

SILICON IN THE ANATOMY AND PHYSIOLOGY OF BANANA PLANT LEAVES UNDER TEMPORARY IMMERSION BIOREACTORS

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ABSTRACT:

Banana is a crop with outstanding economic and social expression throughout the world, therefore, it is important to use appropriate methods of propagation. The micropropagation is an appropriate method because it is disease-free, unlike traditional methods that spread pests and diseases. The objective of this study was to evaluate the effect of silicon on foliar anatomy and chlorophyll content of banana plants cultured *in vitro* using temporary immersion bioreactor technology. Explants of 'Dwarf Cavendish' banana established *in vitro* were inoculated in bioreactors containing MS liquid medium. Treatments included silicon concentration (0 and 1 mL⁻¹) and frequency of immersions (every 4, 6, and 8 hours) lasting 1 minute per immersion. Cultures were maintained in a growth room at 27 ± 2 °C under LED light (50 $\mu\text{mol m}^{-2} \text{s}^{-1}$) with a 16 h of photoperiod for 36 days. After that period, anatomical characteristics and chlorophyll content were evaluated. Higher values were observed for some characteristics validated in plants cultivated with silicon when compared to control. Silicon addition resulted in increased thickness of the leaf's limb tissues, resulting in a larger mesophyll (563.122 μm) which is the set of measurements of all leaf's limb tissues, it also provided greater adaxial and abaxial stomatal density (0.108817 μm and 0.305085 μm) respectively, greater adaxial and abaxial polar diameter/equatorial diameter ratio (1.982 and 2.069) respectively, and increased chlorophyll *a* content (0.000134 mg·g⁻¹ fresh weight) and *a/b* ratio (1.710425) of plants of banana 'Dwarf Cavendish' cultured in bioreactors.

Keywords: *Musa* spp., potassium silicate, chlorophyll, leaf anatomy

SILÍCIO NA ANATOMIA E FISILOGIA DE FOLHAS DE BANANA SOB BIOREATORES DE IMERSÃO TEMPORÁRIA

RESUMO: A banana é uma cultura com notável expressão econômica e social em todo o mundo, por isso, é importante utilizar métodos adequados de propagação. A micropropagação, é um método adequado porque é livre de doenças, diferente dos métodos tradicionais que acabam contribuindo para a disseminação de pragas

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e doenças. O objetivo foi avaliar o efeito do silício na anatomia foliar e no teor de clorofila de plantas de bananeira cultivadas *in vitro* utilizando a tecnologia de biorreator por imersão temporária. Explantes de banana 'Dwarf Cavendish' estabelecidos *in vitro* foram inoculados em biorreatores contendo meio líquido MS. Os tratamentos incluíram concentração de silício (0 e 1 mL⁻¹) e frequência de imersões (a cada 4, 6 e 8 horas) com duração de 1 minuto por imersão. As mesmas foram mantidas em sala de crescimento a 27 ± 2°C sob luz LED (50 μmol m⁻² s⁻¹) com 16 h de fotoperíodo durante 36 dias. Foram avaliadas características anatômicas e conteúdo de clorofila. Foi observado maiores valores para algumas características validadas em plantas cultivadas com silício quando comparado ao controle. A adição de silício proporcionou um aumento da espessura dos tecidos do limbo, resultando em um maior mesofilo (563,122 μm) que é o conjunto das medidas de todos os tecidos do limbo foliar, o mesmo proporcionou também maior densidade estomática adaxial e abaxial (0,108817 μm e 0,305085 μm) respectivamente, maior razão diâmetro polar/diâmetro equatorial adaxial e abaxial (1,982e2,069) respectivamente e aumento no conteúdo de clorofila *a* (0,000134 mg·g⁻¹ peso fresco) e na relação *a/b* (1,710425) de plantas de banana 'Dwarf Cavendish' cultivadas em biorreatores.

Palavras-chave: *Musa* spp., silicato de potássio, clorofila, anatomia foliar

INTRODUÇÃO

Banana (*Musa* spp.) is a crop with outstanding economic and social expression around the world, being considered an important source of food and one of the fruits with the highest consumption and production among tropical fruit trees (Donato et al., 2006).

