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CROP SCIENCE

Acclimatization of Sweet Potatoes Under *in vitro* Application of Diatomaceous Earth

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Abstract: Diatomaceous earth is an organic naturally occurring material rich in silicon. This silicon source can be used in organic agriculture, it also has a great potential of use in the acclimatization of crops. However, there are no reports of the effects of diatomaceous earth supplementation on the micropropagation of sweet potato. Thus, the objective of this study was to evaluate the effects of different concentrations of diatomaceous earth applied *in vitro* on the growth, physiology and anatomy of sweet potato cv. 'Brazlândia Branca' after acclimatization. Four concentrations of diatomaceous earth. After 30 days of *in vitro* growth, the plants were transferred to a greenhouse for acclimatization. After 45 days, leaf number, shoot and root length, fresh and dry shoot and root mass, gas exchange, chlorophyll content, root and leaf anatomy. The experimental design was completely randomized. The supplementation of diatomaceous earth in the *in vitro* cultivation had beneficial effects, increasing the accumulation of mass, improving the photosynthetic apparatus and promoting favorable anatomical characteristics during the acclimatization of the sweet potato plants. In addition, the use of diatomaceous earth achieved adequate seedling development, with higher seedling quality and resistance to biotic and abiotic effects than attained with control treatment.

Key words: *Ipomoea batatas,* Silicon, Plant anatomy, Tissue Culture.

INTRODUCTION

Sweet potato (*Ipomoea batatas*) is a popular crop grown in all Brazilian states. Due to its roots, hardness, high adaptability and productivity, it has a great economic, agricultural and social importance (Flores et al. 2015). This plant originated in Central and South America (Loebenstein 2009).

Sweet potato is propagated via branches or stems and can be produced even under difficult growth conditions. However, some factors can decrease productivity, such as pests and diseases. In the cultivation of sweet potato, 270 species of insects that can attack the crop in the field or in storage have been recorded. Although high levels of losses are not common in well-managed crops, the damage can be high under poor crop management (Wanderley et al. 2004). Some insects cause damage to the leaf surface, roots and pulp (Oliveira et al. 2015).

There are reports in the literature that silicon (Si), in its amorphous silica form, accumulates in the cell wall, forming a protective layer that acts as a barrier of mechanical resistance to the invasion of diseases and pests (Barbosa Filho et al. 2001). This element has high potential in agriculture because its benefits have direct effects on crop productivity, improving significantly plant resistance against pests and diseases (Alves et al. 2015, Reynolds et al. 2016).

Studies of the effects of Si applied to micropropagated sweet potato are scarce. However, studies of *in vitro* cultivation with silicon have been conducted with other crops, such as banana (*Musa* spp.) (Asmar et al. 2013a), strawberry (*Fragaria* x *ananassa*) (Braga et al. 2009), *Anthurium (Anthurium andraeanum)* (Dias et al. 2017), Billbergia (*Billbergia zebrina)* (Martins et al. 2018), yam (*Dioscorea* spp.) (Rodrigues et al. 2017), and orchids (Orchidaceae) (Soares et al. 2011). These studies show that changes to the culture medium, such as the addition of silicon sources, can have beneficial effects on plants. This element also causes structural and physiological changes during acclimatization, improving the photosynthetic apparatus (Asmar et al. 2013b).

The acclimatization process is a critical stage of micropropagation, which is the passage from the *in vitro* phase to the greenhouse, during which the seedlings are influenced by water, phytosanitary treatment, nutrient absorption and photosynthesis, causing variation in plants growth (Silva et al. 2011). During this process, it is necessary for the seedlings to decrease the environments effects and improve the conditions for their growth and development.

Silicon has characteristics beneficial to plants, such as its accumulation in the cell wall; this accumulation creates a barrier that reduces water loss by evapotranspiration and improves plant adaptation to water stress, reducing the demand of plants for water and increasing its resistance to drought (Sangster et al. 2001). During acclimatization, this characteristic lowers the percent mortality of micropropagated plants due to reduced water loss and increased photosynthetic capacity and growth (Epstein 1999).

Diatomaceous earth, as an organic and naturally occurring source of silicon, is used in organic agriculture. It reduces the amount of chemical inputs required during cultivation and improves the growth and development of plants. However, there is little research on the growth and development of horticultural crops treated with diatomaceous earth, which is mainly used for seedling acclimatization.

As diatomaceous earth is a natural compound with high silicon content that can benefit the growth and development of plants and in light of the scarcity of information on its use with sweet potato, the present study aimed to evaluate the effects of different concentrations of diatomaceous earth during *in vitro* cultivation on the growth, physiology and anatomy of sweet potato cv. 'Brazlândia Branca' after acclimatization.

MATERIALS AND METHODS

Plant materials and growth environment

The plant materials in this study were plants propagated *in vitro* through the cultivation of meristems. Parent plants of the cultivar 'Brazlândia Branca' were obtained from the Department of Agriculture (Departamento de Agricultura, DAG) of the Federal University of Lavras (Universidade Federal de Lavras, UFLA), Lavras-MG. At the DAG/UFLA Plant Tissue Culture Laboratory, the plants were multiplied using nodal segments 0.5 cm in length. The segments were inoculated in test tubes containing 15 mL of MS medium (Murashige & Skoog 1962) supplemented with 60 g L^{-1} sucrose and 5.5 g L^{-1} agar, with pH adjusted to 5.8. The tubes were then autoclaved at 121 °C and 1.0 atm for 20 min. Subsequently, the tubes were maintained in a growth chamber for 30 days under a photoperiod of 16 h and a temperature of 25 ± 2 °C, with artificial lighting provided by a white LED lamp yielding a mean irradiance of 49.4 µmol m^{-2} s^{-1}.

