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Acclimatization of coffee (*Coffea racemosa* x *Coffea arabica*) somaclones obtained from temporary immersion bioreactor system (RITA®)

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Abstract

Brazil is the first producer and second consumer of coffee in the world. Besides, coffee production also provides a million direct and indirect jobs throughout the supply chain. To increase productivity and reduce cost in conventional tissue cultures the temporary immersion of somatic embryos in bioreactor system was employed. During this process acclimatization is the key problem to obtain high-quality seedlings that required high cost. Therefore, the aim of this study was to evaluate the efficiency of the acclimatization process of coffee somaclones (*Coffea racemosa x Coffea arabica*) derived from somatic embryogenesis immersed temporarily in bioreactor system (RITA®). Embryos derived from leaves of the 'Siriema 05' cultivar coffee (*Coffea racemosa* x *Coffea arabica*) were used in this experiment. The acclimatization stage of cotyledon embryos was realized in three experiments: Experiment 1 - Different substrates and size of cotyledon embryos; Experiment 2 - Different substrates and Stimulate® concentrations; Experiment 3 - Growth of seedlings in different substrates and Osmocote® concentrations. A higher conversion percentage of cotyledonary embryos into seedlings were obtained from embryos grown in the Plantmax® medium with vermiculite and Plantmax® substrate. Moreover, increasing concentrations of Stimulate® and Osmocote® to a substrate concentration of 10.9 g L^{-1} produced better quality seedlings.

Keywords: *Coffea arabica* L.; Biostimulant; Fertilization; Plant regulator; Substrates.
Abbreviations: P_plantmax; V_vermiculite; CF_coconut fiber; WSS_washed sand and sieved; AP_plant height; DC_stem diameter at the height of the neck; NPF_number of pairs of true leaves; LA_leaf area.

Introduction

Brazil is the number one coffee producer and second largest consumer in the world. Coffee production also provides a million direct and indirect jobs throughout the supply chain, thereby playing an important role in the society. The estimated coffee harvest in 2014 in Brazil reaches 45.35 million 60 kg bags (CONAB, 2014). The south-central area of Brazil is the main coffee-growing region of the country, where the states of Minas Gerais, Espírito Santo and Paraná account for more than 90% of the national coffee production.
The production of clone coffee seedlings can be done in different ways. However, somatic embryogenesis technique is the most promising one. Somatic embryogenesis allows large scale seedling multiplication by using leaves as an explants. Plants obtained by this process exhibit similar genetic and agronomic behavior to the plants grown from seeds, without any limitation for commercial use. The clonal coffee production can be carried out in the tissue culture laboratory adapted for industrial scale production. However, the production of coffee seedlings via somatic embryogenesis is not exploited commercially in Brazil. To increase productivity and reduce the cost of conventional tissue cultures technique, the temporary immersion bioreactor systems has been proposed and allowed high multiplication rate with minimum contamination and low labor cost (Watt, 2012). In temporary immersion bioreactor systems, there is timed immersion of plant tissues in liquid medium to allow culture aeration, which circumvents limitations associated with conventional tissue culture (Balogun et al., 2014). On the other hand, there is still the problem on acclimatization, which is the key problem in high-quality seedlings production in relation to its cost that should be resolved before widespread commercial application. Moreover, most research works done with the aim of increasing the induction, embryo germination rate and conversion into plantlets, but lack research efforts focused on the physiological and molecular processes during acclimatization (Yang et al., 2013). The acclimatization of plantlets is the removal of the plantlets from *in vitro* culture to another type of substrate or the environment with the aim of promoting a gradual adaptation (Moreira et al., 2006). This process is an important step in the program of coffee seedling production by somatic embryogenesis in temporary immersion bioreactors. A significant number of micropropagated plant species cannot survive when transferred from an in vitro environment to greenhouse or field conditions (Hazarika, 2003). Most species grown *in vitro* require an acclimatization process

involving morphological, anatomical and physiological changes to allow plants to survive and grow vigorously in a new environment (Sama et al., 2015).