Banana is exclusively propagated by vegetative methods. However, the use of conventional seedlings, for example, rhizome seedlings, contributes significantly to the spread of pests and diseases (Roels et al., 2005). *In vitro* propagation offers a means for the clonal propagation of uniform, true-to-type, disease-free plant material. In addition, *in vitro*-derived bananas are reported to be more vigorous, with higher yields and produce better quality fruits than those produced by conventional methods (Hwang et al., 1984). Bioreactors involve *in vitro* propagation of cells, tissues, somatic embryos, and plantlets in liquid suspension (Paek et al., 2005). Optimum plant growth conditions can be achieved in a bioreactor by regulating various chemical and physical factors, including gas exchange, pH and hydrodynamic forces (Dong et al., 2013). In temporary immersion bioreactors, the cultures are immersed in the medium for a preset duration at specified intervals (Adelberg e Simpson, 2002). To multiply 'Grande Naine' banana, Alvard et al. (1993) compared five different liquid media culture methods and gelled culture media. They observed the highest multiplication rate (>5 in 20 days) in explants subjected to temporary immersion, and the highest accumulation of dry matter in explants was obtained in an aerated liquid medium and temporary immersion. The same authors observed that leaf development was considerable in the aerated liquid medium and with temporary immersion of explants. Also, no necrosis was observed on the leaves. This experiment shows that the type of liquid medium application greatly influences the development of banana explants in micropropagation.

Silicon (Si) is one of the elements considered beneficial to plants (Vasanthi et al., 2014). The

physiological benefits of Si are not well studied, but it is reported to increase the photosynthetic rate (Dias et al., 2014) and the content of photosynthetic pigments (Dias et al., 2017).

The leaf is an organ with high plasticity, thus the improvement in the interception of light can influence its anatomy. This is confirmed by studies that show that the application of Si resulted in changes in the leaf anatomy of orchids (Soares et al., 2012), banana (*Musa* spp.) (Asmar et al., 2013), anthurium (*Anthurium adreaenum* cv. Rubi) (Dias et al., 2014), cape gooseberry (*Physalis peruviana* L.) (Rezende et al., 2018), cockscomb (*Celosia cristata*) (Assis et al., 2018) and bromeliad (*Aechmea blanchetiana*) (Martins et al., 2019).

Other physiological benefits are derived from silicon nutrition, such as increased chlorophyll content, increased activity of the carboxylation enzyme (RuBisCO), and decreased transpiration (Epstein, 1994). For example, Lim et al. (2012) have indicated that Si significantly increased the chlorophyll content of begonia (*Begonia semperflorens*) and pansy (*Viola × wittrockiana*). As well as Martins et al. (2019) that observed an increase chlorophyll *a* content in bromeliad plants (*A. blanchetiana*) grown *in vitro* with addition of calcium silicate.

The objective of this study was to evaluate the effect of silicon on foliar anatomy and chlorophyll content of bananas plants cultivated *in vitro* using temporary immersion bioreactor technology.

MATERIAL AND METHODS

The experiment was performed at the Laboratory of Ornamental Horticulture and Biotechnology at the Tropical Research and Education Center (TREC) of the University of Florida (UF), in Homestead, Florida, United States.

Explants of 'Dwarf Cavendish' banana established *in vitro* were inoculated in bioreactors containing MS liquid medium (Murashige e Skoog, 1962), supplemented with 30 g L⁻¹ sucrose and 4 mL

L⁻¹ 6- benzyladenine. Potassium silicate (K₂SiO₃) was added to the MS medium at the concentration of 1mL L⁻¹. The MS medium without the addition of silicate was used as the control. The pH was adjusted to 5.7 before autoclaving at 121 °C for 20 min.

Subsequently, in the laminar flow hood, 2-3cm explants (of plants established *in vitro*) were inoculated in bioreactors containing 1000 mL of MS liquid medium. Treatments included silicon concentration (0 and 1 mL⁻¹) and frequency of immersions (every 4, 6, and 8 hours) at the duration of 1 minute per immersion. Cultures were maintained in a growth room at 27 ± 2 °C under LED light (50 µmol m⁻² s⁻¹) with a 16 h of photoperiod for 36 days.