In vitro assay

After the period in the growth chamber, the plants were used as suppliers of explants for the assays. Each nodal segment, at the same size, was inoculated in a test tube containing 15 mL of either MS culture medium supplemented with diatomaceous earth at concentrations of 1, 2, 3 or 4 g L^{-1} or MS medium without diatomaceous earth as a control. Each tube was supplemented with 60 g L^{-1} of sucrose and 5.5 g L^{-1} of agar, with pH adjusted to 5.8, and then autoclaved at 121 °C and 1.0 atm for 20 min. Subsequently, the tubes were maintained in a growth chamber with the same environmental conditions mentioned above.

Acclimatization

After 30 days of *in vitro* cultivation, the plants were removed from the flasks, washed in running water to remove the excess culture medium adhered to the roots, and immediately transferred to 1L pots filled with commercial Tropstrato® substrate. The plants were placed in a greenhouse covered with transparent polyethylene film (150 microns) and 70 % shade cloth and equipped with an intermittent misting system. The plants were maintained in the greenhouse for 45 days, corresponding to the months of September and October.

Plant Phytotechnical analysis

After 45 days, leaf number, shoot and root length (cm), fresh and dry mass of the shoot (g) and fresh and dry mass of the roots (g) were analyzed. The dry mass of the plant material was analyzed after drying in an oven to constant weight at 60 °C for 72 h.

Gas exchange and chlorophyll determination

Gas exchange was measured with an IRGA Infra-Red Gas Analyzer (Licor - 6400XT, Li - Cor, Lincoln, NE, EUA). Completely expanded leaves from eight plants per treatment were selected, and measurements were started at 08h. The photosynthetic photon flux density in the analyzer chamber was fixed to 1000 µmol m^{-2} s^{-1}. The following measurements were recorded: photosynthetic rate *(A) in µmol* CO_2 m^{-2} s^{-1}, transpiration rate *(E) in mol* H_2O m^{-2} s^{-1}, stomatal conductance (*gs*) in *mol* m^{-2} s^{-1} and internal carbon (*Ci*).

For the determination of chlorophyll content, a ClorofiLOG meter (model Cfl 1030) was used, and the measurements were performed on two fully expanded leaves of each plant. The analyses were performed at 08 h am, and the chlorophyll *a* was evaluated.

Anatomical analysis

For the anatomical analysis, the median region of the second fully expanded leaf was used. Samples were collected from four different individuals of each treatment. Subsequently, the samples were transferred to flasks with 70 % alcohol, dehydrated in an alcohol series (70, 80, 90 and 100 %) for 2 h per alcohol concentration and then dried for 15 min under vacuum. Next, the samples were maintained for 24 h in a preinfiltration resin solution composed of 100 % ethanol and liquid resin base (1:1) and then embedded with the HistoResin kit (Leica Microsystems, Heidelberg, Germany).

Cross sections with a thickness of 8 µm were obtained with a semiautomatic rotating microtome, stained with toluidine blue and washed with distilled water to remove excess dye. Subsequently, the slides were mounted with stained glass varnish as mounting medium.

The slides were observed and photographed under an optical microscope (Motic BA 210) with a coupled camera (Moticam 5). For each replicate, ten photographs were taken using six slides of cross sections. The images were analyzed using Motic Images Plus 3.0 image analysis software.

Leaf thickness was assessed using adaxial epidermis thickness (AdET), abaxial epidermis thickness (AbET), palisade parenchyma thickness (PPT), spongy parenchyma thickness (SPT), mesophyll thickness (MT) and leaf blade thickness (LBT). To determine the thickness of root tissues, the following were evaluated: total area (TA), central cylinder area (CCA), epidermis thickness (ET) and cortical parenchyma thickness (CPT).

Silicon concentration

The plant material used to determine silicon concentration was dry shoot, which was attained by drying shoots to constant weight in a ventilated oven at 60 °C for 72 h; after this step, the shoots were ground. The determination of silicon was performed using the methodology proposed by Korndörfer (2004). A total of 0.1 g of sample (dry, ground shoots) was placed in a polyethylene tube, to which 2.0 mL of 30 % (v/v) of H_2O_2 and 3.0 mL of NaOH were added (25 mol L^{-1}). The tubes were sealed and autoclaved for 1 h at 123 °C and 0.15 MPa. After cooling, deionized water was added to complete volume. A 1-mL aliquot of the extract was transferred to a 20-mL flask, and deionized water was added to complete volume. The concentration of silicon was determined by spectrophotometric analysis at 410 nm of the molybdosilicic acid yellow color formed after the reaction between silicon and ammonium molybdate in HCl medium.

Statistical analysis

The experimental design was completely randomized, with four replicates (two seedlings per replicate). The data were subjected to analysis of variance (ANOVA), and means were separated using the Tukey test at 5% probability or by regression according to model fit. The statistical analyses were performed with R software v. 3.2.2 (R Development Core Team 2009).

RESULTS AND DISCUSSION

The diatomaceous earth of various concentrations influenced the growth of acclimated sweet potato seedlings. The silicon levels in the shoot of sweet potato increased with the addition of diatomaceous earth, with an estimated maximum level (5 g kg^{-1}) observed at a concentration of 2.58 g L^{-1} of diatomaceous earth (Figure 1a). Relative to control treatment, 2 g L^{-1} of diatomaceous earth, yielded a 35% increase in silicon in the shoots of plants (Figure 1c). Following the application of calcium silicate in the *in vitro* cultivation of the Grande Naine banana plant (*Musa* spp.), Asmar et al. (2013b) observed an increased silicon content in the shoots of acclimatized seedlings. Similar results were obtained by Almeida et al. (2009) in calla lilies, with silicon yielding an increase in the nutrient content in plant leaves. In a study with bromeliad (*B. zebrina*), in which sodium silicate was used as the silicon source, Martins et al. (2018) found that treated plants exhibited an increase in silicon absorption, resulting in 92% more silicon in treated plants than in controls.

