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Silicon in the Embriogenic Potential of Callus *in vitro* of *Passiflora edulis*

Gabrielen De Maria Gomes Dias[1], David Correia Dos Anjos[2], Bárbara Nogueira De Souza[3], Moacir Pasqual[3], Bruno Grossi Costa Homem[3] & Irton De Jesus Silva Costa[3]

[1] Institute of Rural Development, University of International Integration of Afro-Brazilian Lusophony, Redenção, CE, Brazil

[2] Center of Technological Teaching, CENTEC, Jaguaruana, CE, Brazil

[3] Department of Agriculture, Federal University of Lavras, Lavras, MG, Brazil

Correspondence: Gabrielen De Maria Gomes Dias, Institute of Rural Development, University of International Integration of Afro-Brazilian Lusophony, Redenção, Ceará, Brazil. Tel: 55-85-3332-1155. E-mail: gabriellen@gmail.com

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Abstract

Adding silicon to the culture medium may contribute, to improve many growth parameters including embryogenesis and organogenesis. The objective of this study was to evaluate the embryogenic potential of passion fruit *in vitro* (*Passiflora edulis* f. *Flavicarpa* O. Deg), submitted to different concentrations of silicon. Nodal segments of plants were inoculated at five concentrations of silicic acid added to the culture medium, 0.0; 0.5; 1.0; 1.5 and 2.0 g L^{-1}. After 60 days, the visual characteristics (% contamination, callus, bud, root and oxidation), cytochemical test and microanalysis of X-rays evaluated. Concentrations of 0.5; 1.0 and 2.0 g L^{-1} of silicon did not differ in relation to callus formation. By the cytochemical test, all treatments presented embryogenic cells indicated by the reaction to acetic Carmine and, little reaction to the Evans blue associated with cell death. By the microanalysis of X-rays, the nutrients sulfur and phosphorus, presented significant absorption responses at the concentration of 1.5 g L^{-1} of silicon in comparison to the other concentrations. The silicon added to the culture medium has a response in the formation of cells with embryogenic potential in nodal segments of passion fruit. The concentration of 2.0 g L^{-1} of silicon acid (H_4SiO_4) has a high embryogenic potential in passionflower cells, *Passiflora edulis* f. *Flavicarpa* O. Deg.

Keywords: passionflower, embryogenesis, fruits

1. Introduction

The passion fruit belongs to the genus *Passiflora*, which is considered the largest and most economically important of the Passifloraceae family (Pérez et al., 2007; Braglia et al., 2010). Is an allogeneic plant, has autoincompatibility of the sporophytic type (Ferreira et al., 2010). The propagation of this plant is preferably realized of form sexually, through seed or asexually, by the use of cutting, grafting and tissue culture *in vitro* (Sousa et al., 2010).

Diseases are among the main factors responsible for low yields and losses in commercial passion fruit orchards in Brazil (Salazar et al., 2016). The tissue culture through the micropropagation is an important method to multiply quickly the high production and/or disease genótipos resistant superiors in the physical space and reduced time, very often offering the only alternatives for programs of plant breeding (Zerbini et al., 2008; Silva et al., 2013). However, the regeneration was seeing embryogenesis somatic be shown to be promising and it has the potential to devote it self in the field of the micropropagation, which is the way morphogenic principal of the regeneration in the type Passiflora (Zerbini et al., 2008; Pinto et al., 2010), in the improvement of result of passion fruit.

The inclusion of silicon (Si) in the culture medium has provided callus growth, induction of organogenesis and somatic embryogenesis and especially the improvement of morphological, anatomical and physiological characteristics of the seedlings produced. In addition, the use of silicon has increased the tolerance of plants to different biotic and abiotic stresses, such as salinity, low temperatures, metal toxicity, etc. (Sivanesan & Park, 2014).

During induction of somatic embryogenesis, the tissues involved undergo biochemical and morphological changes, which are strongly related to alterations in the pattern of gene expression (Sahni et al., 2013). The characterization and identification of embryogenic cells, through cytochemical analyzes, are of paramount importance for the study of the process of *in vitro* regeneration of plants. Chemical aspects, such as the mobilization of reserves during somatic embryogenesis, are also relevant because they enable a better understanding, optimization and validation of this morphogenic pathway (Otoni et al., 2013).

