adapted to acidic soils with poor fertility (Rocha et al., 2019). However, Al toxicity may occur in association with Ca and Mg deficiencies. Silicon, in turn, may play a role in alleviating Al toxicity (Hodson &

Sangster, 1999; Hodson and Evans, 2020). More complex interactions between elements can occur (Hodson & Sangster, 1999; Rocha et al., 2019), and the traditional approach of analyzing needle elemental composition may not be sufficient for understanding physiological changes promoted by different management practices. In addition, elements such as Ca can be found in different forms (i.e., oxalate, sulfate, and carbonate crystals) (Pritchard et al., 2000; He et al., 2012; He et al., 2014; Barbosa et al., 2020; Paiva, 2019).

Calcium crystals can have several functions within plants, such as calcium regulation (storage or excretion), heavy metal detoxification, tissue support, and protection against herbivory (Khan et al., 2023). Silicon can also be found precipitated in plant tissues; amount varies by plant part, plant organ age, and interaction with other elements (Mandlik et al., 2020). Silicon is considered beneficial to plants, mitigating the effects of heavy metals and toxic Al, reducing salt and water stress, and providing protection against herbivory and pathogen infection (Zargar et al., 2019).

Microanalysis techniques have been employed to understand the dynamics of elemental forms and distribution in plant tissues (Dinh et al., 2018; Pongrac et al., 2019; Barbosa et al., 2020; Montanha et al., 2020). These techniques can also be used to deepen knowledge concerning the effects of fertilizer management, toxic substances, and even climate change on plant metabolism and morphology (He et al., 2014; Mandlik et al., 2020).

Research exploring the distribution of elements using microanalysis techniques in *P. taeda* needles is incipient, especially under changing plant nutritional status. Thus, the objective of the present study was to use scanning electron microscopy combined with energy-dispersive spectroscopy (SEM – EDS) to analyze the accumulation of elements in needles of *P. taeda* grown in soil receiving cellulosic waste applications.

2. MATERIAL AND METHODS

2.1. Study site and plant material

Needle samples were collected from a *P. taeda* experimental area located in Piraí do Sul, Paraná state, Brazil (24°24'27.36" S and 49º 58' 34.70" W, altitude of 1102 m). According to Köeppen climate classification, the climate of the region is Cfb (Alvares et al., 2013), with annual temperatures varying between 16 and 18ºC, and average annual rainfall of 1400 to 1600 mm.

The regional parent material is sandstone and the relief is undulating. Soil in the experimental area was classified as a typic Orthic Quartzarenic Neosol. Analyses of soil physical and chemical attributes were performed to characterize the area (Table 1). For this purpose, soil samples (0-20 cm deep) were dried and ground to pass through a 2 mm sieve. Subsequently, the pH in CaCl$_2$, potential acidity (H+Al), organic carbon, exchangeable aluminum (Al^{3+}), P, K$^+$, Ca^{2+}, Mg^{2+}, and texture were determined following the procedures of Embrapa (2011)

Table 1. Soil characteristic of the experimental area.

pH CaCl$_2$	C	Ca^{2+}	Mg^{2+}	K$^+$	Al^{3+}	H+Al	P	Clay	Sand	Silt
	g dm^{-3}			cmol$_c$ dm^{-3}			mg dm^{-3}		g kg^{-1}	
4,07	3,97	0,04	0,02	0,03	0,69	3,97	0,20	138,0	788,0	74,0

Source: The author.

The experiment consisted of increasing rates of a composted residue (14, 25, 49, and 60 Mg ha^{-1} of dry material) generated by the cellulose industry. Residue rates were selected to increase soil base saturation by 20, 40, 60, and 80%, respectively. In addition, a control receiving no compost application was included. The residue utilized was a mixture of 70% cellulosic sludge and 30% boiler ash that was composted for ~12 months in the open. *Pinus taeda* seedlings were planted in January 2011 on 2.5 m x 2.5 m spacings. In June of the same year, blocks and experimental plots were laid out; each plot consisted of five rows with five trees per row for a total of 25 trees.