Samples of leaf tissues from 10 plants were fixed in FAA 70% (formaldehyde - glacial acetic acid - 70% ethyl alcohol) for 72 h and later preserved in 70% ethanol (v/v⁻¹). Leafcross sections were obtained from free hand sectioning using a steel blade, and the peridermic sections using a printing technique, which were immersed in a sodium hypochlorite (1% - 1.25% active chlorine), followed by three washes in distilled water, staining with toluidine blue 0.5%, and later mounted on semi-permanent slides with glycerinated water (Kraus e Arduin, 1997).

The slides were observed and photographed under a light microscope (Leica DMLB, Germany) using a SPOT digital camera and SPOT 4.7 version software, respectively. The images were analyzed in Image J analysis software with the measurement of six fields per repetition for each analyzed variable. Within each field, six measurements were taken, and the average was obtained. The thickness of the epidermis, hypodermis of adaxial and abaxial faces, palisade, spongy parenchyma, and mesophyll were determined for the leaf cross sections. For stomata characterization, the stomatal density (number of stomata per mm²) of the adaxial and abaxial faces, the polar diameter (PD), the equatorial diameter (ED), and the PD/ED ratio were analyzed.

Chlorophyll content was assessed using a modified version of the Scopel et al. (2011) protocol. Leaf discs with 1 cm diameter were transferred into test tubes with lids containing 5ml of 80% acetone (v/v) and stored for 24h in a refrigerator at 4°C, in the dark. Extracts were then filtered, and the absorbance readings of the resulting solution (2µL) were performed in a Nanodrop® at 645, 652, and 663 nm. Chlorophyll a, b, and total chlorophyll content were calculated using the obtained readings (Witham et al., 1971). The results were expressed as mg per gram of fresh weight of leaf tissue (mg g⁻¹).

The experiment was established in a 2x3 factorial completely randomized design, being two concentrations of silicon (0 and 1 ml⁻¹) and three immersion frequencies per day (every 4, 6, and 8 hours) at the duration of 1 minute per immersion. Two bioreactors were used per treatment containing 15 explants per bioreactor. The data were submitted to analysis of variance (ANOVA) and the means compared by Tukey's test at the 5% level of significance using the SISVAR statistical program (Ferreira, 2011).

RESULTS AND DISCUSSION

Controls for the immersion times of 4 and 6 hours were lost due to contamination. However, no significant differences were observed between the silicon treatments in 4- and 6-hours immersion compared to the control for 8 hours. This led to the continuing evaluation of the results using the control in the immersion time for 8 hours.

The leaves of the banana displayed an unstratified epidermis on both the adaxial and abaxial faces. Thus, this study confirmed that the species is bifacial or dorsiventral with the palisade parenchyma facing the adaxial epidermis and immediately below the adaxial hypodermis, while the spongy parenchymais directed to the abaxial epidermis (Figure 1), as previously described by Asmar et al. (2013).

