Iron concentrations in the *in vitro* cultivation of native Brazilian orchid *Schomburgkia crispa*

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ABSTRACT

In vitro cultivation is a highly important biotechnological method widely used for the production of orchid seedlings, but it is necessary to study the suitability of the nutrients used in different kinds of formulation, as the nutritional requirement varies according to the species. The objective was to evaluate different concentrations of iron in the *in vitro* cultivation of *Schomburgkia crispa* Lindl seedlings. Seedlings were obtained from seeds germinated *in vitro*. Modified MS culture medium was used with half of the macronutrient concentration. The micronutrients were added according to the original formulation, except for the iron which was added from a stock solution of FeEDTA (FeSO4.7H2O: 5.6 g L^{-1} and EDTA: 7.48 g L^{-1}) at 0.0; 2.5; 5.0; 7.5; 10.0 and 12.5mL L^{-1}. At 200 days after seedling transplantation, shoot height, root length, number of leaves, shoot number, leaf length, leaf width, aerial and root dry mass, chlorophyll a, b and carotenoids content were evaluated. A completely randomized design was used, with six treatments and ten replicates of five seedlings. Regression analysis was performed at 5% of significance. The increase in iron concentration caused a reduction in root length and an increase in the number of leaves and shoots. The concentration of 4.13 mL L^{-1} of FeEDTA was the one that provided the best *in vitro* growth of *S. crispa* plants. High concentrations of iron caused a reduction of initial development, but stimulated an increase in the number of shoots.

Key words: Ferrous sulphate, micropropagation, Orchidaceae, micronutrients.

INTRODUCTION

The *Schomburgkia* genus includes approximately 18 species, mostly epiphytes, found from Tropical America to Western India (Jones 1973). The *Schomburgkia crispa* Lindl. , an epiphyte plant native of Brazil, although non-endemic, is commonly found in gallery forests and dry forests from the Cerrado (Mendonça et al., 1998).

Among the species from the Orchidaceae family from the state of Minas Gerais, the *S. crispa* is included in the Red List of threatened species (Centro Nacional de Conservação da Flora 2017), due mainly to forests illegal extractive activity.

One of the ways to try to take this species off the list of threatened plants is through seedlings production of genetic quality or preservation through *in vitro* propagation (Stancato et al., 2001; Martini et al., 2001).

In vitro propagation is considered a fast and practical vegetative propagation method in the floriculture sector, and together with a set of other biotechnological techniques, allows the collection of a great number of plants with varied authenticity at any time of the year. In the case of orchids, *in vitro* propagation is essential, because their seeds are deprived of endosperm, resulting in a low germination rate in nature (Sorace et al., 2008).

The success of this technique does not depend only on factors related to the vegetal tissue (genetic and physiological), but also on the thermal and luminous conditions which the culture is maintained. It depends on an appropriate culture medium and a nutritional balance to supply culture needs, thus allowing the induction, multiplication and growth of sprouts plus fast growth and development (Nagao et al., 1994).

Therefore, the formulation of the culture medium have a great importance for seed germination and plant development, being normally composed of macronutrients, micronutrients, vitamins, amino acids, sucrose and a gelling agent. Macronutrients (N, P, K, Ca, Mg and S) are essential for seedling nutrition and growth (Sorace et al., 2008). However, the micronutrients also play an important role in the plant development. Among the micronutrients, iron as well as manganese, are absorbed in greater quantity by orchids (Hoshino et al., 2016).

Iron (Fe) is a mineral nutrient that acts on the interior of the cell as a catalyzer to chlorophyll synthesis and is present in a series of essential enzymes. The high affinity of Fe to develop complexes with several binders (organic acids and phosphates) and its capacity to alter its valences are two important characteristic that form the bases for the numerous physiological effects of this nutrient (Mothé 2012).

The use of half of the iron concentration in the MS culture medium brought no significantly different results than those shown by *Desmodium incanum* seedlings grown in a complete MS medium, showing that the lower concentration used was enough to supply the needs of this mineral (Schwalbert et al., 2014). On the other hand, a work carried out with different culture media compositions for the *Laelia cinnabarina* orchid, showed satisfactory results for *in vitro* cultivation in a MS medium, with half of the nutrients concentration (Stancato and Faria 1996).

In view of the above stated, the objective of this work was to evaluate different concentrations of iron in the *in vitro* cultivation of *S. crispa* Lindl. seedlings.

