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Anatomical and structural changes in response to application of silicon (Si) *in vitro* during the acclimatization of banana cv. 'Grand Naine'

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Abstract

This study aimed at evaluating possible effects of silicon application during *in vitro* culture of banana plants (cv. 'Grand Naine') on structural and anatomical characteristics of leaves of acclimatized plants. Shoots previously established *in vitro* were inoculated on Murashige and Skoog (MS) medium with addition of 30 g L^{-1} sucrose and 1 mg L^{-1} NAA (naphthalene acetic acid) and supplemented with three sources of silicon (sodium silicate, potassium silicate and calcium silicate) at a dosage of 1 g L^{-1}. Medium without any source of silicate was used as a control treatment. The cultures were maintained for 45 days at the rooting stage. After this phase, the plants were transferred to the greenhouse where they were held for 60 days. The experimental design was completely randomized with five replicates (a total of fifteen plants per treatment). The presence of silicate in the culture medium favored increased stomatal density on both sides of the leaves and increased the polar diameter/equatorial diameter ratio on the abaxial side. The thicknesses of mesophyll and chlorophyll content were also enhanced in the presence of silicon, mainly when the source used was the calcium silicate. The use of sodium silicate increased the contents of cellulose, hemicellulose and tannins. The application of silicon during the *in vitro* culture provides improvements on anatomical and physiological characteristics of leaves of banana plants on acclimatization phase.

Keywords: *Musa* spp., silicates, tissue culture, micropropagation, anatomy.
Abbreviations: PD_polar diameter, ED_equatorial diameter, PD/ED_diameter polar/equatorial diameter ratio, SD_stomatal density, EAD_epidermis on the adaxial face, EAB_epidermis on the abaxial face; HAD_hypodermis of the adaxial face; HAB_hypodermis of the abaxial face; MP_mesophyll; PP_palisade parenchyma; SP_spongy parenchyma, PP/SP_palisade parenchyma/spongy parenchyma ratio.

Introduction

Banana cultivation plays an important role in the economic and social development around the world because it is considered an important food source as a high-consumption tropical fruit (Donato et al., 2006). Conventional methods of banana plantlets production that are performed in field conditions most often result in low yields (3-8 off-shoots per mother plant in a complete cycle) and they can potentially spread pests and diseases too (Roels et al., 2005). Tissue culture techniques, such as micropropagation through shoot tips, are alternatives to be used instead of traditional propagation methods. *In vitro* techniques have been successfully used because they provide a large amount of plantlets from mother plants with high genetic and phytosanitary patterns. In addition, the multiplication occurs in a short period, in small spaces and without seasonal interruption. However, the acclimatization process is an important stage that deserves special care because during it the plants will become able to go to the field (Gübbük and Pekmezci, 2004). Although the process and technology of *in vitro* buds multiplication is widely used and well-stablished for banana, there is little information about the effects of changes that can occur in the *in vitro* environment, particularly for the use of silicon (Si), or about the manifestation of these effects in the acclimatization process of plants. The acclimatization is the last step of *in vitro* culture. This phase needs special attention to be completed since it is from here that the plants will be taken to grown in field conditions. Therefore, they need to be healthy and uniform. It is known, according to the scientific community, that Si is an element that provides some benefits to the plant, such as its physical accumulation in the plant cell walls, resulting in the reduction of water loss; improvement of plant architecture; and prevention of the penetration of pathogens and insects (Pozza and Pozza, 2003; Santos et al., 2005). Another reported benefits on plants from Si application is the improvement on leaf structure (Smith et al, 2011; 2012), greater photosynthetic activity (Asmar et al, 2013), and induction of a number of metabolic reactions that affect the natural defense of plants, resulting in the formation of phenolic compounds and other chemicals, such as phytoalexins and lignins (Pozza et al., 2004). So many studies have reported that the use of Si in *in vitro* conditions

can be beneficial for vegetative propagation dependent plants due to good effects that it provides. Actually, banana is a specie that have an enormous plantlet production using micropropagated plants. So, any contribution for optimize this process and the subsequent ones becomes effectual.

Therefore, the present study was conducted to verify, through leaf anatomy and the quantification of biocompounds, the morphological differences in acclimatized plants because of the use of Si added to the culture medium *in vitro* cultivation of banana cv. 'Grand Naine'.