In the present study, we selected representative trees from the control, the 14, and the 60 Mg ha^{-1} cellulosic sludge treatments. In June 2016, needles were collected randomly from the middle third of three trees per treatment. Samples were stored in coolers for transport to the laboratory. Subsamples were washed with deionized water, dried (60˚C), and milled (< 1 mm). Samples were prepared for total N concentration by digestion with sulfuric acid, while K, Ca, Mg, P, S, Fe, Mn, Zn, Cu, and B were determined using inductively coupled plasma (ICP-OES) after nitro-perchloric digestion in a microwave oven (Martins & Reissmann, 2007) (Table 2).

2.2. SEM-EDS

Part of the *P. taeda* needle sample was washed with deionized water, placed on a glass plate, and cut with a stainless-steel blade. Sections (~ 2 mm) from central portions of needles were kept in the dark for 24 h (4 °C) in 3 mL tubes containing 1 mL of FAA fixation solution [formalin 5% (v/v), glacial acetic acid 5% (v/v), and 70% ethanol (v/v)]. Needle sections were then stored in 3 mL tubes containing 1 mL of 70% ethanol until being subjected to dehydration by an ethanol series of 80, 90, 95, and 100% (20 min per step). Needle sections were then subjected to dehydration with liquid CO_2 to a critical point and mounted on aluminum stubs coated with double-sided copper tape.

Microanalyses of needle sections were performed using a SEM (Vega3 LM, Tescan) with elements being detected by EDS (X-MaxN 80 mm^2, Oxford). The SEM instrument was a thermionic emission system with 3-nm resolution and a tungsten-heated filament as the electron source. Microscope functions were controlled via VegaTC software. The EDS instrument contained an 80 mm^2 silicon drift detector, and data processing was conducted with AZTech (Advanced) software. Images were obtained with an accelerating voltage of 15 kV in low vacuum. Microanalyses by EDS were conducted with an accelerating voltage of 15 kV, 35º take off angle, and 120 s per image (mapping mode). The mapping mode allowed for the detection and analysis of Ca, Si, and C distribution in needle sections. In the point analysis mode, C, O, Ca, Si, S, and Al were analyzed. These elements were analyzed based on energies and intensities of K- and L-lines and comparison with internal standards ($CaSiO_3$, SiO_2, ascorbic acid, FeS_2, and Al_2O_3).

3. RESULTS AND DISCUSSION

3.1. Ca precipitation in needles

From photomicrograph images, the following tissues were distinguished in *P. taeda* needle cross sections: mesophyll, epidermis, phloem, and xylem (Figure 1A). Only Ca and Si were accumulated in tissues (Figure 1).

Calcium was detected in all needle tissues (Figure 1D, Figure 1E and Figure 1F), primarily as precipitated crystals in phloem (Figure 2). These Ca precipitates were possibly in the form of Ca oxalate (CaOx) since Ca carbonate would be easily dissolved in an acidic medium (i.e., sample fixation solution). Soil acidity and low soil organic matter could have resulted in low S concentrations in needle tissues (Table 3), which would prevent formation of Ca sulfate crystals. Crystals of CaOx have been observed in various tissues and plant organs, and were formed both in intracellular and extracellular environments (Franceschi & Nakata, 2005; Barbosa et al., 2020; Pongrac et al., 2019).