Considering the benefits that silicon promotes to plants and due to the lack of studies in passion fruit, especially with regard to *in vitro* silicon, the objective of the work was to adjust the concentration of silicon in the induction of the embryogenic potential of plant cells in *Passiflora edulis* f. *Flavicarpa* O. Deg.

2. Material and Methods

The work was developed in the Vegetable Tissue Culture Laboratory of the Department of Agriculture (DAG) of the Federal University of Lavras (UFLA). As a source of explants, *Passiflora edulis* f. *Flavicarpa* O. Deg. (Yellow passion fruit) established in a greenhouse.

2.1 Disinfestation of the Material

Prior to removal of the explants, the plants were preventively sprayed with antibiotic Ampicillin (1,000 mg L^{-1}) three times a week for four weeks. In the second week, the systemic fungicide Cerconil WP® (Thiophanate-methyl and chlorothalonil) was added to the sprays at the concentration of 2 g of p.c. L^{-1} water. Next, young nodal segments (second lateral or axillary bud) of the young plants were collected using scissors, then taken to the UFLA Plant Tissue Culture Laboratory.

2.2 In vitro Establishment

The nodal segments of passion fruit plants were submitted to asepsis in 70% alcohol for one minute and in 50% sodium hypochlorite for 20 min, after which four washes were performed with sterilized distilled water. They were inoculated in MS½ modified culture medium (Murashige & Skoog, 1962), added with 30 g L^{-1} sucrose, 0.5 mg L^{-1} BAP, 1.5 mg L^{-1} ANA and solidified with 1.8 g L^{-1} of Phytagel™. Five concentrations of silicic acid (H_4SiO_4) were added to the culture medium, 0.0; 0.5; 1.0; 1.5 and 2.0 g L^{-1}. The pH of the culture medium was adjusted to 5.8 and then autoclaved at 121 °C and 1.2 atm for 20 min.

Subsequently, in a laminar flow chamber, nodal segments containing a 1 cm long were inoculated in a test tube containing 15 mL of the culture medium with the respective treatments. The tubes were maintained in a growth room, with photoperiod of 16 h, temperature of 25±2 °C, with luminous intensity of 52.5 W m^{-2} s^{-1}.

2.3 Visual Evaluations

Were performed at 60 days after inoculation, observing the percentage of contamination, callus formation, number of shoots, number of roots and percentage of oxidation.

2.4 Cytochemical Analyzes

Cytochemical analyzes are performed to confirm if the tissue comes from cells with meristematic characteristics. Were performed on a fraction of the regenerated material. For this, 100 mg of three-tube callus fractions were collected for each treatment, which were submitted to the Evans blue/acetamide staining according to the methodology described by Steiner et al. (2005). At the end, slides were assembled and photomicrographs were taken with a digital camera coupled to an Olympus BX 60 light microscope at the Anatomy Laboratory of the Department of Biology of UFLA. The image analysis was performed by the RGB technique (Red, Green, Blue) and by means of the histogram tool of Adobe Photoshop® CS6 software version 10.0. For this analysis the experimental design was the completely randomized, with five treatments, consisting of three slides/treatment, each slide analyzed in 8 different regions.

2.5 X-Ray Microanalysis

Microscopic analysis by x-ray microanalysis maps and quantifies the elements present in the tissues. Was performed at the Laboratory of Electron Microscopy in the Department of Phytopathology of UFLA. Three tube beads per treatment were kept in desiccator with silica gel for 3 days, after which they were assembled in stubs, and metallized with CEC 020 Baltec carbon. Afterwards, it was analyzed the LEO-EVO 40 XVP Zeiss Scanning Electron Microscope and quantified the chemical composition by Spectroscopy of X-ray Dispersive Energy in the Quantax XFlash 5010 Bruker apparatus, following the protocol of Alves and Perina (2012).