Results

The anatomical observations in leaves of banana cv. 'Grand Naine' micropropagated using silicon sources on culture medium showed that Si provided significant effects on the frequency and shape of stomata, thickening and organization of tissues, as well as in the chlorophylls and biocompounds formed by the cells, such as cellulose, hemicellulose and tannins.

The stomatal complex of banana leaves are tetracytic and the stomata are present on both faces of the epidermis (Figure 1), but are more frequently on the abaxial face (Table 1). Plants grown under Si sources showed a higher number of stomata per mm^2 on both faces of epidermis compared to the treatment without Si (Table 1; Figure 1). A larger polar diameter (PD) of the stomata on the adaxial face of the epidermis was observed in the presence of K_2SiO_3 and $CaSiO_3$ (29.1 and 30.0, respectively), although the equatorial diameter (ED) and the PD/ED ratio showed no significant differences (Table 2). However, on the abaxial face, which is the face with the highest number of stomata, the PD/ED ratio was significantly different from the control, with the highest values for treatments, which had Na_2SiO_3 and K_2SiO_3 in medium. The thickness of the epidermis on the adaxial face of treatments that had Si was higher when compared to the control (Table 3). The thickness on the abaxial face was already higher in leaves of plants grown in medium with $CaSiO_3$ (32.6 µm). The increased thickness of the hypodermis on the adaxial face was obvious in plants grown under K_2SiO_3 and $CaSiO_3$ (86.00 and 82.36 µm, respectively), which is a 13.2% increase compared with the other treatments (Table 3). However, we did not find any effect of Si on increase of the thickness of hypodermis on the abaxial face, so that plants grown in the absence of the element had a 33.06% thicker hypodermis. We observed a thicker palisade parenchyma (117.80 µm) when we use $CaSiO_3$ in culture medium, and this tissue increased 16.33% compared to the control (Table 3). This increase was also accompanied by an increase of 35.79% in the spongy parenchyma using the same source. $CaSiO_3$ generated increased leaf mesophyll thickness when compared to the other treatments. The anatomical observations in leaves of banana cv. 'Grand Naine' showed that leaves of plants grown in medium containing silicate, in particular $CaSiO_3$, presented a development in the leaves structure (frequency and shape of stomata, thickening and organization of tissues) with a higher potential for photosynthesis, allowing these plants to adapt to the heterotrophic environment *ex vitro*. Leaves of micropropagated plants with Si sources showed higher levels of chlorophyll a, b and total. Treatment with $CaSiO_3$ added to the medium, resulted in leaves with 22.47% more chlorophyll a (8.01 mg mL^{-1}) compared to other treatments at the end of acclimatization. Treatments with K_2SiO_3 and $CaSiO_3$ showed plants with higher levels of chlorophyll b (3.03 and 2.85 mg mL^{-1}, respectively) and total chlorophyll (10.83 and 10.86, respectively) compared to the Na_2SiO_3 and the control (Table 4). The K_2SiO_3 and $CaSiO_3$ treatments showed a significant increase in the production of chlorophyll b (40.59%) and total chlorophyll (26.24%) compared with the other treatments.

The biocompounds cellulose, hemicellulose and tannins were significantly influenced by application of different sources of Si. Higher contents of cellulose (1.9%) and tannins (838.95%) were obtained with the use of Na_2SiO_3, and this source increased by 24.21% and 36.84% respectively, when compared to the other treatments. There was a higher hemicellulose content (50.23% more) when we used any Si source compared to the control treatment. The lignin content did not differ among treatments (Table 5).