The optimization of the acclimatization process involves the nutrient supply, use of suitable substrates, use of growth regulators and control of the culture environment. An ideal substrate meets the chemical and physical requirements of seedlings and provides an adequate content of nutrients for their development. Moreover, an ideal substrate should provide uniform composition, low density, high porosity, high cation exchange capacity, and good water retention. An ideal substrate should also not contain pests, pathogens or seeds, and it should be abundant, operationally and economically feasible. Various materials have been used in the production of seedlings in tubes (Dias and Melo, 2009), either alone or in mixtures, including vermiculite, manure, "chaff" coal, sawdust, bagasse, acicula, pine bark, composted eucalyptus bark, carbonized rice, earthworm humus and peat.

The complementation of the substrate with nutrients for seedlings in tubes is usually made with slow release fertilizer to reduce problems of excess solubility and leaching losses of nutrients. Osmocote®, Nutricote® and sulfur-coated urea are examples of this type of fertilizer (Adams et al. 2013).

Thus, the aim of this study was to evaluate the efficiency of the acclimatization process of coffee somaclones (*Coffea racemosa x Coffea arabica*) derived from somatic embryogenesis and obtained in temporary immersion bioreactor system (RITA®).

Results and Discussion

The interaction between the substrates and the size of cotyledonary embryos was significant for the conversion percentage of cotyledonary embryos into seedlings at 1% probability. The use of Plantmax Forestry® increased the conversion of cotyledonary embryos into seedlings in medium-sized (1.50 to 2.51 cm) embryos (Table 1). These results were similar to those obtained by Barry-Etienne et al. (2002), who evaluated substrates and cotyledonary embryo sizes and found that the highest percentage of conversion was obtained using embryos between 1.5 and 5.0 cm in Plantmax® vegetable substrate. These authors also reported that this substrate results in high seedling vigor and greater accumulation of fresh matter. The same authors studied the morphological types of somatic embryos from small and medium cotyledons, which were more representative in the bioreactor, with an embryo to cotyledonary seedling conversion percentage of 47-63%. Small cotyledonary embryos (0.50 to 1.50 cm) showed a low conversion rate (from 16.0 to 32.25%) during acclimatization. However, large embryos (2.51 to 5.00 cm) also showed a conversion percentage lower than expected, which may have been due to the shading of seedlings resulting in lower stem diameter and greater height. The coconut fiber substrates had a lower conversion percentage compared to the Plantmax® Forestry substrate as shown in Table 1. Significant effects for substrate, Stimulate® and the interaction of substrate and Stimulate® were found at a 1% probability. The regression curves (Fig 1) indicated that there was an increasing trend in the conversion of cotyledonary embryos into seedlings with higher concentrations for all substrates, which may have been caused by the Stimulate® phytoregulator. However, the best results were obtained with the combination of the substrate composed of 2/3 Plantmax forest® and 1/3 vermiculite with increasing concentrations of Stimulate®. These data confirmed the results obtained in the application of growth regulators as an agronomic technique to optimize the production of seedlings in different cultures (Dantas et al., 2012), especially in coffee. The use of plant growth regulators for the acclimatization of coffee is still not a common practice even though the plant growth regulators have achieved a high technological level. However, the use of these substances may influence the growth of plants, enabling a more balanced relationship between shoots and roots.

There were significant differences in leaf area and stem diameter among the substrates. Doses of Osmocote® influenced all traits significantly ($P<0.01$). The significant substrates and Osmocote® doses interaction was observed only for the plant height and leaf area. Increased numbers of true leaves were obtained using 5.44 to 8.16 g $L^{-1}$of Osmocote® and these fertilizer doses (Fig 2A) provided improved plant growth. Similar results have been obtained by Melo et al. (2001), who found that the best development of coffee seedlings originating from reproductive propagation is obtained with the application of 450 g of Osmocote® fertilizer (15-10-10 + micronutrients) in 55 liters of the commercial substrate, Plantmax®, corresponding to the concentration of 8.18 g L^{-1}.