The silicon present in plants of treatments that did not receive diatomaceous earth possibly originated from the water used in the experiment. However, this possibility could not be confirmed, because the water used to prepare the culture medium or used in the irrigation system in the greenhouse was not tested for silicon. According to Lazzerini & Bonotto (2014), silicon is a very abundant element in nature, being present everywhere, including in water. The amount of absorbed silicon in the control plants was low relative to the amounts in plants from treatments that received diatomaceous earth.

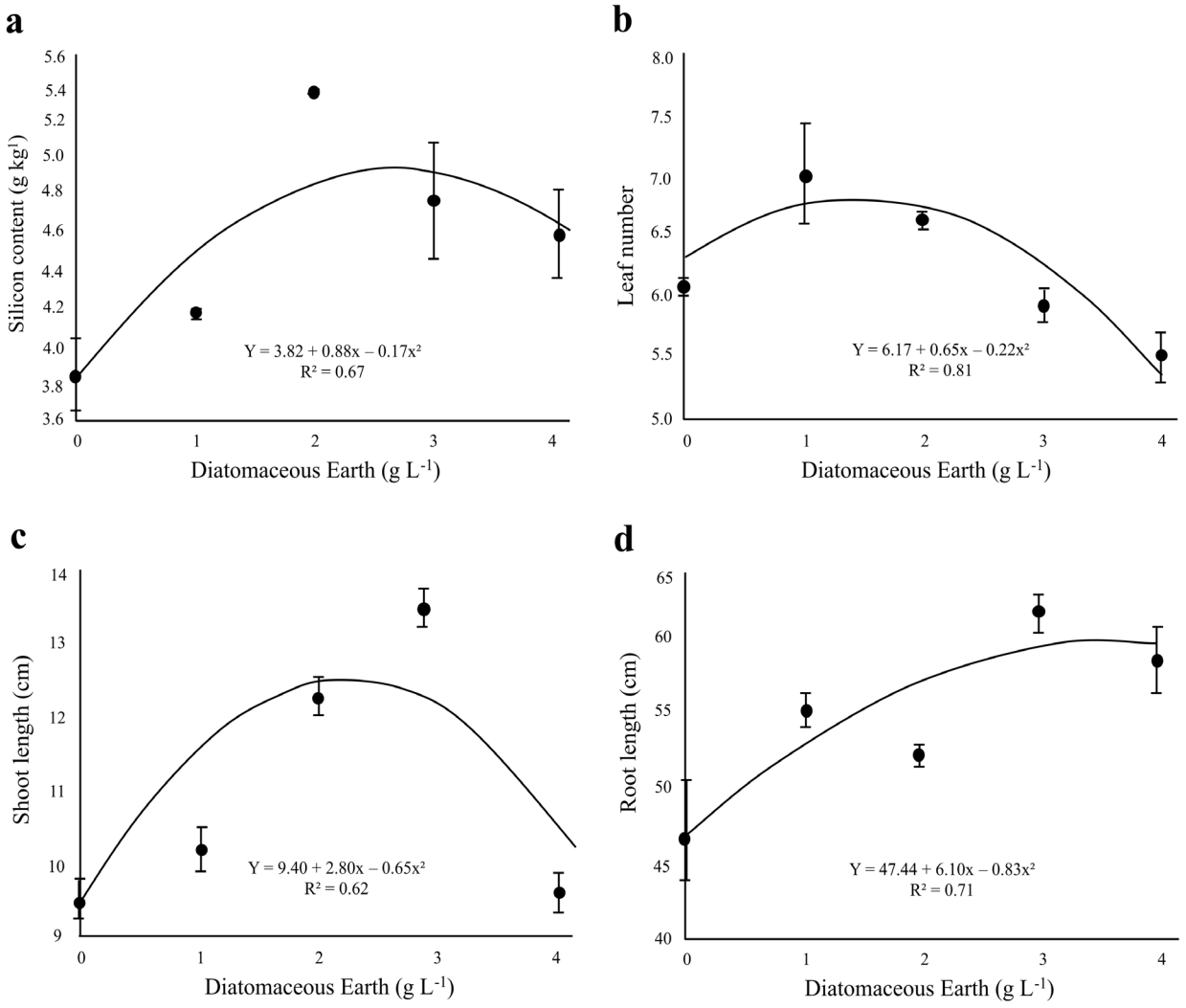

Figure 1. (a) Silicon content, (b) leaf number, (c) shoot length and (d) length of the largest root in sweet potato plants subjected to different concentrations of silicon (diatomaceous earth) and acclimated for 45 days.

There were significant effects of the concentration of diatomaceous earth on the leaf number (Figure 1b), shoot length (Figure 1c) and length of the largest root (Figure 1d), with maximum values achieved with estimated additions of 1.52, 2.08 and 3.67 g L^{-1}, respectively, of diatomaceous earth. Similar results were obtained by Dias et al. (2017), who observed increases in leaf number and shoot length in response to the addition of sodium silicate in the *in vitro* cultivation of anthurium. Furthermore, Pasqual et al. (2011) observed a similar response in leaf number in two orchid species when calcium silicate was used as the silicon source. When studying the effect of the type of silicon source added to MS medium for yam micropropagation, Rodrigues et al. (2017) observed differences in the leaf number between silicon-supplemented medium and control medium.