MATERIALS AND METHODS

This study used *Schomburgkia crispa* Lindl. seeds grown *in vitro* in a MS culture medium (Murashige and Skoog 1962), with half of the macronutrients concentration. Seeds were taken from mature capsules obtained by plants self-pollination grown in a greenhouse located at Universidade Estadual de Londrina Plant Science Department.

Sixty days after seeding, the protocorms were sub-cultivated in a MS culture medium modified with half of the macronutrients concentration. The micronutrients were maintained according to the original formulation, with the exception of iron that was added from a stock solution of FeEDTA ($FeSO_4.7H_2O$: 5.6 g L^{-1} and EDTA: 7.48 g L^{-1}) at concentrations of 0.0; 2.5; 5.0; 7.5; 10.0 and 12.5mL L^{-1}.

Medium base included 30 g L^{-1} of sucrose, 1 g L^{-1} of activated charcoal and 7.5 g L^{-1} of agar, with the pH adjusted to 5.8 ± 0.2 before the addition of the agar. Glass flasks of 250 mL received 50 mL of the culture medium and were properly autoclaved at 120 ºC and pressure of 1.05 Kg cm^{-2}, for twenty minutes.

After transplanting the seedlings to the culture medium, the flasks were kept in a growth room at the temperature of 25 ± 2 ºC under a photoperiod of 16 hours of lighting and 35µmol m^{-2} s^{-1} of luminous intensity.

At 200 days after seedlings transplantation, the following phytometric characteristics were analyzed: a) aerial part height: measured from the neck of the plant to the superior extremity of the greater leaf; b) root length: measured from the neck of the plant to the extremity of the greater root; c) number of leaves: number of leaves per plant; number of shoots; d) leaf length: measured from the larger leaf, from the leaf insertion until its extremity; e) leaf width: measured on the larger leaf with the help of a digital pachymeter; f) aerial part dry matter; and g) root dry matter (dry matter was measured after being dried in a forced ventilation greenhouse at 60 ºC).

Chlorophyll a, b and carotenoids content quantification followed the methodology described by Meschede et al. (2011), where samples of 0.2 g of fresh foliar tissue were placed in the tube with lids containing 10 mL of acetone 100% (v/v). Extracts were filtered and readings were done by a spectrophotometer at wavelengths of 663, 645 and 434 nm for chlorophyll a, b and carotenoids, respectively. Chlorophyll levels determinations (mg gmf^{-1}) were based on the equations described by Whitham et al. (1971): chlorophyll a = (11.24 x A663 – 2.04 x A645), chlorophyll b = (20.13 x A645 – 4.19 x A663) and carotenoids = (1000 x A434 – 1.90 Chlorophyll a - 63.14 Chlorophyll b)/214, where A is the absorbance in the recommended wavelength.

The experimental design was entirely randomized, with six treatments and ten replications with five seedlings each. The analysis of variance was conducted by the F test, and, when significant, it was submitted to a regression analysis at 5% of significance. The relationship between the characteristics was detected by the Pearson's correlation, and a multivariate of key factors analysis was realized in the software R.

RESULTS AND DISCUSSION

Seedling length showed quadratic behavior with the increase in Fe concentration in the culture medium (Figure 1 A), with the maximum point at 4.07 mL L^{-1}, and, from the point on the increase in concentration reduced the seedling size.