A quadratic response for plant height was observed for Osmocote® concentrations (Fig 3A) with a significant interaction for the substrates. The seedling height increased with an increase in the dose of slow release fertilizer (Osmocote®) for both substrates, and the highest value was recorded at a dose of 10.9 g L^{-1} Osmocote® (Fig 3A), which suggests that the substrates do not provide the necessary nutrients for proper plant development.

Figure 3B shows the effects of the interaction between the substrates and Osmocote® on the leaf area of coffee plants. Increasing doses of Osmocote® in both substrates corresponded with an increase in leaf area, where the maximum obtained with 10.9 g L^{-1} Osmocote® (Fig 3B). Similar results were obtained by Marcuzzo et al. (2005) when evaluating coffee seedlings produced by seeds in tubes. These authors observed a linear response from Osmocote® slow release fertilizer at concentrations above 10.9 g L^{-1}.

Micropropagated plants with greater leaf area at the time of transplanting to the field have faster initial growth due to higher production and higher allocation of assimilates to other plant organs (drains). However, the shoot dry matter is also a good indicator of resilience to the adverse conditions of the seedlings after planting in the field (Lima et al., 2009).

Significant effects on stem diameter were found for the substrates and Osmocote®, and the best dose of Osmocote® added to the substrate composed of vermiculite and Plantmax® was between 8.17 and 10.9 g L^{-1} (Fig 4 and 5). Stem diameter can be an indicator of shading for the seedlings. The concentration range of 8.17 to 10.9 g L^{-1} may be ideal because values higher than these can result in stem diameters smaller than the acceptable standard for seedlings, which results in smaller banding and consequently promotes low fixation of plants under field conditions. Barbizan et al. (2002) evaluated the influence of Osmocote® on the development of coffee seedlings from seeds in plastic tubes, and they observed that the stem diameter increases with the application of slow release fertilizer to a maximum of 3.46 mm, which corresponds to a concentration of 7.7 g L^{-1} Osmocote®. These results were similar to those obtained in the present experiment. Commercial substrates containing pine bark significantly affect the stem diameters of coffee seedling. Pine bark is widely used in the composition of commercial substrates that are virtually organic matter, and its decomposition occurs as it is used. The main features of pine bark are low density, easy drainage and granulometry.

Table 1. Conversion of different sized cotyledon embryos into coffee plant seedlings (%) in different substrates.

Size of cotyledonary embryos	Conversion of cotyledonary embryos into seedlings			
	Substrates			
	CF	$^{2}/_{3}$CF+$^{1}/_{3}$WSS	P	$^{2}/_{3}$P+$^{1}/_{3}$WSS
Small	16.00Ca	14.25Dc	31.25Bc	37.25Ab
Medium	20.25Da	37.75Ca	78.00Aa	70.50Ba
Large	18.25Da	28.50Cb	51.00Ab	36.00Bb

The means followed by the same capital letter in the horizontal line and the same lowercase letter in the vertical line belong to the same group and do not differ significantly at 5% probability according to the Scott-Knott test.

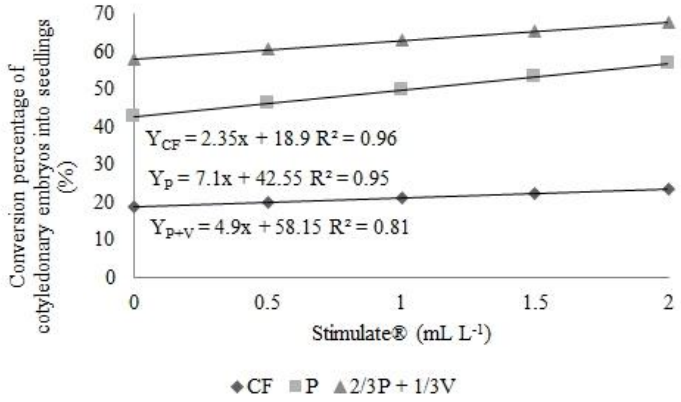

Fig 1. Conversion percentage of cotyledonary embryos into seedlings in different substrates and concentrations of the Stimulate® phytoregulator. Legend: Coconut Fiber (CF), Plantmax Forest® (P) and vermiculite (V).