Discussion

In relation to stomatal characteristics, the observations obtained with this study agree with information published by Sandoval et al. (1994) who classified the banana as an amphi-hypostomatic species. According to Alkynyl et al. (2006), the tetracytic stomata is evident in numerous families of monocotyledons. In this type of stomatal complex, the stomata are surrounded by only four subsidiaries cells, of variable size and shape, of which two are polar and two lateral in position (Prabhakar, 2004). Changes in stomatal density may be due to environmental factors or the culture medium in which seedlings are raised (Souza et al., 2010, Grisi et al., 2008). These results demonstrate that the increase in the number of stomata was generated by the use of Si, though most likely indirectly, during cultivation *in vitro* and that this improvement was maintained at acclimatization; the plants grown without the addition of this element had smaller numbers of stomata on average. An increase in stomatal density can facilitate the flow of CO_2 into the leaf (Castro et al., 2009), which is beneficial because this gas is one of the most limiting factors for photosynthesis (Zhou and Han, 2005). The addition of silicates may have promoted an increase in photosynthetic rate, causing the plant to increase the number of stomata, and thus allowing CO_2 could more easily stream into the leaf. This increase in the number of stomata may have occurred even *in vitro* because, despite the condition of low CO_2 availability, Asmar et al. (2013) demonstrated that the addition of $CaSiO_3$ in culture medium increased photosynthesis *in vitro* in the banana cv. 'Maçã'. Si occurs more often in regions of the plant where the loss of water occurs in large quantities, i.e., in the leaf epidermis near the guard cells of stomata (Currie and Perry, 2007). Thus, a greater number of stomata and a greater deposition of Si contribute to increase the transpiration rate efficiency, i.e., less water will be lost. This fact indicates that banana could be successfully cultivated even under a water deficit. The polar diameter/equatorial diameter ratio (PD/ED ratio) is associated with the shape of the guard cells and is important for the functionality of the stomata; the elliptical shape (higher PD/ED) is characteristic of functional stomata, while the rounded shape is associated with abnormal stomata functionality (Khan et al., 2002). Thus, in this study, although the difference in the PD/ED ratio of the epidermis on the adaxial face was not significant, the stomata in the

Table 1. Stomatal density on adaxial and abaxial faces of epidermis of leaves of banana 'Grand Naine' cultured *in vitro* with different silicon sources and acclimatized for 60 days.

Source of silicon	Stomata number per mm^2	
	Adaxial face	Abaxial face
Control	19bB	94bA
$MS+Na_2SiO_3$	29aB	112aA
$MS+K_2SiO_3$	30aB	113aA
$MS+CaSiO_3$	33aB	113aA
CV (%)	26.58	19.56

Upper case letters represent significant differences between the adaxial and abaxial epidermis and lower case letters represent differences among the silicon source, according to Scott-Knott test at 5% probability

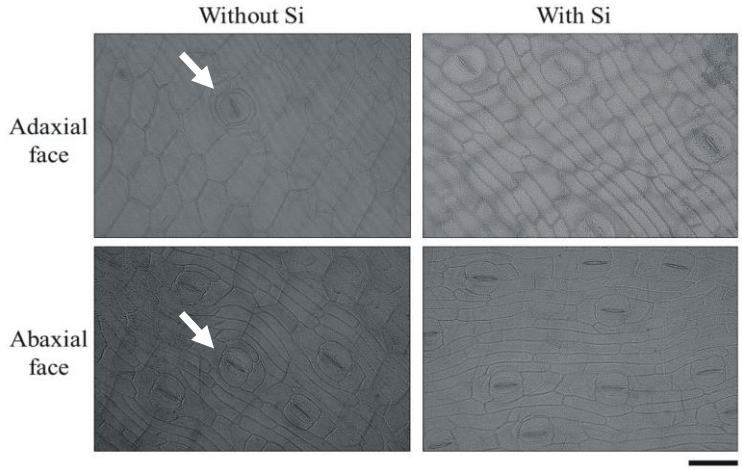

Fig 1. Photomicrographs of paradermic sections of leaves of banana plants acclimatized for 60 days, showing that the application of silicon during the *in vitro* culture contributes to increase the stomatal density of banana plants cv. 'Grand Naine' on acclimatization phase. Bar = 100 µm. Arrow: stomata tetracytic.

Table 2. Polar diameter (PD) and equatorial diameter (ED) of stomata and PD/ED ratio of banana leaves ('Grand Naine') cultivated *in vitro* with different silicon sources and acclimatized for 60 days.