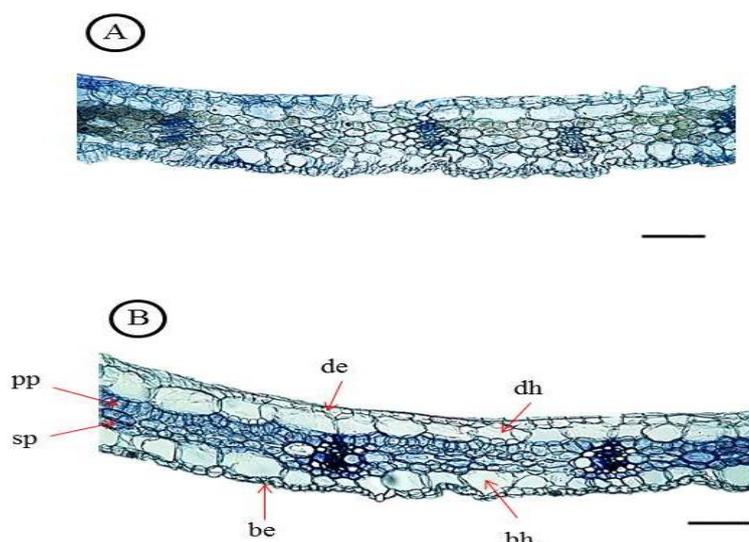


Figure 1. Photomicrograph of *Musa* spp. leaves grown *in vitro* under temporary immersion bioreactor technology without potassium silicate (control; A) and with $1 \text{ mL}\cdot\text{L}^{-1}$ potassium silicate (B). (bar = $50\mu\text{m}$). Adaxial epidermis (de), adaxial hypodermis (dh), palisade parenchyma (pp), spongy parenchyma (sp), abaxial epidermis (be) and abaxial hypodermis (bh). Fotomicrografia de folhas de *Musa* spp. cultivadas *in vitro* sob tecnologia de biorreatores de imersão temporária sem silicato de potássio (Controle, A), e com $1 \text{ mL}\cdot\text{L}^{-1}$ desilicato de potássio (B). (barra = $50\mu\text{m}$). Epiderme adaxial (de), hipoderme adaxial (dh), parênquima paliçádico (pp), parênquima esponjoso (sp), epiderme abaxial (be), e hipoderme abaxial (bh).

Our study, observed a significant change in the epidermis characteristics for banana leaves under silicon treatments, including increased thickness in the epidermis, hypodermis, palisade parenchyma, and mesophyll compared to control (Table 1). This change indicates the plasticity of the plant leaves and the potential effects of silicon accumulation in them, as Epstein reported (1999). Several studies have also found similar changes in the anatomical characteristics of leaves due to the application of calcium silicate *in vitro*, including orchids (Soares et al., 2012), strawberry (Braga et al., 2009) and banana (Asmar et al., 2013, 2015). Martins et al. (2018) observed that the addition of sodium silicate and calcium silicate provided greater thickening of the adaxial and abaxial epidermis, respectively, in bromeliad leaves grown *in vitro*. However, negative effects of sodium silicate were observed in anthurium (Dias et al., 2014). The primary function of the epidermis is as a covering, and the

arrangement of the cells complicates the action of mechanical shock and penetration of pathogens, in addition to restricting the loss of water (Castro et al., 2009). Most of the Si is incorporated into the cell wall, especially in the epidermis cells, stomata, and trichomes (Currie e Perry, 2007). Thus, the thickening of the epidermis on both faces generated by deposits of Si can alleviate the effects of nature biotic, and abiotic stress. This happens because the deposition of Si near the cuticle of leaves provides protection to plants by reducing the transpiration rate (Currie e Perry, 2007).

The advantages of thicker leaf tissues in plants include a higher chance of survival during transfer to an *ex-vitro* environment, an increased tolerance to stresses resulting from a shifting cultivation environment, and consequently improving acclimatization of *in vitro*-derived plants (Asmar et al., 2015).

Table 1. Thickness of adaxial epidermis (de), abaxial epidermis (be), adaxial hypodermis (dh), abaxial hypodermis (bh), palisade parenchyma (pp), spongy parenchyma (sp) and mesophyll (M) of *Musa* spp leaves of grown *in vitro* under temporary immersion bioreactors technology with potassium silicate (K_2SiO_3) supplement. Espessura da epiderme adaxial (de), epiderme abaxial (be), hipoderme adaxial (dh), hipoderme abaxial (bh), parênquima em paliçádico (pp), parênquima esponjoso (sp) e mesofilo (M) de folhas de *Musa* spp cultivadas *in vitro* sob tecnologia de biorreatores de imersão temporária com suplemento de silicato de potássio (K_2SiO_3).