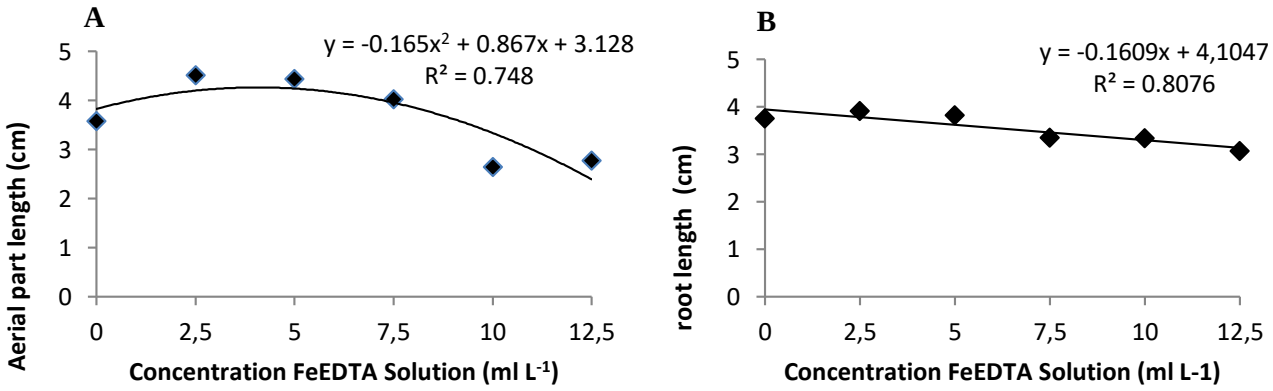

Figure 1. Aerial part length (cm) (A), and root length (cm) (B) of *Schomburgkia crispa* Lindl. seedlings submitted to different iron solution concentrations in a MS culture medium, modified by half concentration of macronutrients, 200 days after cultivation.

Under ideal concentrations, Fe favors photosynthesis, respiration, nitrogen fixation and DNA and vegetal regulators synthesis (Sahrawat 2004a), improving plant development. However, at concentrations above the ideal, Fe induces the development of oxygen reactive species (Jucoski et al., 2013), resulting in severe reductions in plant growth and development, reaching a critical level of toxicity, which varies according to plant species, age

and nutritional status.

Fe concentration increase in the culture medium resulted in the linear reduction of root length (Figura 1 B). Jucoski et al. (2016) reported a similar result, an increase of iron concentration in the nutritive solution resulted in a reduction in root length of *Eugenia uniflora* L. In irrigated rice plants, Fe toxic concentrations caused a reduction in root number and length, and they became rough and brownish (Sahrawat 2004b).

Root growth was more sensitive to Fe dosages than the aerial part development, which may be related to the low translocation of Fe from the roots to the aerial part. It was suggested that this fact could be a defense mechanism against the stress caused by the toxic levels of Fe (Stein et al., 2008).

The aerial part dry matter (Figure 2 A) showed the maximum point at 5.67 mL L^{-1}, and from this concentration, the increase in Fe contents resulted in a reduction in dry matter accumulation. In regards to root dry matter, it showed a quadratic behavior with the maximum concentration of 4.27 mL L^{-1}, with a reduction in dry matter accumulation after this point (Figure 2 B).

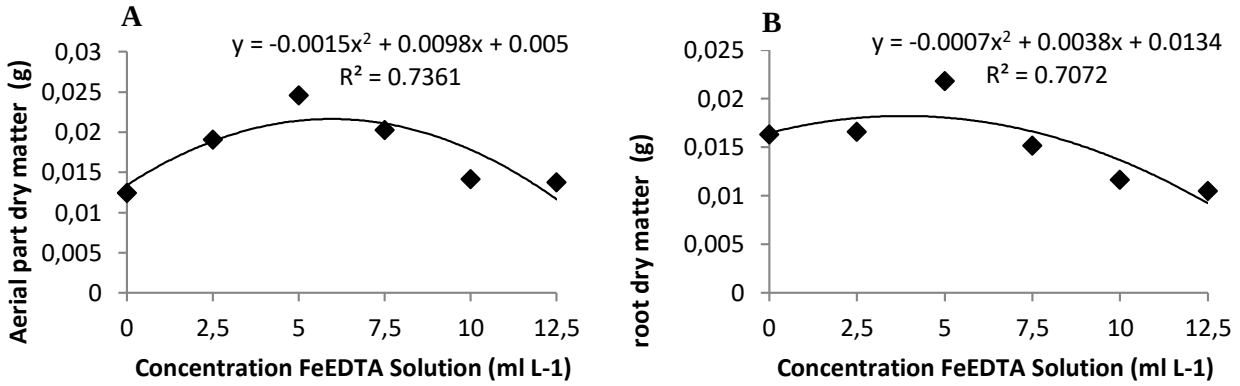

Figure 2. Aerial part dry matter (g) (A), and root dry matter (g) (B) of *Schomburgkia crispa* Lindl. seedlings submitted to different iron solution concentrations in a MS culture medium, modified by half concentration of macronutrients, 200 days after cultivation.