2.6 Experimental Design and Statistical Analysis

The experimental design was a completely randomized design, with five treatments and 25 replications, totaling 125 test tubes, each tube containing one explant. The data were transformed to and submitted to analysis of variance. When possible, dose dependent regressions of silicic acid were used to demonstrate the results. The regression models were selected using the coefficient of determination (R^2) with a probability of 5%, according to the F test. Variables that did not fit the regression models were compared by the Scott-Knott test ($p \leq 0.05$). The statistical analysis was performed through Program R, version 3.0.3 (Ferreira et al., 2011).

$$Yi = \mu + ASi + \gamma i \quad (1)$$

Where, Yi = value observed at the ith dose of AS (silicic acid); μ = general mean; ASi = fixed effect associated with the i-th AS dose, i = 0.0, ... 2.0; Γi = random effect associated with the ith dose of AS (silicic acid), assuming $\gamma i \sim N(0, I\sigma 2\gamma)$, where, $I\sigma 2\gamma$ is the identity matrix of variance and covariance, since it assumes independence of the residues.

3. Results and Discussion

The decontamination methodology of the segments was efficient for *in vitro* establishment, presenting less than 2% of contamination. No endophytic contamination was present in any of the treatments until the final analysis.

For the oxidation, no type of preventive control was performed, even though with a low oxidation rate, the control and the concentration of 2.0 g L^{-1} of silicon acid (H_4SiO_4), which did not differ statistically between the other concentrations (Table 1).

Table 1. Percentage of contamination, callus formation, root induction, sprouting and oxidation in the *in vitro* establishment of nodal segments of *Passiflora edulis* Sims. f. *Flavicarpa* O. Deg. With addition of silicon (H_4SiO_4)

Silicon (g L^{-1})	Contamination	Callus	Root	Bud	Oxidation
0.0	8.0 a*	1.0 b	12.0 a	36.0 a	44.0 a
0.5	16.0 a	20.0 a	10.0 a	56.0 a	28.0 a
1.0	20.0 a	36.0 a	8.0 a	32.0 a	20.0 a
1.5	20.0 a	8.0 ab	10.0 a	44.0 a	32.0 a
2.0	8.0 a	20.0 a	10.0 a	36.0 a	44.0 a
EPM	2.71	4.11	2.0	4.83	5.13
P-Value	0.4307	0.0469	0.1649	0.5696	0.5393

Note. * Means followed by the same lowercase letters in the column do not differ significantly from each other, by the Scott-Knott test, at 5%.

The culture medium used in this study contained fixed concentrations of cytokinin and auxin, which influenced the activation of cell division of explants in different treatments. According to Navroski et al. (2012), the use of BAP and ANA in the culture medium promotes the increase in the formation of callus in Segurelha (*Satureja hortensis* L.).

In the control observed the presence of calluses in few repetitions, which did not allow enough material for the analysis in this work (Table 1). Concentrations of 0.5; 1.0 and 2.0 g L^{-1} of silicon did not differ in relation to callus formation in nodal segments of passion fruit (Table 1). According, Máthé et al. (2012) obtained embryogenic calli in nodal segments of Caniço (*Phragmites australis*) using 0.5 mg L^{-1} of sodium silicate.

The formation of shoots and roots in the different concentrations of silicon did not present statistical difference between them (Table 1). However, for Máthé et al. (2012), the use of sodium silicate (Na_2O_3Si) in the induction of calli in Caniço (*Phragmites australis*), induced root formation in all explants at the concentration of 0.5 mg L^{-1}.

By the cytochemical test, all the treatments presented embryogenic cells indicated by the reaction to acetic Carmine (reddish coloration), and little reaction to the Evans blue associated with cell death (bluish coloration) (Figure 1). Steiner et al. (2005) state that the use of Evans blue and acetic Carmine dyes allows the differentiation of embryogenic cultures. Cells that react strongly to Acetic Carmim and weakly to Evans blue are embryogenic and cells with weak reaction to the first and intermediate to the second are callus cells.