Occurrence of Ca in phloem increased as a function of cellulosic sludge rate (Figure 2). Twenty-four crystals were counted in the control and 14 Mg ha^{-1} treatments, while 30 crystals were found in the 60 Mg ha^{-1} treatment (Table 3); this was possibly due to increased total Ca concentration in needles from the highest treatment (Table 2). The presence of Ca crystals in needles was not expected since the soil concentration was very low. Increases in crystals occurred despite large increases in plant growth, which often results in Ca dilution within tissues (Sass et al., 2020) and increased K and Mg found in tissue (Table 2). The size of the transect cut of needles confirms that enhanced growth had occurred (Figure 2 A, Figure B and Figure C).
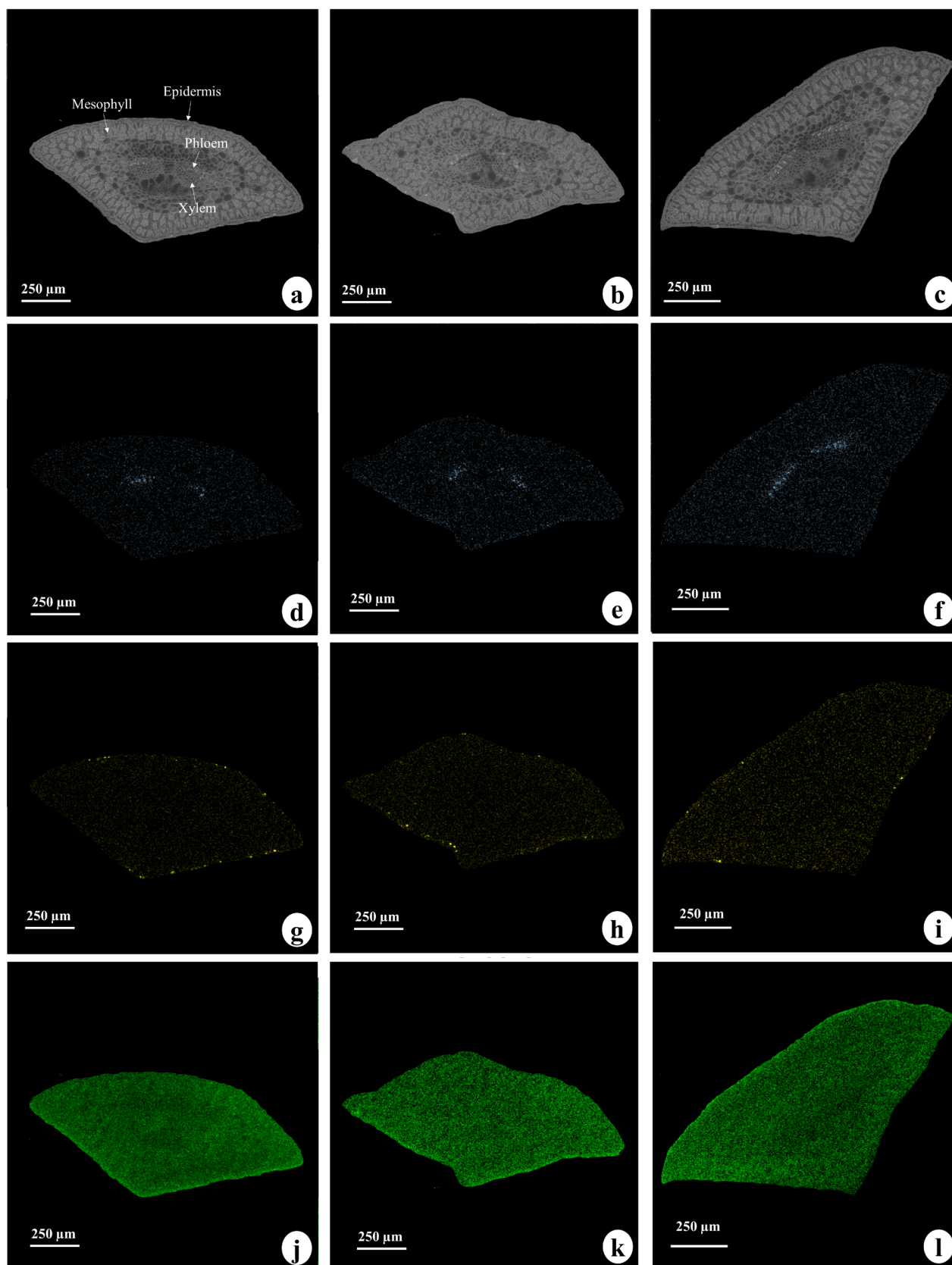
Figure 1. Photomicrograph (a – c) and distribution of Ca (d – f), Si (g – i), and C (j – l) in needles of *Pinus taeda* grown in soil receiving applications of 0 (a, d, g, and j), 14 (b, e, h, and k), and 60 Mg ha^{-1} (c, f, i, and l) of cellulosic residue. Source: The author.
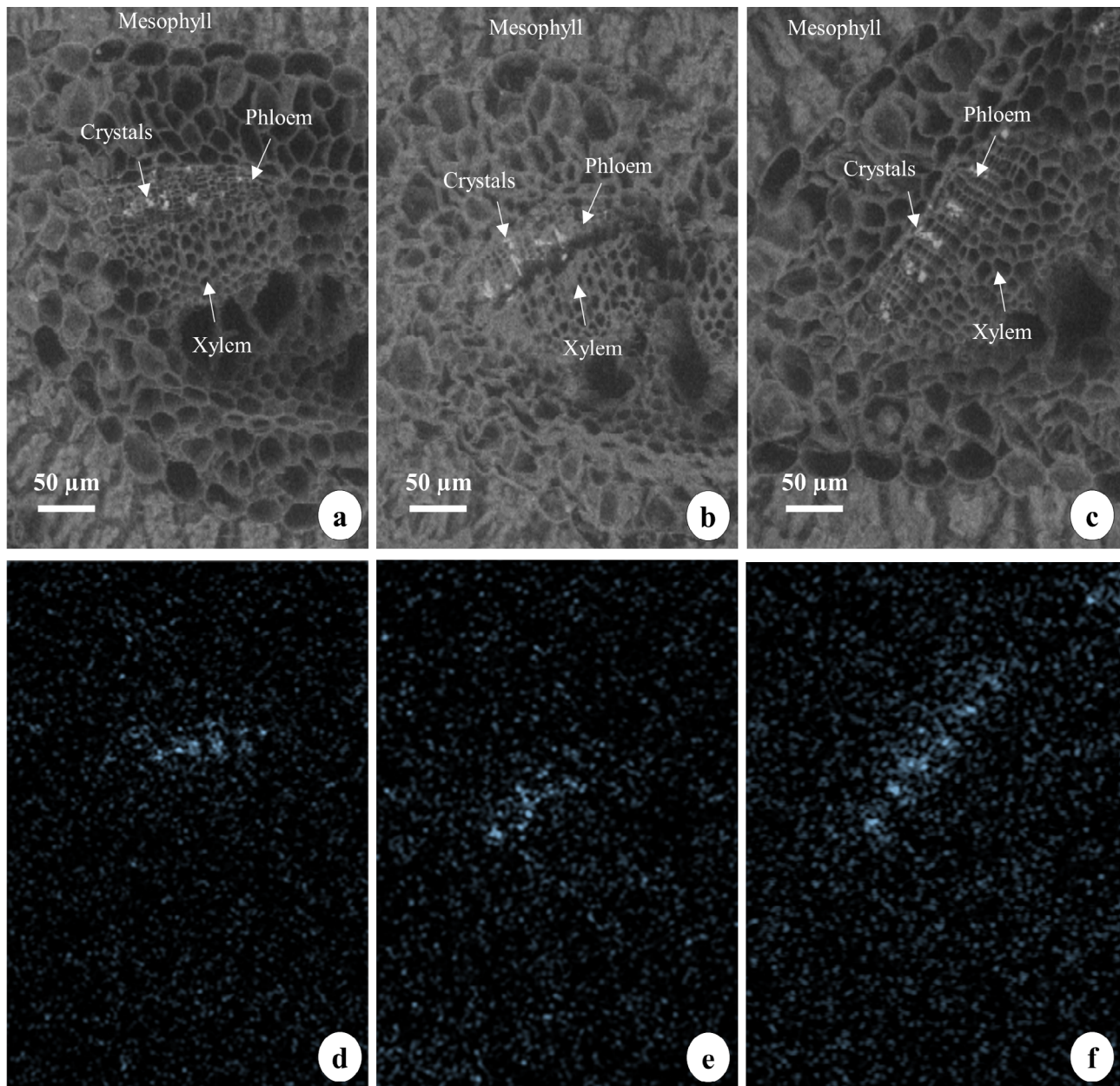
Figure 2. Photomicrograph (a – c) and Ca distribution (d – f) in the vascular bundle region of *Pinus taeda* needles grown in soil receiving applications of 0 (a, d), 14 (b, e), and 60 Mg ha^{-1} (c, f) of cellulosic waste. Source: The author.