Source of silicon	Adaxial face			Abaxial face		
	-------- µm--------			-------- µm--------		
	PD	ED	PD/ED	PD	ED	PD/ED
Control	24.36 b	11.97a	2.25a	27.74a	14.42a	1.95b
$MS+Na_2SiO_3$	26.16 b	10.87a	2.30a	26.17a	11.37b	2.35a
$MS+K_2SiO_3$	29.10 a	13.07a	2.29a	25.77a	10.33b	2.56a
$MS+CaSiO_3$	30.00 a	12.54a	2.42a	22.65b	12.59a	1.86b
CV (%)	11.65	21.47	15.76	10.37	18.15	18.56

PD = polar diameter; ED = equatorial diameter; PD/ED = ratio polar and equatorial diameter.
Means followed by the same letter in columns do not differ by the Scott-Knott test at 5% probability.

Table 3. Thickness of leaf tissues of banana ('Grand Naine') cultivated *in vitro* with different sources of silicon and acclimatized for 60 days.

Source of silicon	EAD	EAB	HAD	HAB	PP	SP
	---------- µm----------					
Control	19.56b	29.11b	74.65b	84.44a	98.56b	43.49b
$MS+Na_2SiO_3$	25.53a	29.73b	79.05b	59.80b	99.48b	32.63c
$MS+K_2SiO_3$	27.53a	29.01b	86.00a	64.85b	102.18b	34.05c
$MS+CaSiO_3$	26.08a	32.60a	82.36a	56.52b	117.80a	50.82a
CV (%)	31.40	15.43	10.68	13.52	12.38	18.34

EAD = epidermis on the adaxial face; EAB = epidermis on the abaxial face; HAD = hypodermis on the adaxial face; HAB = hypodermis on the abaxial face; PP = palisade parenchyma, SP = spongy parenchyma.
Means followed by the same letter in columns do not differ by the Scott-Knott test at 5% probability.

Table 4. Chlorophyll a, b, total and a/b ratio of leaves of banana 'Grand Naine' cultivated *in vitro* with different silicon sources and acclimatized for 60 days.

Source of silicon	Chlorophyll (µg mL^{-1})			
	a	b	Total	a/b
Control	7.04b	2.46b	9.50b	2.86b
MS+Na_2SiO_3	6.21c	1.80c	8.01c	3.57a
MS+K_2SiO_3	7.80b	3.03a	10.83a	2.58b
MS+$CaSiO_3$	8.01a	2.85a	10.86a	2.80b
CV%	0.71	2.80	0.78	2.96

Means followed by the same letter in columns do not differ by the Scott-Knott test at 5% probability

Table 5. Levels of cellulose, hemicellulose, lignin and tannin in banana leaves ('Grand Naine') cultivated *in vitro* with different silicon sources and acclimatized for 60 days.

Silicon source	Cellulose	Hemicellulose	Lignin	Tannin
	---------- % ----------			
Control	1.44c	1.05b	1.14a	532.61c
MS+Na_2SiO_3	1.90a	1.83a	1.18a	838.95a
MS+K_2SiO_3	1.72b	1.93a	1.25a	748.27b
MS+$CaSiO_3$	1.73b	2.11a	1.12a	529.87c

Means followed by the same letter in columns do not differ by the Scott-Knott test at 5% probability.