The addition of diatomaceous earth enhanced mass accumulation in the shoots of the sweet potato plants, with maximum accumulation of fresh (Figure 2a) and dry mass (Figure 2c) achieved under the estimated addition of 2.16 and 2.44 g L^{-1}, respectively, of

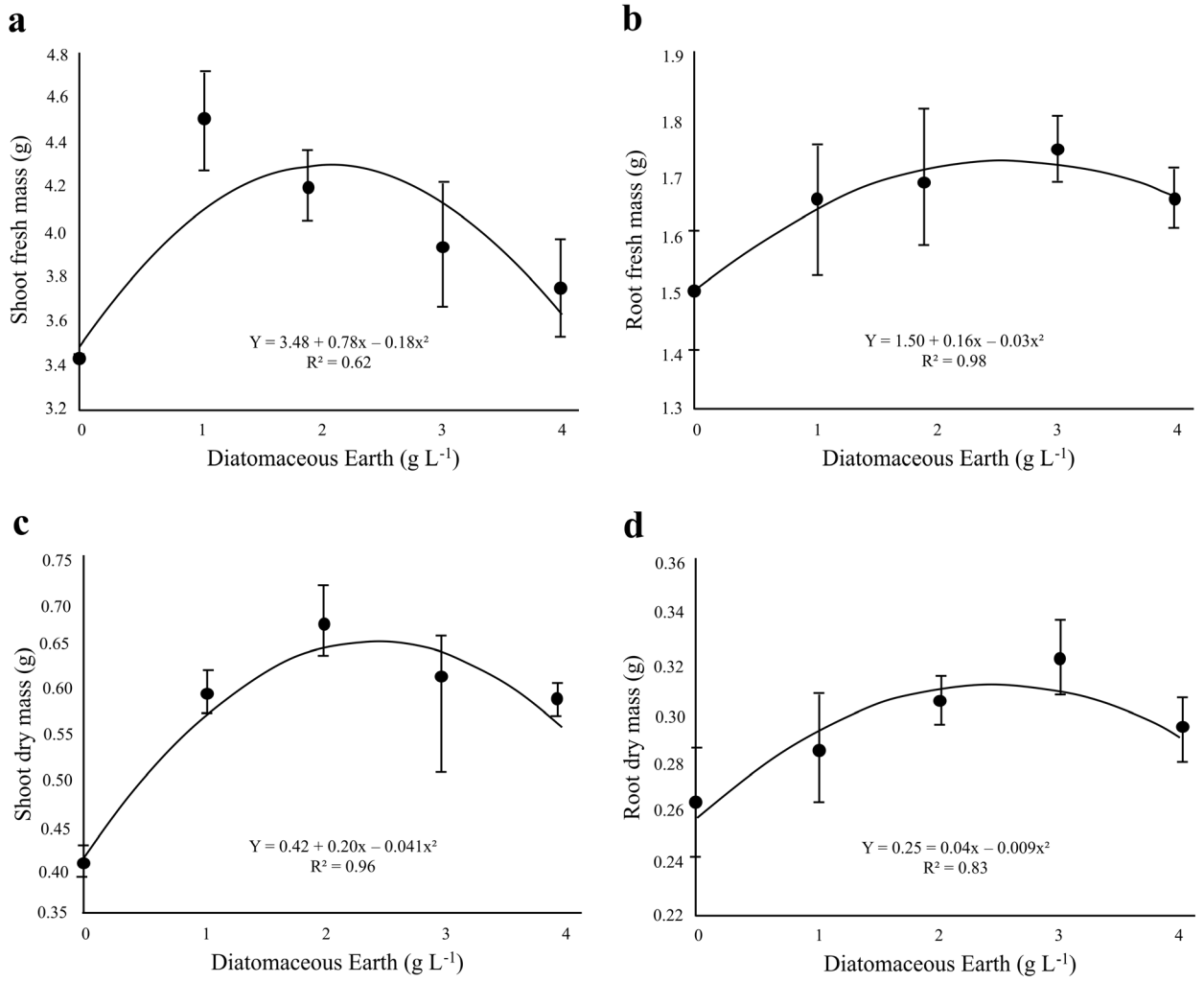

Figure 2. (a) Shoot fresh mass, (b) root fresh mass, (c) shoot dry mass and (d) root dry mass of sweet potato plants subjected to different concentrations of silicon (diatomaceous earth) and acclimated for 45 days.

diatomaceous earth. The use of this material at a dosage of 1 g L^{-1} increased fresh shoot mass (Figure 2a) relative to the control amount by 37 %. Relative to that under control treatment, the dry shoot mass (Figure 2c) under a concentration of 3 g L^{-1} of diatomaceous earth was increased by almost 67 %.

Root fresh (Figure 2b) and dry mass (Figure 2d) increased with increasing diatomaceous earth supplementation up to 2.66 and 2.22 g L^{-1} of the product, respectively, demonstrating a quadratic relationship with diatomaceous earth concentration, and achieving gains of 15 % and 23 % relative to the control values. Asmar et al. (2013b) found increased shoot levels of silicon in banana with the addition of calcium silicate to the *in vitro* growth medium, which also favored higher mass accumulation in the shoots and roots of the plants.

Decrease in mass were observed at higher concentrations of diatomaceous earth, possibly because any nutrient, including silicon, taken in excess may cause nutritional imbalances (Dias et al. 2017). However, there is a lack of studies in the literature that approaches the deleterious effects regarding the excess of silicon in the plants.

The mass results indicate that diatomaceous earth provides several benefits to plant development, increasing the capacity of nutrient absorption, producing seedlings of higher quality and with greater resistance to biotic and abiotic effects, thereby increasing the chances of cultivation success. Thus, diatomaceous earth can lead to higher seedling production, shorter cultivation time and, consequently, higher producer profit. Sandhya et al. (2018) found that the use of silicon increased the grain yield of rice plants, grown in pots immersed and at field capacity, as the doses of diatomaceous earth increased. However, studies about the effects and absorption mechanisms still need to be clarified.