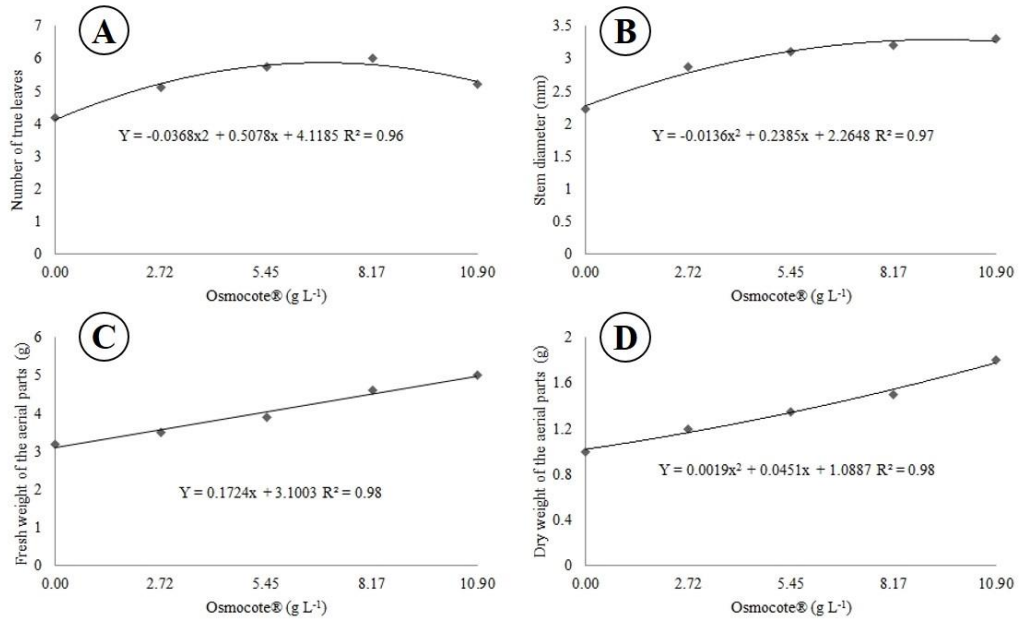

Fig 2. Number of true leaves (A), stem diameter (B) fresh weight (C) and dry weight (D) of the aerial parts in coffee plants grown in different doses of Osmocote®.

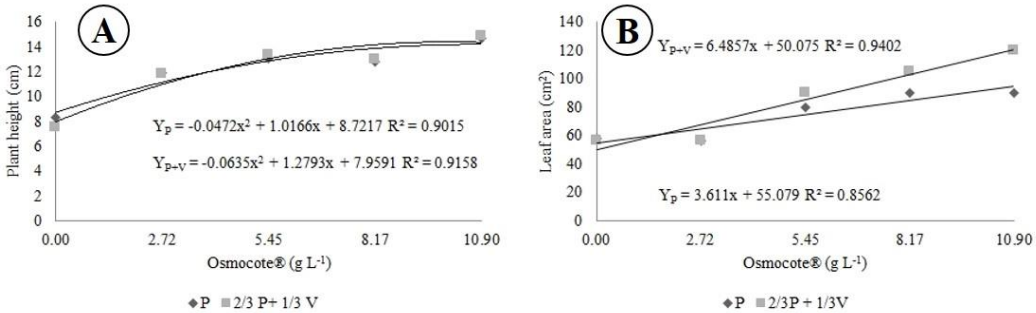

Fig 3. Plant height (A) and leaf area (B) of coffee grown on different substrates and doses of Osmocote®.