| Treatments | de (μm) | be (μm) | dh (μm) | bh (μm) | pp (μm) | sp (μm) | m (μm) |
|-----------------------------------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|---------------------|
| Control | 33.195 b | 102.123 b | 30.050 b | 74.178 b | 61.098 b | 130.018 a | 454.405 b |
| K_2SiO_3 ($1\text{mL}\cdot\text{L}^{-1}$) | 68.127 a | 125.390 a | 48.348 a | 124.332 a | 88.528 a | 145.669 a | 563.122 a |
| CV(%) | 12.51 | 20.98 | 22.23 | 18.58 | 10.86 | 18.94 | 9.21 |

*Means followed by the same letter within columns are not significantly different by Tukey's test ($p \leq 0.05$). Médias seguidas pela mesma letra dentro das colunas não são significativamente diferentes pelo teste de Tukey ($p \leq 0,05$).

Potassium silicate also promoted a significant difference in stomatal characteristics in banana leaves, such as increased adaxial stomatal density (SD) and increased polar/equatorial diameter ratio (PD/ED) on both the adaxial and abaxial leaf surfaces compared to the control (Table 2).

The increase in stomatal density and PD/ED ratio with silicon application has been previously reported. Sodium and calcium silicate resulted in a higher stomatal density of the adaxial surface, and sodium and potassium silicate resulted in a higher PD/ED ratio in the adaxial surface of banana cv.

'Maçã' cultivated *in vitro* (Asmar et al., 2013). In addition, potassium, sodium, and calcium silicate also resulted in a higher stomatal density of both surfaces of the epidermis and sodium and potassium silicate increased the PD/ED ratio of the abaxial surface of banana cv. 'Grand Naine' (Asmar et al., 2013). A consequence of a higher PD/Ed ratio is elliptical-shaped stomata, which enhances its functionality (Sha Valli Khan et al., 2002), such as higher CO_2 uptake, increased photosynthetic potential, and consequently reduced transpiration rate (Castro et al., 2009).

Table 2. Stomatal density (SD) and polar/equatorial diameter ratio (PD/ED) of leaves of *Musa* spp. grown *in vitro* under temporary immersion bioreactors technology supplemented with silicon. Densidade estomática (SD) e relação diâmetro polar/equatorial (PD / ED) de folhas de *Musa* spp. cultivadas *in vitro* sob tecnologia de biorreatores de imersão temporária suplementado com silício.

| Treatments | Adaxial SD | Abaxial SD | Adaxial PD/ED | Abaxial PD/ED |
|-----------------------------------------------|--------------------------|------------|---------------|---------------|
| | stomata mm^{-2} | | | |
| Control | 0.079383 b | 0.275680 a | 1.570 b | 1.706 b |
| K_2SiO_3 ($1\text{mL}\cdot\text{L}^{-1}$) | 0.108817 a | 0.305085 a | 1.982 a | 2.069 a |
| CV(%) | 11.52 | 9.64 | 13.98 | 12.53 |

*Means followed by the same letter within columns are not significantly different by Tukey's test ($p \leq 0.05$). Médias seguidas pela mesma letra dentro das colunas não são significativamente diferentes pelo teste de Tukey ($p \leq 0,05$).

The variation in the size and the density of the stomata is evidence that plants possess the capacity to rearrange these epidermal structures in response to environmental changes by increasing their active involvement in gas exchange and transpiration (Rossatto et al., 2009).

From a practical point of view, these results are also extremely important because they can provide a higher survival rate of these *ex vitro* plants.

Since the palisade parenchyma is rich in chloroplasts and therefore is the primary tissue

related to photosynthesis, greater leaf thickness might contribute to greater efficiency in the photosynthetic process (Castro et al., 2009). Furthermore, the increase in chlorophyll content results in an increase in light absorption and hence higher electron transmission in the photochemical phase of photosynthesis (Taiz et al., 2017). According to Asmar et al. (2013), the absorption of silicon by seedlings and its location in the banana leaves grown *in vitro* show that this element may be directly involved with the structure of photosynthetic tissues and, therefore, be beneficial to many plant physiological processes and the adaptation to the *ex vitro* environment. Photosynthesis corresponds to the basic input of energy for plants, is essential for plant growth, and is

linked directly to the structure of leaves (Castro et al., 2009).