The photosynthetic efficiency (*A*) of the plants increased when the plants were cultivated in the presence of diatomaceous earth, with the highest efficiency observed at the concentration of 2 g L^{-1} (Figure 3d). The maximum point estimated by the regression curve for this trait was 2.16 g L^{-1}. This increase was a result of the increased stomatal conductance (*gs*) (Figure 3c) with the increase in internal carbon (Figure 3a) concentration and the higher content of

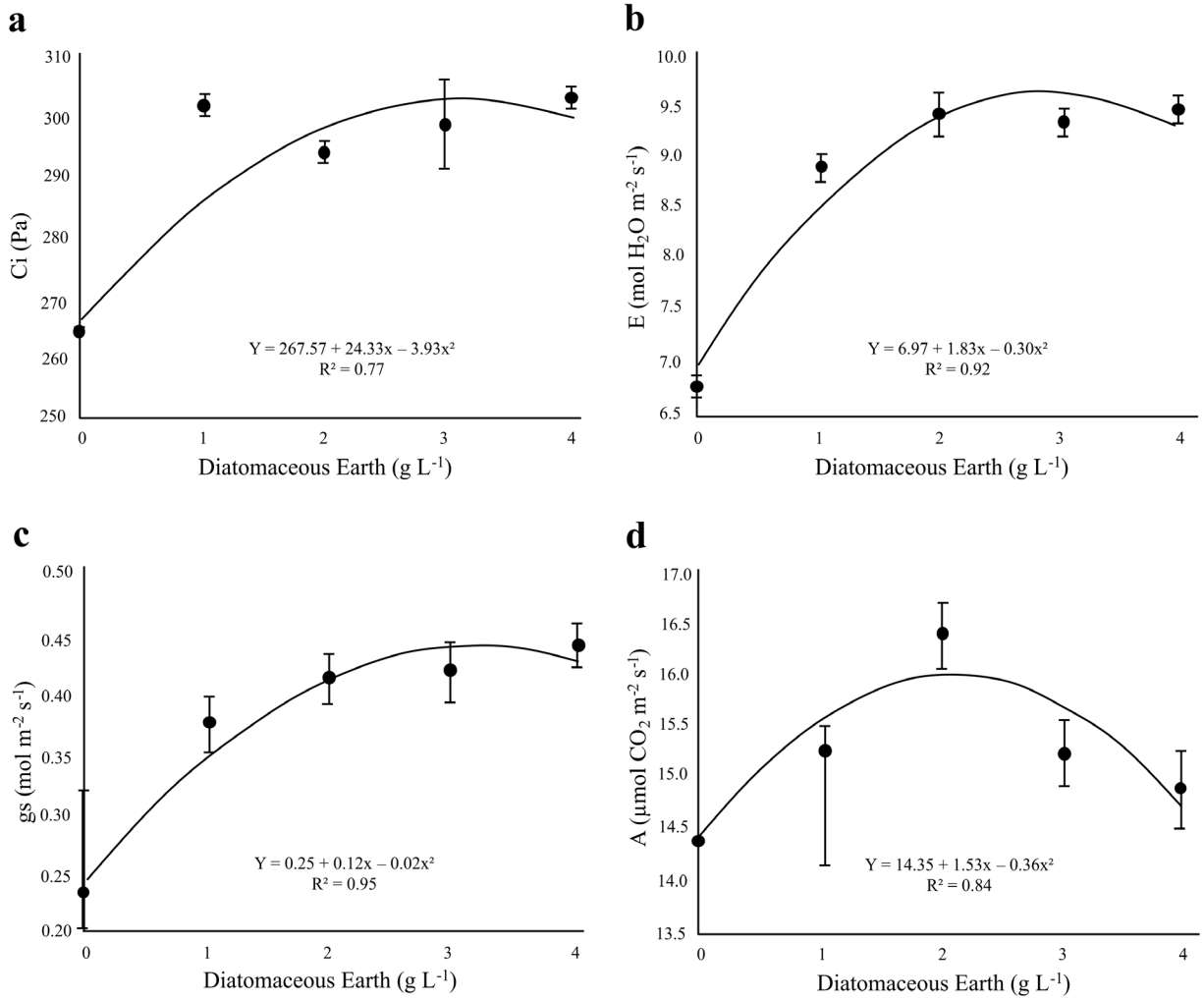

Figure 3. (a) Internal carbon (Ci); (b) transpiration (E); (c) stomatal conductance (gs) and (d) photosynthesis (A) in sweet potato plants subjected to different concentrations of silicon (diatomaceous earth) and acclimated for 45 days.

chlorophyll *a* (Figure 4). Plant transpiration (*E*) (Figure 3b) was also increased in the presence of diatomaceous earth, with the highest rates observed at concentrations above 2 g L^{-1}. The pattern of *E* was consistent with that of *gs*, demonstrating a connection between these processes, transpiration is important in the regulation of internal temperature and affects plant growth by controlling the flow of sap within the plant.

Stomatal conductance regulates gas exchange and is directly related to the photosynthetic process, thereby influencing plant growth and development (Paiva et al. 2005). The results of the present study revealed a higher photosynthesis rate and greater stomatal conductance under treatment with diatomaceous earth than under control treatment, revealing an influence of diatomaceous earth on gas exchange.