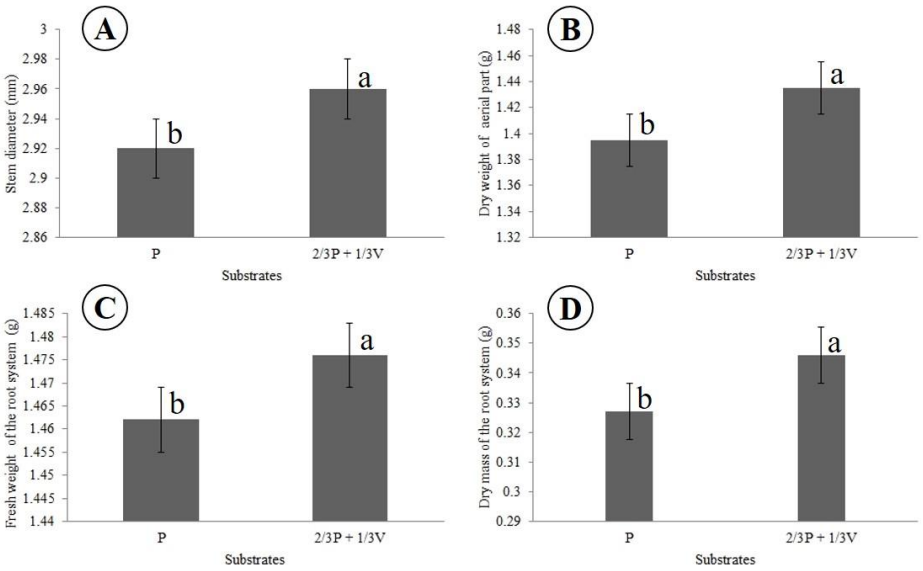

Fig 4. Stem diameter (A), dry weight (B) of aerial part, fresh weight (C) and dry weight (D) of the root system of coffee plants grown in different doses of Substrates®.

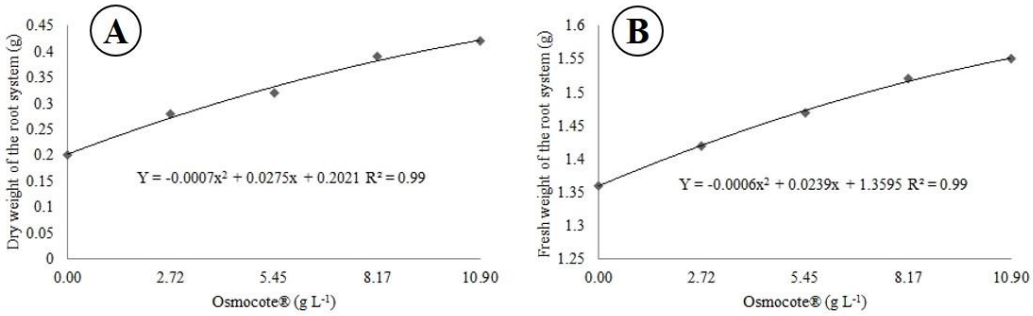

Fig 5. Dry weight (A) and fresh weight (B) of the root system of coffee plants grown in different doses of osmocote®.