Table 2. Elemental composition of *Pinus taeda* needles as function of cellulosic residue applied to soil in Piraí do Sul, Paraná state, Brazil.

| Nutrient	Units	Treatment		
		Control	14 Mg ha^{-1}	60 Mg ha^{-1}
N	g kg^{-1}	15.1±0.5	15.4±0.4	13.9±0.2
K	g kg^{-1}	4.5±0.5	4.6±0.2	5.5±0.3
Ca	g kg^{-1}	1.2±0.4	2.9±0.4	2.7±0.4
Mg	g kg^{-1}	0.3±0.2	0.7±0.1	0.9±0.1
P	g kg^{-1}	1.4±0.1	1.5±0.2	1.6±0.3
S	g kg^{-1}	0.8±0.1	0.9±0.1	0.9±0.0
Fe	mg kg^{-1}	54.2±5.3	62.8±4.0	60.3±9.0
Mn	mg kg^{-1}	1075±261	877±79	499±98
Zn	mg kg^{-1}	33.7±6.3	31.1±2.5	25.0±3.0
Cu	mg kg^{-1}	6.0±0.7	5.5±0.5	5.4±0.3
B	mg kg^{-1}	13.8±2.6	13.6±1.6	15.5±1.7

Source: The author.

The formation of CaOx crystals results from biological activity and environmental conditions, and depends on the amount of Ca available to the plant (Weiner and Doeve, 2003; Smith, 2009). Brown et al. (2013) found a positive relationship between the amount of Ca present in leaves of *Acacia phyllodes* and levels of insoluble CaOx; this dynamic was regulated by the concentration of Ca in soil solution.

Potassium deficiency also promotes greater Ca absorption, which led to Ca precipitation in *Araucaria angustifolia* leaves (Barbosa et al., 2017). These crystals act like a Ca storage system, regulating Ca stocks in plant tissue (Franceschi & Nakata, 2005; Karabourniotis et al., 2020). Under Ca deficient conditions, dissociation of CaOx crystals can result in remobilization of Ca (Volk et al., 2002; Turpault et al., 2019).

Pinus taeda normally has two annual needle flushes (in some cases, three) (Motta et al., 2014). We hypothesize that, when plants are not in the flush phase, it is possible that increased Ca levels in phloem provides for the formation of CaOx crystals; this may be especially true if increased amounts of Ca are available in soil. Calcium is very immobile in plants and a pivotal structural component of cell walls (Hawkesford et al., 2012). However, formation of crystals related to Ca regulation and stocks in plant tissues may be a consequence of high Ca levels in phloem of *P. taeda* needles from high rates of cellulosic sludge application (Figure 2). Specific studies are required to expand knowledge on Ca dynamics in phloem tissue during *P. taeda* needle flush development.