The amount of internal carbon (*Ci*) was positively influenced by the addition of diatomaceous earth; a higher concentration of *Ci* is associated with a greater carboxylation capacity of plants, which might have contributed to the increased photosynthetic rate observed in plants grown in the presence of diatomaceous earth. An increase in plant *gs* in response to diatomaceous earth was also observed; gs directly influences several parameters, such as *Ci*, *A* and *E*. Greater stomatal opening is associated with an increased capacity of gas exchange, facilitating CO_2 influx to the plant, which increases the levels of *Ci*, and the outflow of H_2O to the external environment, contributing to higher transpiration rates, as seen in the graphs below. Pinto et al. (2012) applied potassium silicate to a cocoa crop and found that with 3 ml L^{-1} potassium silicate, the photosynthetic rate was increased by 44 % relative to the control rate, whereas stomatal conductance, transpiration and internal carbon were not influenced by silicon addition.

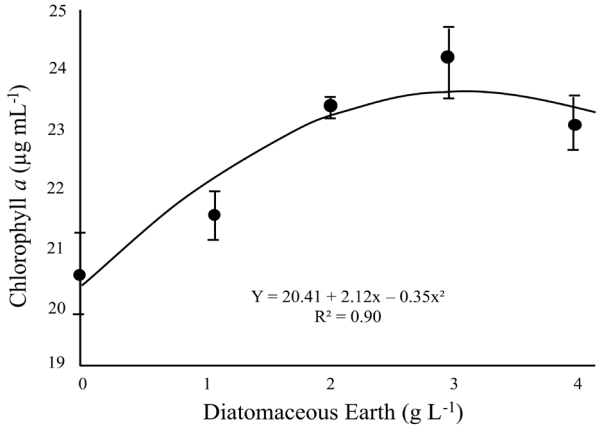

Figure 4. Chlorophyll *a* content of sweet potato plants subjected to different concentrations of silicon (diatomaceous earth) and acclimated for 45 days.

Chlorophyll content increased with increasing concentration of diatomaceous earth from 2 g L^{-1} and above (Figure 4). This result is consistent with the higher photosynthetic rate observed in plants grown in the presence of diatomaceous earth, as the presence and concentration of chlorophyll are directly related to the photosynthetic process. Chlorophyll *a* is the pigment directly involved in the photochemical phase of photosynthesis, according to Asmar et al. (2013b), the absorption of silicon brings several benefits, such as an increase in chlorophyll levels, thereby improving photosynthetic efficiency.

The treatments with higher photosynthetic rates, transpiration rates and chlorophyll contents had the highest accumulation of mass. Mass accumulation directly influences the growth and development of the plant throughout the cultivation cycle, culminating in higher productivity at the end of the plant cycle.

The results of the present study demonstrated the efficacy of silicon application in enhancing chlorophyll content in sweet potato (*Ipomoea batatas*), consistent with findings of Al-aghabary et al. (2005), who observed an increase in chlorophyll content in response to

silicon supplementation. Similarly, Braga et al. (2009) reported higher chlorophyll contents in 'Oso Grande' strawberry (*Fragaria* x *ananassa*) when the medium was supplemented with a silicon source.

Analysis of the cross sections of the leaves of sweet potato (*Ipomoea batatas*) seedlings grown *in vitro* and acclimatized in a greenhouse revealed significant effects of diatomaceous earth on all measures of leaf tissue thickness at all concentrations (Figure 5). The use of diatomaceous earth at 3 g L^{-1} resulted in thicker adaxial epidermis (Figure 5a) than did other treatment concentrations, and a significant difference was observed between the control treatment and the 1 g L^{-1} treatment. Relative to control treatment, the 3 g L^{-1} concentration of diatomaceous earth increased the adaxial epidermis thickness by 34% (Figure 5a).

Greater abaxial epidermis thickness was observed with the dose of diatomaceous earth at 1 and 2 g L^{-1} than under the other concentrations; however, significant differences were observed only between these treatments and the control