Jucoski et al. (2016) observed that the increase in iron concentration in the nutritive solution caused a reduction in dry matter accumulation in *Eugenia uniflora*. According to Chatterjee et al. (2006), the reduction in dry matter accumulation by toxic levels of Fe may have occurred due to an interference of this nutrient in the carbohydrates metabolism, resulting in a smaller biosynthesis of N protein. However, the toxic effect of Fe can be more complex, involving the development of species reactive to oxygen, and consequently the establishment of oxidative stress (Jucoski et al., 2013) or interfering in the absorption and use of some essential mineral elements (Fageria et al., 2008).

Therefore, it is believed that after reaching an ideal concentration for plant development, the increase in Fe concentration turns into something harmful to plant development both for the development of species reactive to oxygen and the small protein synthesis, as well as for inhibiting the absorption of other nutrients like P, K, Ca, Mg and Zn, resulting in multiple nutritional disorders (Benckiser et al., 1984).

Research has shown that part of this Fe interference in the absorption of other nutrients is related to the development of an oxide ferric crust around the roots, denominated “iron plate” (Siqueira-Silva et al., 2012). Although these iron plates on the root surface are considered part of a tolerance mechanism to high Fe levels (Becker and Asch 2005), they may cause a nutritional imbalance due to the high capacity of functional groups of Fe hydroxide to immobilize nutrients and metals by adsorption and/or co-precipitation (Howeller 1973). They also inhibit both absorptions as well as transport and use of several minerals (Fageria et al., 2008).

The increase in Fe concentration in the culture medium resulted in a linear increase of the number of leaves (Figure 3 A). This may be related to the imbalance of vegetal regulators caused by the Fe, since the reduction in root length caused by an increase in Fe concentration may be related to the smaller auxin/cytokinin relation in the tissues, caused by greater peroxidase enzyme activity. This greater cytokinins relations inhibited root growth and stimulated the initiation of stem buds, causing an increase in number of leaves and shoots.

This finding is based on the fact that increases in Fe concentrations induce an increase in phenolic compounds production (Siqueira-Silva et al., 2012) and greater peroxidase activity in the plant’s roots (Jucoski et al., 2013). According to Taiz and Zeiger (2013), in experiments conducted *in vitro*, the AIA auxin, responsible for root growth, is degraded by the peroxidase enzyme and some pigments such as the riboflavin. Therefore, greater peroxide activity results in a lower concentration of AIA in the root tissues.

Leaf length also showed quadratic behavior with maximum concentration point of 3.15 mL L^{-1} (Figure 3 B). Leaf width, on the other hand, showed maximum Fe concentration point of 3.52 mL L^{-1} (Figure 4).

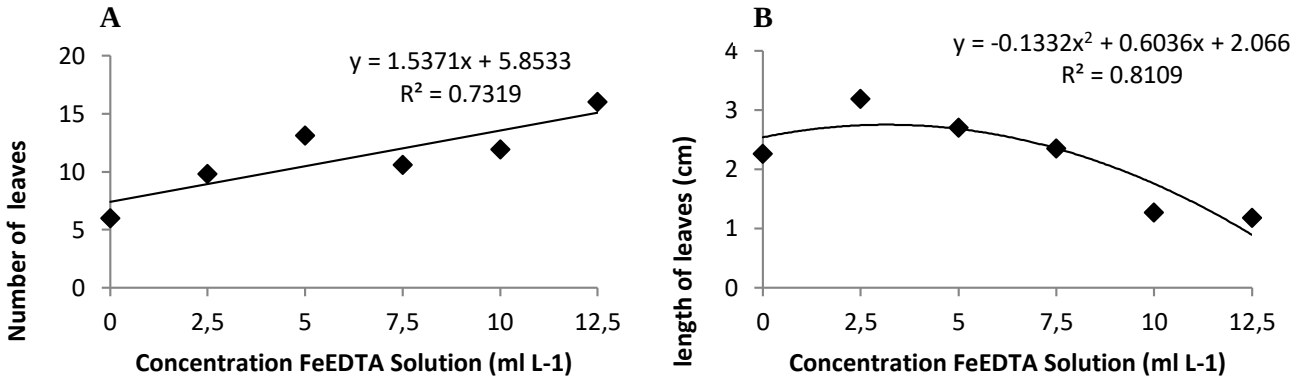

Figure 3. Number of seedlings leaves (A), and length (cm) of the *Schomburgkia crispa* Lindl. seedlings submitted to different iron solution concentration in a MS culture medium modified by half of the macronutrients concentration, 200 days after cultivation.