Phloem crystals were prismatic in nature (Figure 3). There were five morphological variations of CaOx crystals based on shape: druse, needle, styloid, sand crystals, and prismatic (Pierantoni, 2019). Extracellular crystals are generally rhombohedral or prismatic in shape, while crystals formed in intracellular spaces can present any of the habits mentioned above (Franceschi & Nakata, 2005).
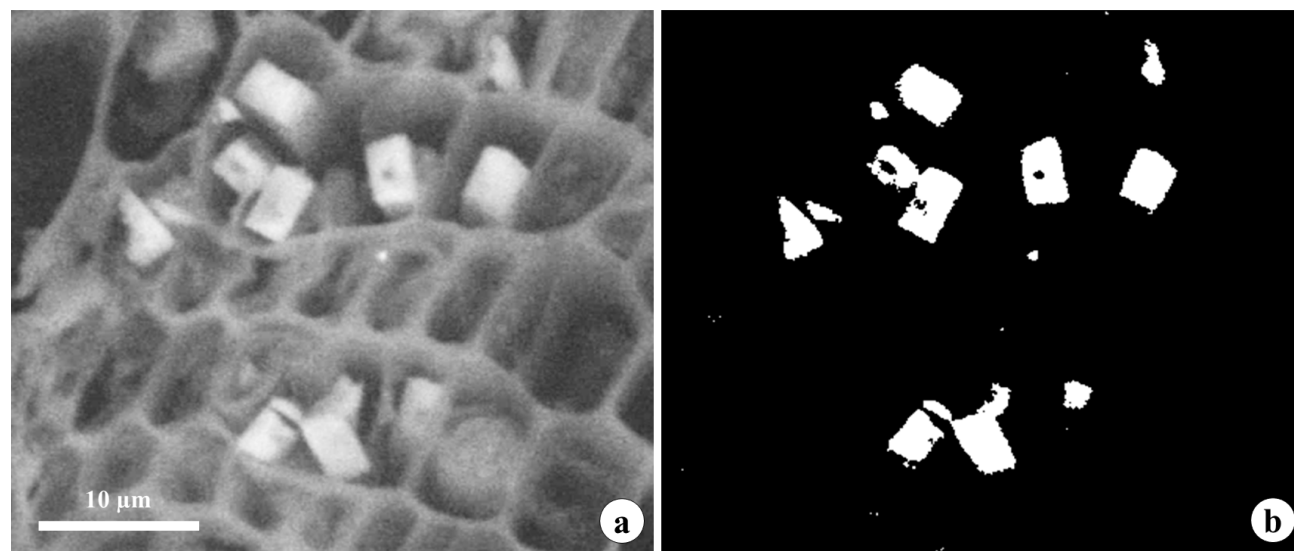
Figure 3. Details of Ca crystals found in the phloem of *Pinus taeda* needles cultivated in soil receiving cellulosic waste. a – Normal photomicrograph; b – Black and white photomicrograph. Source: The author.

According to He et al. (2012), prismatic crystals are typically five to ten μm in length, and are associated with phloem fibrous cells, or cells associated with them. Up to 50% of cells of specific tissues had one or two crystals. Crystals of CaOx can occur inside vacuoles (usually in a cell specialized in crystal formation), or in the apoplast (associated with the cell wall) (Franceschi & Nakata, 2005). Fink (1991) evaluated the presence of crystals in needles of several conifer species. In *Pinus* spp., intracellular crystals are preferentially formed in the phloem, and rarely extracellular crystals are formed in the mesophyll. This indicates that Pinaceae species are more evolved than other conifer species, since only extracellular crystals have been found in Araucariaceae, Taxodiaceae, and Taxaceae (Fink, 1991).

In addition to Ca regulation, the formation of crystals containing Ca in plants can perform several other functions such as detoxification of metals, harnessing light, and protection against herbivore attack (Franceschi and Nakata, 2005; Karabourniotis et al., 2020). Since Ca crystals did not occur near or on the surface of needles, they were likely not related to luminosity. Although the low Al concentrations detected in crystals (Table 3) could affect Al dynamics, it is important to assess whether increases in Al availability would increase this element in crystals.

The main function generally conferred to calcium crystals is Ca storage and regulation (Khan et al., 2023), with druses being the main crystal form related to this function (Nakata, 2003). However, depending on crystal shape, they can perform different functions within the plant (Lawrie et al., 2023). Druses and prismatic crystals can improve photosynthesis by remitting light and making better use by photosynthetic cells (Gal et al., 2012; Pierantoni et al., 2017), suggesting a possible function of these forms found in needles (Figure 3). Raphides, which were not found in *Pinus* needles in this experiment (in addition to regulating calcium storage), are the main form responsible for providing plant defense against herbivores (Nakata, 2003; Paiva, 2021).

Table 3. Number of crystals and percentage of Ca, C, and O in crystals formed in the phloem of *Pinus taeda* needles in response to application of cellulosic residue to soil.