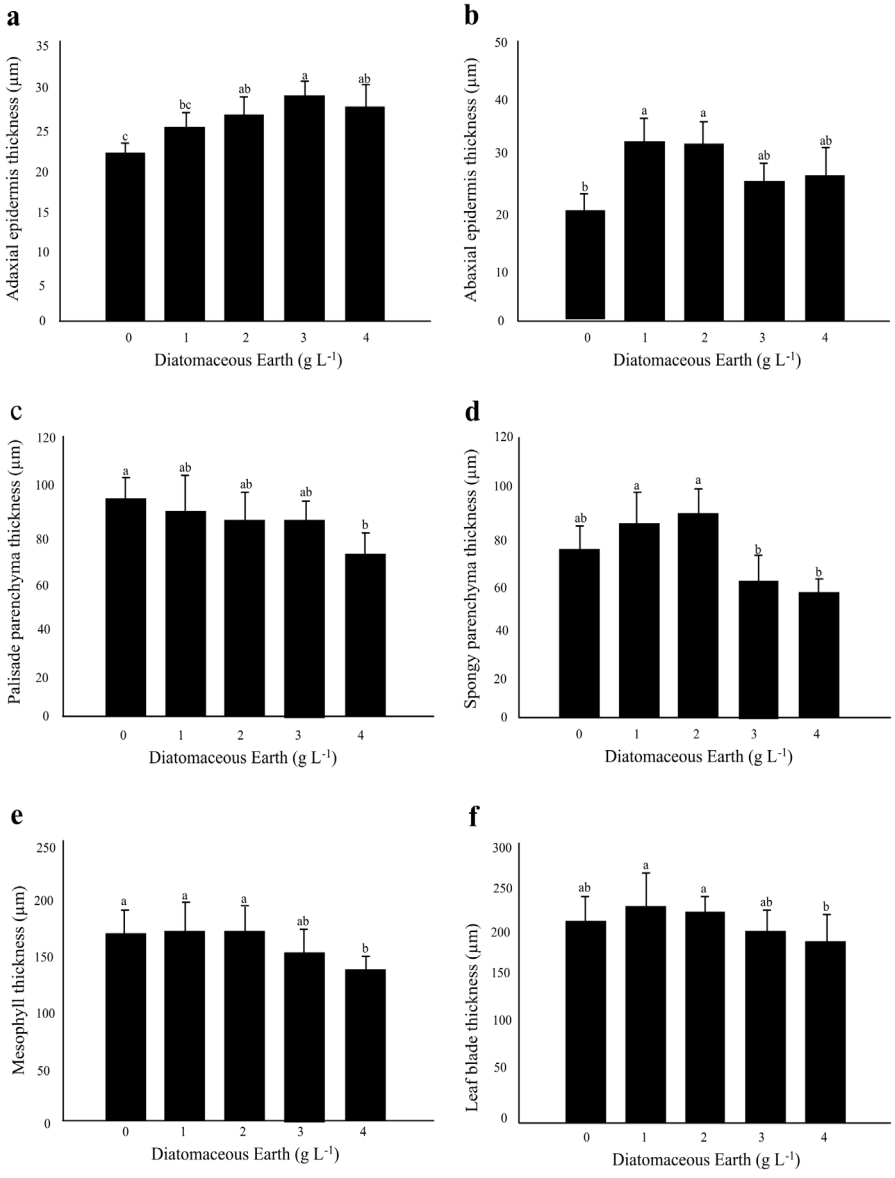

Figure 5. Adaxial epidermis thickness (a), abaxial epidermis thickness (b), palisade parenchyma thickness (c), spongy parenchyma thickness (d), mesophyll thickness (e) and leaf blade thickness (f) of sweet potato plants subjected to different concentrations of silicon (diatomaceous earth) and acclimatized for 45 days. Means represented by the same letter do not differ significantly by Tukey's test at 5% probability.

(Figure 5b). In the *in vitro* cultivation of 'Maçã' banana (*Musa* spp.) plants, greater thickness of the abaxial epidermis was obtained when using calcium silicate than when using other sources of silicate (Asmar et al. 2013a). The responses of silicon accumulation and absorption in plants vary with the source and concentration used, how it is available, and plant species (Asmar et al. 2013b, Martins et al. 2018).

The deposition of silicon in the cell wall leads to its accumulation in the leaves. This accumulation creates a protective barrier, making the plant more resistant to fungi and insects, preventing the loss of plant water, decreasing transpiration and aiding the acclimatization of micropropagated plants, which may increase the survival rate during acclimatization (Pasqual et al. 2011, Silva et al. 2008).

For palisade parenchyma (Figure 5c) and mesophyll thickness (Figure 5e), the highest concentration of diatomaceous earth yielded values significantly lower than the control. The doses of 1 and 2 g L^{-1} of diatomaceous earth resulted in higher thicknesses of the spongy parenchyma (Figure 5d) and leaf blade (Figure 5f) than did the other treatments; however, there were no significant differences from the control values (Figure 5). Braga et al. (2009), working with strawberries *in vitro* with different silicon sources, also observed increased thickness of the leaf blade under silicon supplementation. Plants with greater leaf blade thickness have higher chances of survival at the time of transfer to the greenhouse, with blade thickness being a determining factor in tissue culture (Barboza et al. 2006).

The greatest influence of diatomaceous earth on leaf anatomy was observed for epidermal thickness, both adaxial and abaxial, with lower values observed in the control treatment than in all of the other treatments. For the other variables, no increases were observed under the addition of diatomaceous earth. High concentrations of diatomaceous earth tended to reduce the thickness of the internal tissues of the leaf.

These changes in the leaf anatomy of acclimatized seedlings are directly related to photosynthetic rate and transpiration, contributing positively to the growth and development of sweet potato seedlings. Furthermore, such changes enhance adaptation to the greenhouse during the acclimatization process and, consequently, the responses to field conditions (Silva et al. 2012).

Significant effects of diatomaceous earth addition were observed for all parameters related to root tissue thickness (Figure 6). Epidermis (Figure 6a) and exodermis thickness (Figure 6b) increased with supplementation with diatomaceous earth, with largely equal values across treatments, with significant differences from the control values. Epidermis thickness at the concentration of 2 g L^{-1} was increased by 37% relative to the control value (Figure 6b).

The highest values of cortical parenchyma thickness (Figure 6c), central cylinder area (Figure 6d) and total area (Figure 6e) were observed with doses of diatomaceous earth of 2 and 3 g L^{-1}; these values which were significantly different from the corresponding control values and those at the dose of 4 g L^{-1}. The increases in these variables with 3 g L^{-1} of diatomaceous earth relative to the control values were greater than 50% (Figure 6).

The images of the anatomical sections of the acclimatized sweet potato seedlings (Figure 7) reveal differences among treatments in the thicknesses of the leaf and root tissues, corroborating the quantitative results and demonstrating the desirable responses achieved using diatomaceous earth. The silicon-induced changes in the thicknesses of the tissues, both leaf and root, influenced the accumulation of