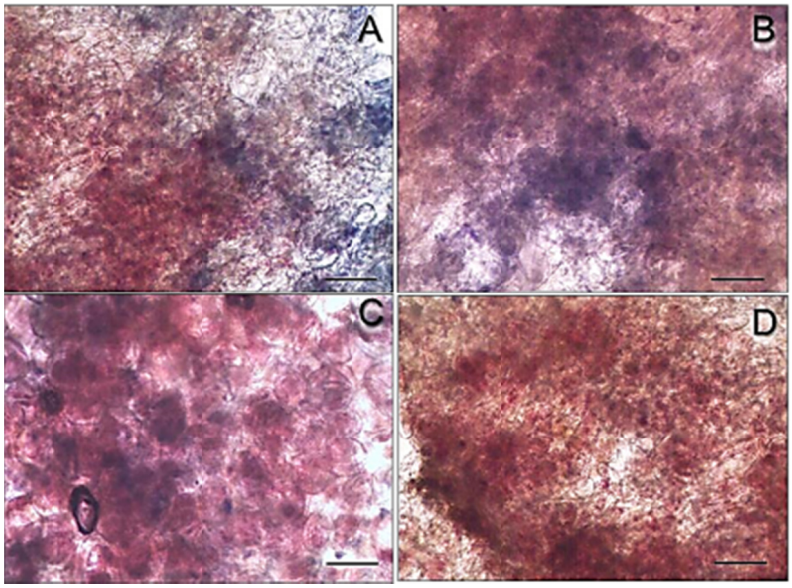

Figure 1. Calogenic masses of *Passiflora edulis* f. *Flavicarpa* O. Deg., Cultivated in: A) 0.5 g L^{-1}; B) 1.0 g L^{-1}; C) 1.5 g L^{-1}; D) 2.0 g L^{-1} silicic acid (H_4SiO_4), reacting strongly with acetic Carmine. (Bar = 20 µm)

This distinction is possible, because the embryogenic cells that present dense cytoplasm are isodiametric and reactive to acetic Carmine, resulting in reddish coloration. This reaction is associated with cellular competence for embryogenic development and chromosomal integrity (Steiner et al., 2005). On the other hand, Evans's blue cells penetrate through ruptures of the membrane, cells that are in the process of cell death or, according to Silva and Yuffá (2006), dead cells.

By the RGB analysis of the blades made by the double Carmine acetic/blue color of Evans, it was observed that the level of red differed statistically between the silicon concentrations applied to the culture medium (Figure 2). Therefore, the embryogenic cells adquired by the 2.0 g L^{-1} concentration of silicic acid, gave a morphogenic response to the explants. According to Máthé et al. (2012), silicon prevents the lignification and subsequent senescence of embryogenic tissue, and this provides an increase in the viability, that is, the morphogenic capacity of somatic embryogenesis.

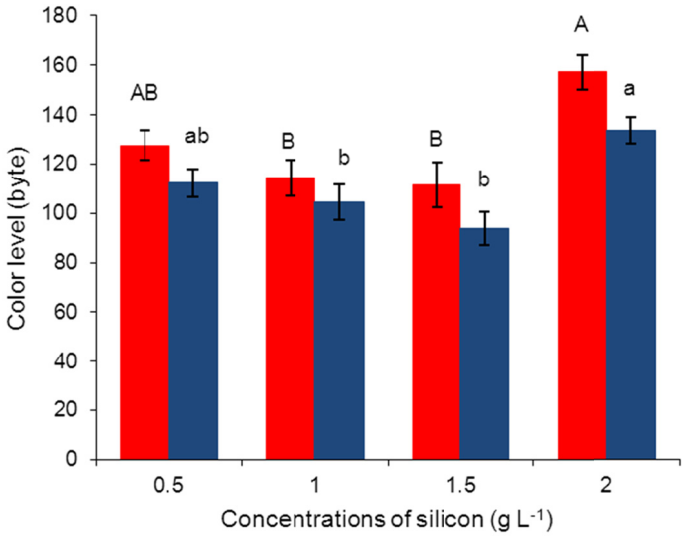

Figure 2. Red and blue levels obtained by image of calogenic masses of *Passiflora edulis* f. *Flavicarpa* O. Deg., Grown under different concentrations of silicic acid (H_4SiO_4), stained with Carmine acetic and Evans blue

Note. * Equal letters in the columns (uppercase-red and lowercase-blue) do not differ significantly from each other, by the Scott-Knott test, at 5%.