treatments with K_2SiO_3 and $CaSiO_3$ were more elongated compared to the other treatments. Positive results for the use of Si were also found by Braga et al. (2011), in terms of increased number of stomata, polar diameter and PD/ED ratio in the micropropagation of pineapple cv. 'Gomo-de-mel'. Asmar et al. (2013) also found interesting results for *in vitro* culture of banana cv. 'Maçã', where the use of K_2SiO_3 resulted in a higher polar diameter and PD/ED ratio for both faces of epidermis. Plants that presents increased tolerance to drought have xeromorphic characteristics, such as higher stomatal density and stomata with smaller size. These characteristics allow stomata to stay open for a shorter time but still capture the same amount of CO_2 (Castro et al., 2009). Thus, the addition of K_2SiO_3 may have promoted the formation of much smaller stomata in the adaxial and abaxial faces of epidermis in greater numbers. Interestingly, this result during the acclimatization period, would allow a greater flow of CO_2, stimulate drought tolerance, and still be a physiological characteristic that could optimize the productivity of the species. The main 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 cells of the epidermis, stomata and trichomes (Currie and 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 because the deposition of Si near the cuticle of leaves provides protection to plants by reducing the transpiration rate (Currie & Perry, 2007). Investment in thickness of the epidermis on both faces was also observed in banana leaves of the cultivar 'Maçã' with the use of $CaSiO_3$; according to the authors of that study, this feature can assist in the acclimatization of the species (Asmar et al., 2013). The hypodermis tissue may be specialized in storing water or acting as a barrier to excessive transpiration (Castro et al., 2009). Therefore, the thicker hypodermis of the adaxial face observed in treatments containing K_2SiO_3 and $CaSiO_3$ can protect the leaves against excessive water loss because radiation directly contacts this surface, compared with the hypodermis on the abaxial face, which has less tissue thickness, and promotes a structure to endure more xeric conditions. Shade leaves generally present thinner mesophyll than sun leaves, as thickening of the mesophyll is a feature that contributes to xeromorphic sclerophylly (Castro et al., 2009; Boeger and Wisniewski, 2003). However, this characteristic varies depending on environment in which plants are grown (Grisi et al., 2008; Batista et al., 2010; Souza et al., 2010). So, the addition of $CaSiO_3$, even *in vitro* phase, caused changes in the thickening of the mesophyll (palisade and spongy parenchyma), and this could contribute to development of sclerophyllous leaves during acclimatization, which provide features similar to xeromorphic leaves and changes the internal structure, favoring leaves of banana 'Grand Naine'. Inferences about the beneficial effects of silicon *in vitro* were performed initially with orchid (Soares et al., 2012) and subsequently with banana cv. 'Maçã' (Asmar et al., 2013). Our study demonstrated that changes induced *in vitro* can be expressed in *ex vitro* conditions. The increase in thickness of the mesophyll is important because specialization of chlorophyll tissue leads to improvement in photosynthetic efficiency of the plant since these tissues contain the most chloroplasts (Castro et al., 2009). The increase in chlorophyll production in plants grown with addition of Si is in agreement with the results reported by Yao et al. (2010), Asmar et al. (2013) and Braga et al (2009). Chlorophylls tend to accumulate, particularly in the palisade parenchyma, and they are the key molecule involved in photosynthesis. Additionally, the photosynthetic capacity is related to the amount of chlorophyll present in the tissues of leaves (Castro et al., 2009). Thus, both the increase in thickness of the spongy and palisade parenchyma may have been due to an increase in the rate of photosynthesis, which, in turn, led to changes in anatomical structure, and most likely due to the most efficient use of CO_2, thereby generating chlorenchyma rich in chlorophyll. The presence of Si has been found to reduce various types of abiotic and biotic stresses, leading to the incorporation of silicates in many fertilizers (Currie and Perry, 2007). The mechanisms of the beneficial effect of Si have not been fully elucidated, although some studies have suggested a role in physical and/or biochemical defense systems (Fauteux et al., 2005). It is known that Si acts in the natural defense of plants, resulting in the formation of tannins and other chemicals such as lignins (Keeping and Kvedaras, 2008). Thus, in this study, Si participated directly or indirectly in the synthesis of cellulose, hemicellulose and tannins because its presence in the medium led to higher levels of these biomolecules in leaves of banana 'Grande Naine'. These plants, besides presenting more anatomically

efficient structures for gathering CO_2 as noted previous, had chemical substances on plant resistance to pathogens, once the limitation to action of them was generated by forming a physical barrier.

Materials and Methods

The study was conducted in the Laboratories of Plant Tissue Culture (Department of Agriculture), Plant Anatomy (Department of Biology) and Plant Products (Department of Food Science) at the Federal University of Lavras, MG, Brazil.

Culture medium

The medium used was MS (Murashige and Skoog, 1962), with the addition of 30 g L^{-1} sucrose and 1 mg L^{-1} NAA (naphthalene acetic acid), and after solidified with 1.8 g L^{-1} Phytagel™. Three sources of Si were tested: sodium silicate (Na_2SiO_3), potassium silicate (K_2SiO_3) and calcium silicate ($CaSiO_3$), at a dose of 1 g L^{-1}. Medium without any source of Si was used as control. The pH was adjusted to 5.8 before autoclaving at 121 °C and 1.2 atm for 20 minutes.