Treatment	Mean±sd	Maximum	Minimum
Number			
Control	24±5	28	18
14 Mg ha⁻¹	24±12	37	14
60 Mg ha⁻¹	30±9	40	24

Table 3. Continued

Treatment	Mean±sd	Maximum	Minimum
Ca (%)			
Control	4.1±0.9	5.9	2.9
14 Mg ha⁻¹	3.6±0.9	4.7	2.5
60 Mg ha⁻¹	3.7±0.5	4.5	2.8
C (%)			
Control	62±2	65	58
14 Mg ha⁻¹	63±3	69	60
60 Mg ha⁻¹	63±1	65	61
O (%)			
Control	34±2	39	31
14 Mg ha⁻¹	33±2	37	29
60 Mg ha⁻¹	33±2	36	30
Al (%)			
Control	0.04±0.01	0.06	0.03
14 Mg ha⁻¹	0.07±0.03	0.10	0.04
60 Mg ha⁻¹	0.06±0.04	0.12	0.00
S (%)			
Control	0.05±0.01	0.06	0.04
14 Mg ha⁻¹	0.05±0.01	0.06	0.04
60 Mg ha⁻¹	0.04±0.01	0.06	0.04

Source: The author.

3.2. Si precipitation in needles

Silicon was present in all tissues, with preferential accumulation in the epidermis in the absence of cellulosic residue (Figure 1G, Figure 1H, Figure 1I – I and Figure 4). Silicon is considered a beneficial element for plants, affecting several mechanisms of protection and mitigation of abiotic and biotic stresses (Luyckx et al., 2017; Broadley et al., 2012). This element is absorbed by roots, transported via transpiration flow, and accumulates in aerial plant parts, usually in epidermal tissues (Barbosa et al., 2020; Guerriero et al., 2020).
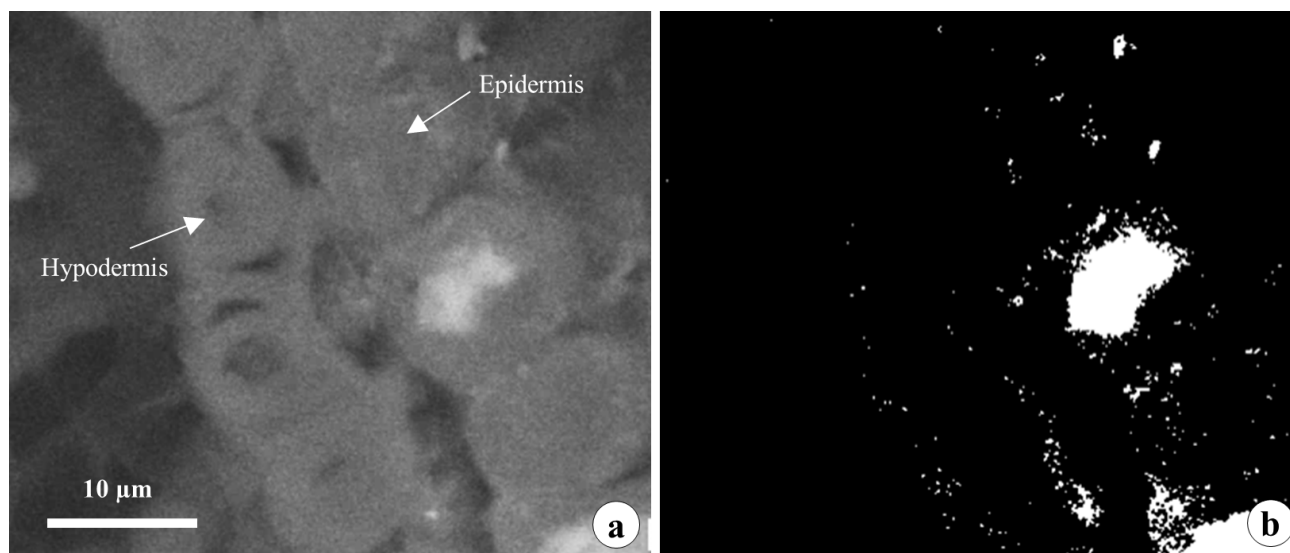
Figure 4. Details of Si crystals found in the epidermis of *Pinus taeda* needles cultivated in soil receiving cellulosic waste. a – Normal photomicrograph; b – Black and white photomicrograph. Source: The author.