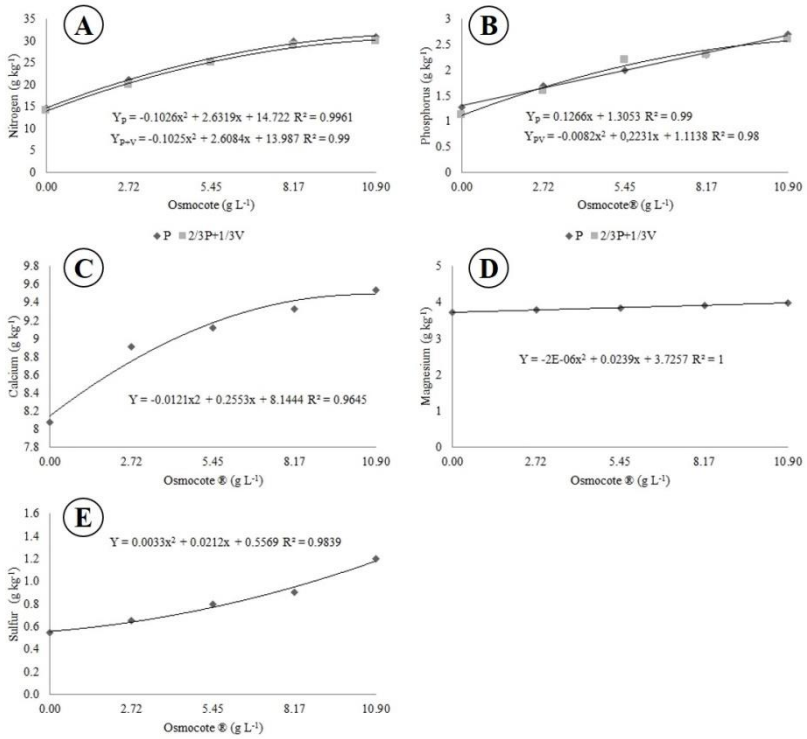

Fig 6. Foliar nitrogen (A), phosphorus (B), calcium (C), magnesium (D) and sulfur (E) in coffee plants grown on different substrates and doses of Osmocote®.

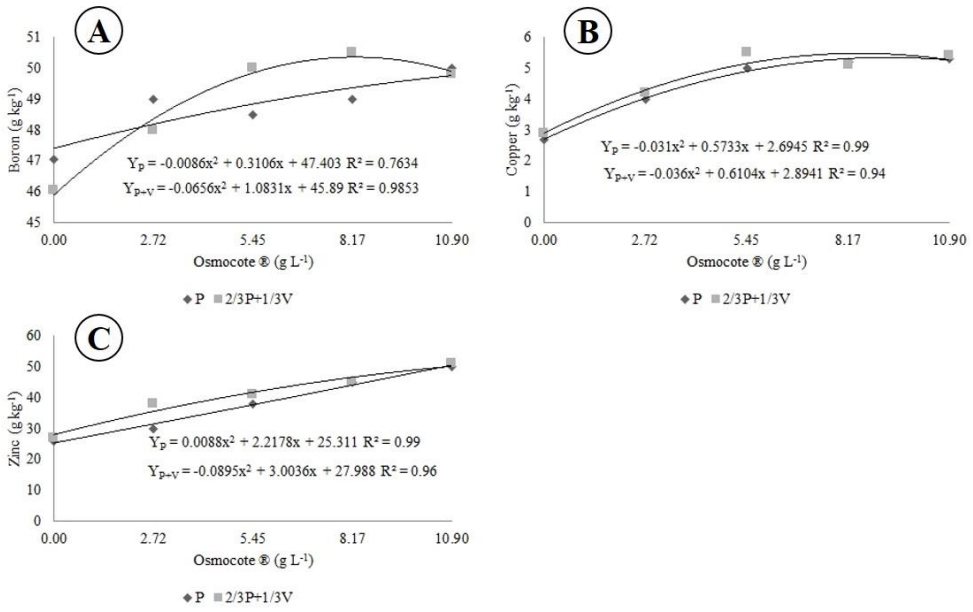

Fig 7. Foliar boron (A) copper (B) and zinc (C) in coffee plants grown on different substrates and doses of Osmocote®.