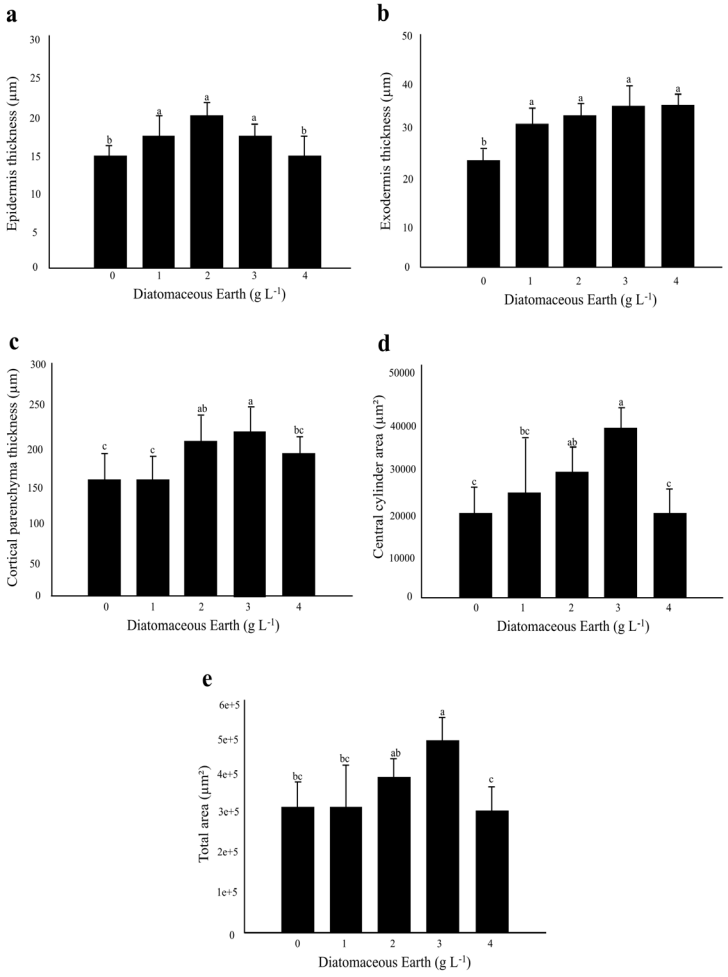

Figure 6. Epidermis thickness (a), exodermis thickness (b), cortical parenchyma thickness (c), central cylinder area (d) and total area (e) of the root system of sweet potato plants subjected to different concentrations of silicon (diatomaceous earth) and acclimatized for 45 days. Means represented by the same letter do not differ significantly by Tukey's test at 5% probability.

plant mass and improved nutrient absorption, cell rigidity and plant water maintenance. These effects improved the acclimatization ability of the seedlings, allowing the plants to maintain adequate growth and development (Asmar et al. 2011).

Supplementation with diatomaceous earth at various concentrations had a great influence on the performance of sweet potato seedlings during acclimatization to *in vitro* cultivation, with results superior to control treatment. Changes in the anatomical structure of leaf and root tissues were observed in the seedlings, in addition to increased photosynthetic rates, chlorophyll contents and mass, leading to improved production of the sweet potato seedlings. These findings highlight the importance of this study. However, high concentrations of diatomaceous earth did not have positive effects on seedling development.

CONCLUSIONS

The use of diatomaceous earth *in vitro* at 2 g L^{-1} provides benefits during the acclimatization process by increasing mass, photosynthetic rate and chlorophyll *a* content and improving the anatomical parameters of sweet potatoes, which directly influence the growth and development of the plants throughout the production cycle. The use of diatomaceous earth is strongly encouraged because it results in anatomical characteristics that favor the adaptation of seedlings to field conditions.

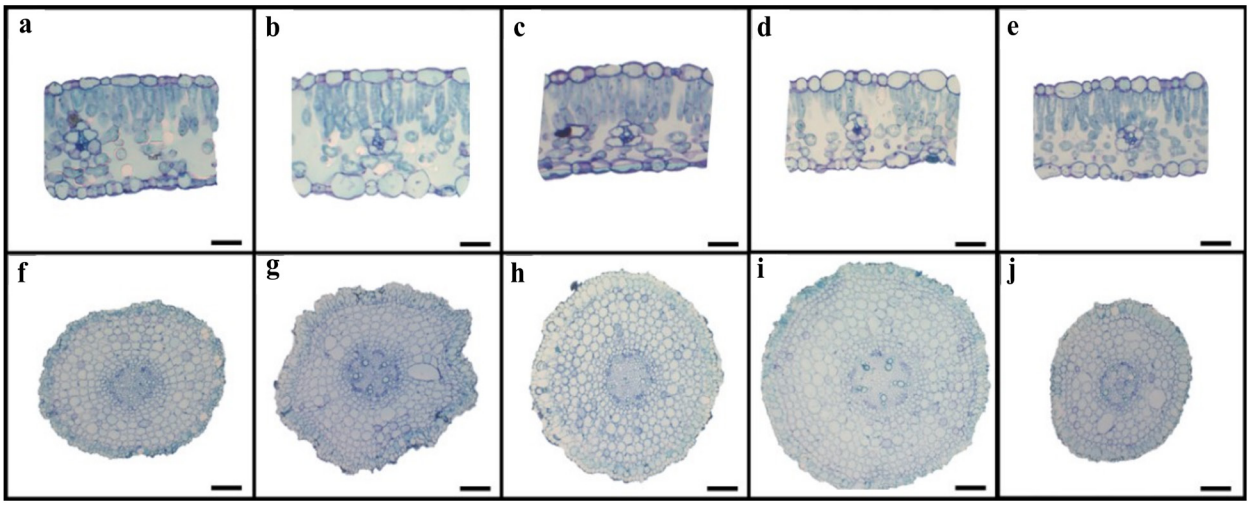

Figure 7. Cross sections of leaves (a to e) and roots (f to j) of sweet potato cv. 'Brazlândia Branca' grown with different concentrations of diatomaceous earth *in vitro* and acclimatized for 45 days (a and f, control; b and g, 1 g L^{-1}; c and h, 2 g L^{-1}; d and i, 3 g L^{-1}; e and j, 4 g L^{-1}). Bar = 100 µm.

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