Silva et al. (2012), Santos et al. (2015), and Lopes et al. (2016), used the double staining cytoskeletal test with Carmine acetic and Evans blue in the identification of callus masses with embryogenic potential in small Murici (*Byrsonima intermedia* Juss A), Jatropha (*Jatropha curca*s L.) and Pitaia (*Hilocereus undatus*), Respectively. This proves the efficacy of the cytochemical test of cells with embryogenic or non-embryogenic potential.

With the X-ray microanalysis it was possible to quantify the nutrients absorbed by the explants in callus formation (Figure 3). In this work the absorption of the silicon element was not observed by this method. This is due to the sensitivity of the device that does not detect much lower elements.

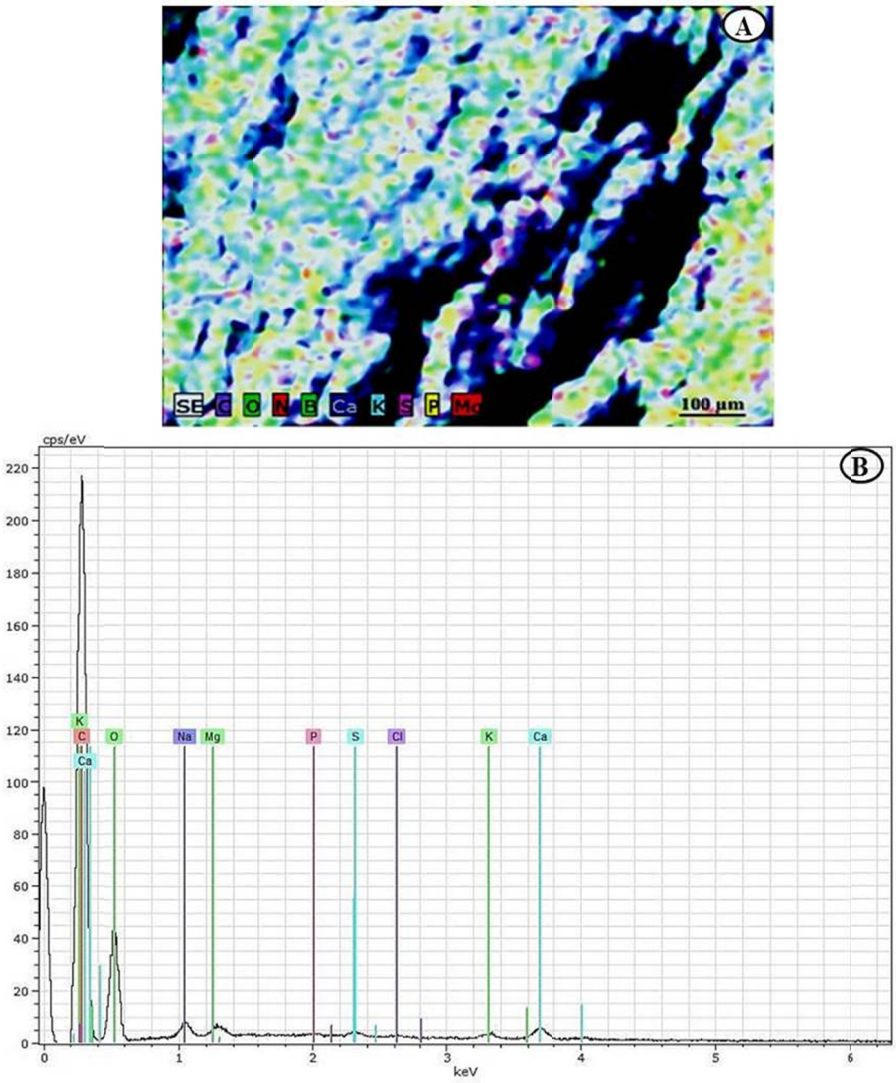

Figure 3. A) Callus mapping; B) Quantification of nutrients in calli *in vitro* of *Passiflora edulis* f. *Flavicarpa* O. Deg. with the presence of several elements and absence of silicon

The nutrients calcium (Ca), potassium (K), magnesium (Mg), iron (Fe) and boron (B), did not present significant differences among treatments (Table 2). However, the sulfur (S) and phosphorus (P) nutrients presented significant absorption responses at the concentration of 1.5 g L^{-1} of silicon in comparison to the other concentrations (Figure 4).