In this study, potassium silicate promoted an increase in the chlorophyll *a* and *a/b* ratio content compared to the control, but there were no significant differences between treatments for chlorophyll *b* and total chlorophyll content (Table 3). The chlorophyll content is related to the ability of plants to photosynthesize (Streit et al., 2005). The higher production of chlorophylls in plants grown in the presence of silica agrees with the results of (Yao et al., 2011). This ability may be related to the fact that the supply of Si can improve the bioaccumulation of Mn, which acts directly on the synthesis of chlorophyll (Marschner 2011; Patil et al. 2018).

Table 3. Chlorophyll *a* and *b*, total chlorophyll ($\text{mg}\cdot\text{g}^{-1}$ fresh weight), and *a/b* content ratio of leaves of *Musa* spp. grown *in vitro* under temporary immersion bioreactors technology supplemented with silicon. Clorofila *a* e *b*, clorofila total ($\text{mg}\cdot\text{g}^{-1}$ peso fresco) e relação do conteúdo de *a/b* em folhas de *Musa* spp. cultivadas *in vitro* sob tecnologia de biorreatores de imersão temporária suplementado com silício.

| Treatments | Chlorophylla | Chlorophyllb | Total Chlorophyll | <i>a/b</i> |
|-------------------------------------------------------------|--------------|--------------|-------------------|------------|
| Control | 0.000090 b | 0.000080 a | 0.000160 a | 0.93537 b |
| K_2SiO_3 ($1\text{mL}\cdot\text{L}^{-1}$) | 0.000134 a | 0.000096 a | 0.000214 a | 1.710425 a |
| CV (%) | 13.83 | 14.60 | 21.49 | 22.75 |

*Means followed by the same letter within columns are not significantly different by Tukey's test ($p \leq 0.05$). Médias seguidas pela mesma letra dentro das colunas não são significativamente diferentes pelo teste de Tukey ($p \leq 0,05$).

In addition, Asmar et al. (2013) and Braga et al. (2009) found that sodium and potassium silicate sources provided higher contents of chlorophyll *a*, *b* and total chlorophyll of *in vitro* banana cv. 'Maça' and *in vitro* strawberry, respectively. Similar results were found by Dias et al. (2017) who observed a linear increase in chlorophyll *a*, *b*, and total chlorophyll content using different concentrations of sodium silicate in anthurium plants cultivated *in vitro*.

Rezende et al. (2018) induced salt stress in *in vitro* cape gooseberry plants and observed that the addition of silicic acid to the culture medium with the lowest concentration of NaCl (0.5%) increased the chlorophyll *a*, *b* and total content. However, these results are different from those found in this

study since an increase only in chlorophyll *a* content and *a/b* ratio were observed. The chlorophyll content in the leaves is frequently used to estimate the photosynthetic potential. The chlorophyll *a* participates directly in the photochemical stage (the first stage of the photosynthetic process - energy transfer), chlorophyll *b* and carotenoids constitute the so-called accessory pigments that aid in the light absorption (Taiz et al., 2017).

This study observed that silicon provided greater content of chlorophyll *a* and *a/b* ratio of bananas cultivated *in vitro*. This type of chlorophyll (is?) the main one responsible for photosynthesis, therefore all other pigments are called accessory pigments. These results are likely due to the accumulation of Si in the leaf epidermis, which can

improve plant structure allowing for better light capture, increasing the concentration of chlorophylls (Barbosa et al. 2015).

As demonstrated in this study, Si addition resulted in changes in leaf anatomy and the chlorophyll content of *in vitro* bananas. The changes observed are related to improved photosynthesis and an increased functionality of the stomata. These characteristics can result in plants with enhanced vigor and tolerance to diseases, and increased survival of plantlets once transferred to the *ex-vitro* environment.

CONCLUSIONS

The potassium silicate provides increased thickness of the leaf's limb tissues, stomatal density, polar diameter/equatorial diameter ratio, increased chlorophyll *a* content and *a/b* ratio of plants of 'Dwarf Cavendish' banana cultivated in a bioreactor.

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