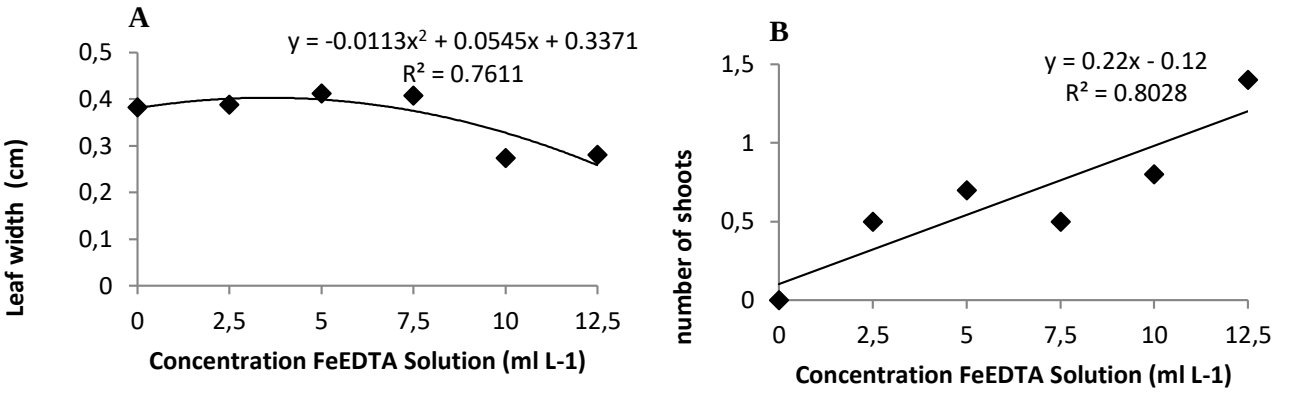

Figure 4. Leaf width (cm) (A), and number of shoots (B) of *Schomburgkia crispa* Lindl. seedlings submitted to different iron solution concentrations in a MS culture medium, modified by half concentration of macronutrients, 200 days after cultivation.

The number of shoots showed increasing linear behavior with the increase in Fe concentration in the culture medium. Possibly, this fact may also be related to the smaller auxin/cytokinin relation in the vegetal tissues caused by the increase in Fe concentration, as previously discussed.

Chlorophyll contents showed maximum concentration point of 10.52 mL L^{-1} for chlorophyll b and maximum point of 9.30 mL L^{-1} for chlorophyll b and carotenoids was 11.0 mL L^{-1} (Figure 5).

This increase in chlorophyll contents is related to the fact that Fe is part of the biosynthesis of this pigment, being important for the δ-aminolevulinic acid (ALA) formation phase, later for the coproporphyrinogem and protoporphyrinogen reduction and for the reduction of Mg protoporphyrin and protochlorophyllidea (Kirkby and Römheld 2007).

According to Mehraban et al. (2008), Fe in excess does not affect only chloroplastid pigment biosynthesis but also the luminous reactions of the photosynthesis, resulting in assimilation rates, and, consequently, plant growth (Adamski et al., 2011).

Characteristic iron deficiency symptoms are: reduction of the leaf's chlorophyll and carotenoids biosynthesis (Abadía et al., 1999); reduction of the CO_2 liquid assimilation rate (Sharma 2007); disorganization of the photosynthetic complex (Timperio et al., 2007) and increase in the stomatal resistance, together with a decline in the transpiration rate (Nenova 2009). However, high Fe concentrations can also promote growth and reduction in the chlorophyll contents (Chatterjee et al., 2006), as well as maximize oxidative stress through the development of reactive oxygen species (Robello et al., 2007).

Adamski et al. (2011), assessing sweet potato plants growth under excessive Fe conditions observed that exposition to high Fe concentrations caused an increase in the chlorophyll rate but a decline in the liquid photosynthesis rate.

The effect of excessive Fe can be associated with loss of connectivity or groupings among Active PSII units or the disorganization of the thylakoids membrane structure (Strasser and Stirbet 1998).