Explants and cultivation environment

Shoots of banana cv. 'Grand Naine', obtained by culture of stem apices and multiplied *in vitro* in a laminar flow chamber, were inoculated in 200 mL bottles containing 30 mL of MS medium modified according to the silicate treatments. The bottles were sealed with polypropylene caps and plastic parafilm. After inoculation, plants were kept in a conventional growth room with a photoperiod of 16 h, at 25 ± 2°C, and with light intensity of 52.5 W m^{-2} s^{-1} provided by white fluorescent lamps. After 45 days of *in vitro* culture, the plants were removed from the bottles and washed in running water to remove excess culture medium from the roots. Immediately the plants were transferred individually to 0.3 L tubetes filled with Plantmax® commercial substrate.

The plants stayed in a greenhouse for 60 days (corresponding to the months of July and August) and covered with transparent polyethylene film (150 microns) with 70% shade and an intermittent mist system. The experimental plot consisted of three plants (one per tube), with five replicates, in a total of fifteen plants per treatment in a completely randomized design (CRD) with four treatments (the same ones used during *in vitro* phase).

Anatomical analyzes

For anatomical studies, leaves collected from five different plants per treatment were fixed in FAA 70% (formaldehyde, acetic acid, ethyl alcohol 70%) (Johansen, 1940) for 72 hours and thereafter kept in 70% ethanol ($v.v^{-1}$). The middle third of the second fully expanded leaf was the default region to obtain the sections. The cross sections were obtained using a microtome type LPC table, and parademic sections were made by freehand using a steel blade. After sectioning the material, samples were clarified with sodium hypochlorite (1% - 1.25% active chlorine), followed by a triple-rinsed in distilled water, stained with safrablau solution (astra blue 0.1% and safranin 1%) for cross sections, and 1% safranin for parademic sections. The process was completed with the sections assembled into semi-permanent slides with glycerol 50% (v/v) (Kraus & Arduin, 1997). The slides were observed and photographed under a light microscope (model Olympus BX 60) coupled with a Canon A630 digital camera. The images were analyzed using the image analysis software UTHSCSA-ImageTool, with the measurement of five fields per replicate for each variable analyzed. The following anatomical features were evaluated: stomatal density (number of stomata per mm^2) on the adaxial and abaxial faces, polar (PD) and equatorial (ED) diameters of the stomata; polar diameter/equatorial diameter ratio (PD/ED) of stomata; thickness of epidermis on the adaxial face (EAD) and abaxial face (EAB); hypodermis of the adaxial face (HAD) and abaxial face (HAB); mesophyll (MP); palisade parenchyma (PP); spongy parenchyma (SP), and the ratio of the palisade and the spongy parenchyma (PP/SP). The design was completely randomized with 25 fields per treatment. Data were analyzed using the statistical program Sisvar 4.3 (Ferreira, 2011) and means were compared using the Scott-Knott test at 5% probability.

Determination of chlorophylls content

Contents of chlorophyll a, b and total were quantified according to the method of Arnon (1949). Five leaves of each treatment were collected, and 0.5 g of leaf tissues were macerated in liquid nitrogen and solubilized in 80% acetone. The material was centrifuged at 8000 xg for 15 minutes. The supernatant was collected for determination of the contents of pigments using a spectrophotometer (663 nm and 645 nm).

Determination of contents of cellulose, hemicellulose, lignin and tannin

The contents of cellulose, hemicellulose and lignin were determined using the method of Soest (1967). The extract for the quantification of tannins was obtained following the method of Deshpande et al. (1986) and was performed by colorimetric determination (Association of Official Analytical Chemists, 1990).

Conclusions

The use of Si *in vitro* results in structural and anatomical changes that are beneficial to banana plants cv. 'Grand Naine' during the acclimatization process. Silicates act positively in relation to stomatal density on both faces of leaf, as well as provide an increased polar diameter/equatorial diameter ratio of stomata. The mesophyll and its tissues are also favored by the addition of Si to the culture medium, especially when the source used is $CaSiO_3$. Silicates also show their activity in the increased formation of biocompounds, especially in the presence of Na_2SiO_3. The application of Si during *in vitro* culture of banana cv. 'Maçã' is recommended because its effects are beneficial for plants during acclimatization phase.

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