Table 2. Quantification of nutrients in calli in nodal segments *in vitro* of *Passiflora edulis* Sims. f. *Flavicarpa* O. Deg. With addition of silicon (H_4SiO_4)

Concentrations of silicon	Ca^{2+}	K^+	Mg^{2+}	Fe	B
---------- g L^{-1} ----------	---------- g Kg^{-1} ----------			---------- mg Kg^{-1} ----------	
0.5	1.39a	1.76a	0.52a	1.16a	0.52a
1.0	1.57a	1.62a	0.70a	1.05a	0.52a
1.5	1.76a	1.97a	0.64a	1.05a	0.52a
2.0	2.36a	1.31a	0.71a	0.94a	0.51a
CV (%)	24.35	59.82	23.70	39.21	1.36

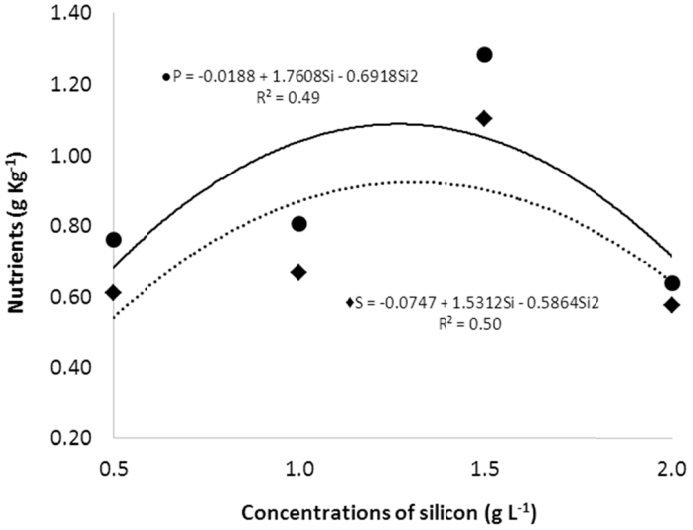

Figure 4. Regression graph of the phosphorus (P) and sulfur (S) elements in the different concentrations (0.0; 0.5; 1.0; 1.5 and 2.0 g L^{-1}) of silicic acid (H_4SiO_4) of silicon in calli *in vitro* of *Passiflora edulis* f. *Flavicarpa* O. Deg.

In addition to the elements considered essential, silicon can promote the absorption of other nutrients, among them calcium (Sarvas et al., 2002). Although the Ca element did not show differentiation in the treatments (Table 2), can observe a gradual accumulation of this nutrient as the increase of silicon concentration in the culture medium.

Ca is indispensable for growth regions (meristems) where continuous mitotic division is performed, well as root development, acting on the processes of cell stretching, hydrogen ion detoxification and cell division (Taiz & Zeiger, 2013). After the nitrogen (N) and potassium (K), calcium (Ca) is the third most accumulated macronutrient in the matter of yellow passion fruit, in most cultivated plants (Malavolta et al., 2006).

Arruda et al. (2000) reported the action of calcium on the development of somatic embryos in callus *Eucalyptus urophylla* and *Citrus*, stating that the macronutrient was accumulated in large quantities in materials with embryogenic potential. For Trevizam et al. (2011) working with *Eucalyptus urophylla*, observed that calli cultivated in rich Ca culture medium combined with B, were the most favored to root formation.

Culture media rich in silicon present positive responses in the formation of calli with embryogenic potential in nodal segments of passion fruit. This same response was observed by Mathé et al (2012) working with different types of Caniço explants (*Phragmites australis*), observed that silicon (sodium silicate-Na_2O_3Si) influences the formation of embryogenic calli and is dependent on the type of explant used.

4. Conclusion

The silicon added to the culture medium has a response in the formation of cells with embryogenic potential in nodal segments of passion fruit, *Passiflora edulis* f. *Flavicarpa* O. Deg.

The concentration of 2.0 g L^{-1} of silicon acid (H_4SiO_4) has a high embryogenic potential in passionflower cells, *Passiflora edulis* f. *Flavicarpa* O. Deg.

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