The composition of the pine bark and vermiculite mixture provides a coarser texture to the substrate, thereby reducing the compression level and increasing the internal drainage and aeration of the root system. Vermiculite has the primary function to increase water absorption to allow a balance between the aeration and water storage, which promotes root development and, consequently, the stem diameter of coffee seedlings (Lana et al., 2002). There were significant effects of the substrates on the shoot and root dry weight as well as on the root fresh weight. The Osmocote® concentrations significantly influenced all traits at 1% probability. No significant interaction effect between substrates and Osmocote® was observed for any of the traits. The fresh and dry weight showed a linear response to increasing concentrations of slow release fertilizer (Fig 2C and 2D). Figure 4B shows that the 2/3 P + 1/3 V substrate mixture produced more shoot dry weight. By analyzing Figures 2, 3, 4, 5, we noted that maximum growth points corresponded to the dose of 10.9 g L^{-1} Osmocote® for plant height, stem diameter, fresh weight and dry weight, even when considering higher concentrations. However, one should take into account that high concentrations of Osmocote® can promote etiolation of seedlings and other problems after the time of deployment of the coffee crop.

Marana et al. (2008) analyzed the behavior of coffee seedlings produced in tubes and observed that the maximum growth rate points corresponded to slow release fertilizer concentrations of approximately 13.31 g L^{-1} for stem diameter to 15.28 g L^{-1} for shoot dry matter. Lana et al. (2002) demonstrated the positive effect of the combined use of commercial substrates with slow release fertilizers, which may have been due to combining the characteristics of substrates that favor higher growth of coffee plants with gradual and continuous release of nutrients by the fertilizer for plants. The fresh weight of the root system was significantly affected by the substrate (Fig 4C), and the dry weight of the root system was influenced by the levels of Osmocote® (Fig 5A) and substrate (Fig 4D). A quadratic response of slow release fertilizer concentrations was observed for these characteristics. The best Osmocote® dose for dry weight (Fig 5A) and fresh weight (Fig 5B) of the root system was 10.9 g L^{-1}, and these results were similar to those reported by Marcuzzo et al. (2005), who used an Osmocote® concentration of 8.64 g L^{-1}. To analyze the seedling leaves of coffee plants, we quantified the levels of macro- and micronutrients in the dry matter, and statistical analyses were performed on the levels of nutrients in relation to the dose of slow release fertilizer (Osmocote®), substrates and the interaction between Osmocote® and substrates. A significant effect of the interaction between substrates and Osmocote® concentrations was found for nitrogen and phosphorus. The calcium, magnesium and sulfur significantly differed only for concentrations of slow release fertilizer. There was no significant effect on potassium levels in the coffee seedlings, but the obtained values were considered suitable for grown coffee plants. Increasing Osmocote® concentrations resulted in corresponding increases in the levels of nitrogen, phosphorus, calcium, magnesium and sulfur, and the highest values were obtained when using an Osmocote concentration of 10.9 g L^{-1} in both Plantmax forest® (P) and forest Plantmax® + vermiculite® substrates (Fig 6). As expected, the nitrogen content (g kg^{-1}) in leaves increased with increasing Osmocote® concentrations, reaching levels of 34.44 and 33.43 g kg^{-1} (Fig 6A), the values lower than this was found by Assis et al. (2015) in which the nitrogen levels ranged between 24.23 and 33.61 g kg^{-1}. While Santos et al. (2015) observed from 30.16 to 41.64 g kg^{-1}in different genotypes of coffee plants. In this study, the production of shoot and root dry weight of coffee seedlings were significantly affected by increasing concentrations of Osmocote® slow release fertilizer. Nitrogen is one of the most required nutrients by coffee for the successful development and productivity of coffee, which justifies higher dry weight accumulation in the treatments with higher concentrations of Osmocote®. Phosphorus is an essential nutrient for plant growth. For coffee seedlings, however, the requirements of phosphorus for growth and development are relatively small when compared to other macronutrients. Foliar phosphorus levels were between 1.12 and 3.01 g kg^{-1} depending on the availability of slow release fertilizer as shown in Figure 6B. On the other hand, Silva and Lima (2014) analyzed the leaf phosphorus content in different species of the genus *Coffea* and found that the level of this nutrient ranged from 0.8 to 2.3 g kg^{-1}. The calcium content in coffee leaves had a quadratic relationship where the best results were obtained using 10.9 g L^{-1} Osmocote® (Fig 6C) .