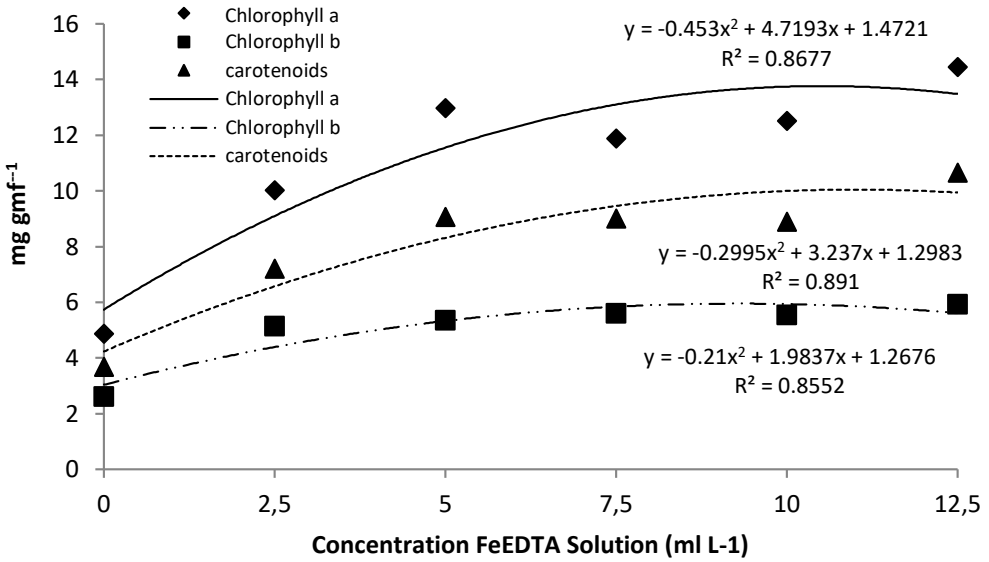

Figure 5. Chlorophyll a and b contents, and carotenoids of *Schomburgkia crispa* Lindl, seedlings submitted to different concentrations of iron solutions in a MS culture medium, modified by half concentration of macronutrients, 200 days after cultivation.

Another hypothesis is that during the electrons cyclic flow, of the PSI photochemical reactions (Munekage and Shinakai 2005), the ferredoxin transfers electrons to the plastoquinone, with the simultaneous production of ATP (Shikanai 2007). This flow of electrons may have been intensified in plants that receive high concentration of (9.00 mM), thus assuming that the transportation chain maximized the development of reactive oxygen species (ROS), especially O_2 (Bowler et al., 1992). ROS can be highly destructive because they induce serious lesions in several cell components, including lipids, proteins, carbohydrates and nucleic acids, which may lead to morphological, biochemical and physiological changes (Fang et al., 2001) that affect plants growth and development.

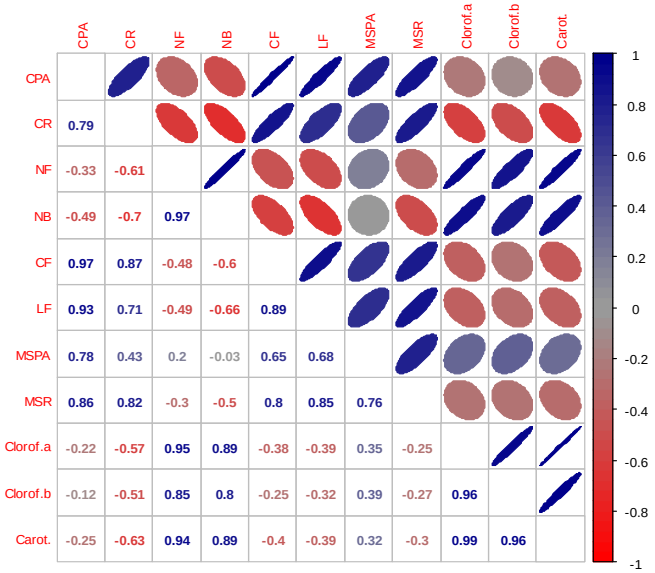

Figure 6. Pearson correlation coefficient between variables, aerial part length (APL), root length (RL) , number of leaves (NL), number of shoots (NS), leaf length (LL), leaf with (LW), aerial part dry matter (APDM), root dry matter (RDM), chlorophyll a (Chlorop. a), chlorophyll b (Chlorop. b) and carotenoids (Carot.) of *Schomburgkia crispa* Lindl. seedlings submitted to different iron solution concentration in a MS culture medium , modified by half concentration of macronutrients, 200 days after cultivation.