Preferential accumulation of Si in the form of precipitates occurred throughout the epidermis of *P. taeda* needles (Figure 1 G, Figure 1H, Figure 1I and Figure 4). Deposition can be quite diverse, whether in the form of precipitates inside the cells or as amorphous material impregnating the middle lamella of cell walls in the epidermis, stomata, mesophyll, and tracheids (Sangster et al., 1997; Mvondo-She and Marais, 2019; Barbosa et al., 2020). Analyzing needles from 12 *Pinus* species, Sangster et al. (1997) found that four species showed accumulation in tracheids (*P. koraiensis, P. parviflora, P. peuce*, and *P. strobus "radiata"*), and three displayed accumulation in isolated epidermal cells (*P. cooperi, P. strobus "radiata",* and *P. sylvestris*). Contrary to observations of Hodson and Sangster (2002) and Niemiec et al. (2017) *for P. strobus*, at this work we did not observed Al accumulation in needles, likely due to low Al concentration in plant tissue (Table 4).

The control treatment had 19 Si crystals, while the 14 and 60 Mg ha⁻¹ treatments had 18 and 13 crystals, respectively (Table 4). Greater tree growth was favored by the 14 Mg ha⁻¹ cellulosic residue application, with little increase observed at 20 Mg ha⁻¹ to 60 Mg ha⁻¹ (Sass et al., 2020). Thus, increasing cellulosic residue application rate probably diluted Si concentration in needles and consequently decreased amount of Si precipitates. Species belonging to gymnosperms are considered low Si accumulators; this genetic factor may also have influenced the low amount of Si precipitated in needles (Zargar et al., 2019).

Table 4. Number of crystals and percentage of Si, C, and O in crystals formed in the epidermis of *Pinus taeda* needles in response to application of cellulosic residue to soil.

Treatment	Mean±sd	Maximum	Minimum
Number			
Control	19±2	21	17
14 Mg ha⁻¹	18±1	19	17
60 Mg ha⁻¹	13±1	14	13
Si (%)			
Control	4.4±0.5	5	4.1
14 Mg ha⁻¹	4.0±0.3	4.2	3.8
60 Mg ha⁻¹	4.0±0.6	4.5	3.9
C (%)			
Control	63±3	65	60
14 Mg ha⁻¹	66±2	68	65
60 Mg ha⁻¹	65±3	66	60
O (%)			
Control	32±2	35	30
14 Mg ha⁻¹	29±1	30	28
60 Mg ha⁻¹	30±2	34	29
Al (%)			
Control	0.06±0	0.06	0.06
14 Mg ha⁻¹	n.d.	n.d.	n.d.
60 Mg ha⁻¹	0.03±0	0.03	0.03
S (%)			
Control	0.03±0	0.03	0.03
14 Mg ha⁻¹	0.03±0.01	0.04	0.03
60 Mg ha⁻¹	0.02±0	0.02	0.02

n.d. denotes not detected.
Source: The author

Silicon accumulation in plant tissue promotes greater resistance to attack by fungi and herbivores when Si precipitation occurs in the epidermis (Mandlik et al., 2020; Broadley et al., 2012). Thus, Si precipitation in the epidermis of *P. taeda* may contribute to increased tolerance of pests (especially insects). If Si precipitation occurred in isolated cells in the epidermis, a null effect against fungal infection would be expected. However, further studies are required to broaden our knowledge regarding the ecological and physiological interactions associated with Si precipitation in *P. taeda*.

4. CONCLUSIONS

The use of SEM – EDS microanalysis illustrated different patterns of elemental accumulation that may have possible physiological and ecological roles in *P. taeda*. Calcium and Si precipitation occurred in needles of *P. taeda*, with Ca accumulating in the phloem, while Si accumulated in epidermal cells. Application of 14 Mg ha^{-1} of cellulosic sludge did not affect precipitation of these elements compared to the control treatment. However, with the application of 60 Mg ha^{-1}, Ca crystals tended to be formed in the phloem, and the number of Si crystals in needle epidermal tissue decreased; changes were probably due to variations in the availability and absorption of these elements. Analysis of crystal composition did not indicate significant changes in concentrations of Ca and Si.

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Shizuo Maeda: Writing - original draft (Equal).
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DATA AVAILABILITY
Data not available.

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