According to the Pearson's correlation (Figure 6), there is a positive correlation between aerial part length and root length, leaf length and width and root dry mass and aerial part. The number of leaves, however, showed positive correlation with the number of shoots, chlorophyll a, chlorophyll b and carotenoids. It is believed that this positive high correlation between the number of shoots and number of leaves is related to the reduction of the auxin/cytokinin relation caused by the Fe and by the degradation of AIA by peroxidase. This greater cytokinin relation stimulated the development of lateral buds, and, consequently, a greater number of leaves and shoots.

The high correlation between number of shoots, number of leaves and chlorophyll and carotenoids contents is

probably related with the smallest auxin/cytokinin relation caused by Fe application. According to Taiz and Zeiger (2013), the cytokinins, besides promoting cell division, they also act on the retardation and/or reduction of senescence. They also reduce chlorophyll and cellular protein degradation speed, considering that Fe is directly related to chlorophyll development.

Aerial part dry matter showed the positive correlation with leaf length, aerial part and root length and chlorophyll a, chlorophyll b and carotenoids contents. This correlation may be related to the fact that chlorophylls have the direct relation with photosynthesis, and, consequently, greater carbon fixation, resulting in greater plant growth and dry matter accumulation.

According to the principal components analysis (Figure 7), which showed 93% of explanation, it is possible to observe the group of Fe dosages used, where zero dosage (control) was isolated from the others, forming group 1. Dosages of 2.5; 5.0 and 7.5 mL L^{-1} of Fe solution formed another group (group 2), which showed a strong correlation with the aerial part dry matter (APDM) and root dry matter (RDM), aerial part length (APL) and root length (RL), as well as leaf length (LL) and leaf width (LW). On the other hand, dosages of 10.0 and 12.5 mL L^{-1} of Fe solution, formed another group (group 3), showing the negative correlation with the variables reported in group 2.

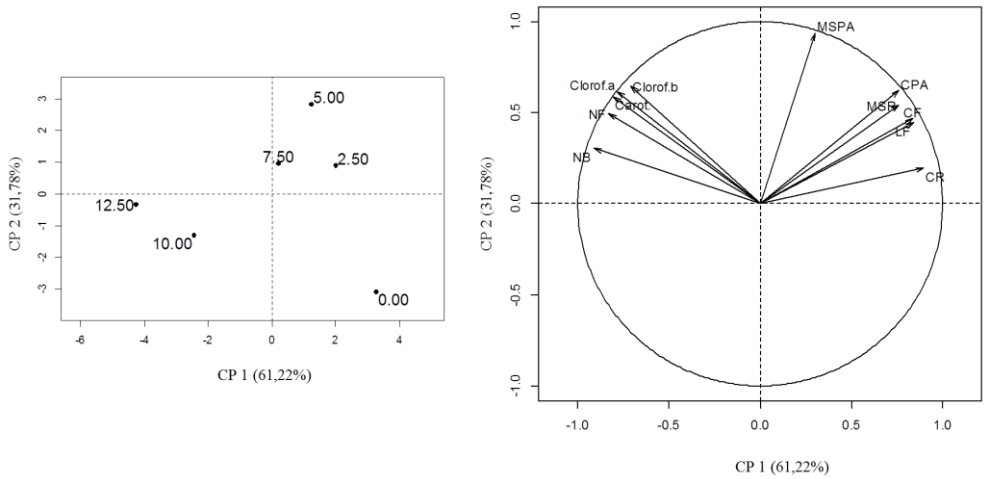

Figure 7. Principal components analysis (PCA) between variables, aerial part length (APL), root length (RL), number of leaves (NL), number of shoots (NS), leaf length (LL), leaf width (LW), aerial part dry matter (APDM), root dry matter (RDM), chlorophyll a (Chlorop a), chlorophyll b (Chlorop. b) and carotenoids (Carot.) of *Schomburgkia crispa* Lindl. seedlings submitted to different concentration of iron solution (0.0; 2.5; 5.0; 7.5; 10.0 and 1.,5 mL L^{-1}) in a MS culture medium, modified by half concentration of macronutrients, 220 days after cultivation.

The number of shoots (NS), number of leaves (NL), chlorophyll a and b and carotenoids variables showed the negative correlation with the absence of Fe application, showing the importance of this nutrient in the development of chlorophylls, leaves and shoots.

CONCLUSIONS

The concentration of 4.13 mL L^{-1} of FeEDTA *in vitro* resulted in better *Schomburgkia crispa* Lindl plant development. High iron concentrations cause reduction in the initial development; however, they stimulate an increase in the number of shoots.

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