The observed foliar calcium levels (from 8.07 to 10.88 g kg^{-1}) were close to the limit set by Santos et al. (2015), who indicated that suitable calcium levels for adult plants are between 11.42 and 16.94 g kg^{-1}. The highest magnesium content was 4.40 g kg^{-1} (Fig 6D). This level is below the amount suitable for coffee (from 6.25 to 9.28 g kg^{-1}), as suggested by Santos et al. (2015). The highest sulfur content (0.54 to 1.36 g kg^{-1}) (Fig 6E) was below the recommended optimal levels (1.57 to 1.78 g kg^{-1}) by Ogeh and Ipinmoroti (2014). There are no references in the literature of this relationship with the development of coffee, but sulfur plays an important role in plant metabolism because it is incorporated into amino acids, proteins, enzymes, vitamins, oils and ferredoxins (Anjum et al., 2012) and may thus influence the development of coffee seedlings. A significant effect on the level of boron, copper and zinc was found for the interaction between substrates and Osmocote® concentrations (Fig 7), but there was no significant effect on iron and manganese levels. In general, there was an increase in boron, copper and zinc concentrations for both substrates when Osmocote® concentrations were high (Fig 7). The boron contents ranging from 46.03 to 50.36 mg kg^{-1} were considered suitable. According to Santos et al. (2015) the critical ranges of boron ranges from 62.58 to 130.58. The addition of slow release fertilizer (15-10-10 + micronutrients Osmocote®) to substrates has great importance in the acclimatization of coffee produced *in vitro*. Boron (Fig 7A) is among the micronutrients added, and it is important since it is a mobile nutrient in plants that requires constant supply to meet the needs of coffee. Copper levels ranged from 2.6 to 5.8 mg kg^{-1} in the present study (Fig 7B). Critical ranges of copper found by Santos et al. (2015) ranged from 0.19 and 2.40 mg kg^{-1} in coffee leaves. The highest zinc levels were achieved at 10.9 g L^{-1} concentration of Osmocote®. The zinc levels obtained in *C. arabica* plantlet leaves using different substrates and Osmocote® concentrations ranged between 25.9 and 55.5 mg kg^{-1} (Fig 7C). Santos et al. (2015) established critical ranges of foliar zinc for the production of plants as 8.92-19.04 mg kg^{-1} that is lower than the result reported in the present study.

Materials and Methods

Plant materials

Embryos from cotyledon calluses derived from leaves (indirect somatic embryogenesis) of the Siriema 05 plant matrix population (*Coffea racemosa* x *Coffea arabica*) were kept in temporary immersion bioreactor system (RITA®). These embryos were transferred to 128-cell polyethylene trays in a greenhouse with 90% relative humidity, an average temperature of 25°C, 50% brightness and equipped with automatic misting system. During the acclimatization stage of the cotyledonary embryos, three experiments were performed.

Experiment 1: substrates and size of cotyledonary embryos

Cotyledonary embryos were classified into three based on their sizes as follows: small (0.50 to 1.50 cm), medium (1.51 to 2.50 cm) and large (2.51 to 5.00 cm). The embryos were placed in trays containing the following substrates: coconut fiber (CF); 2/3 coconut fiber + 1/3 washed sand and sieved (2/3 CF + 1/3 WSS); Plantmax forest® (P); and 2/3 Plantmax forest® + 1/3 washed sand and sieved (2/3 P + 1/3 WSS).

Experiment 2: substrates and Stimulate® phytoregulator

Cotyledonary embryos were transferred to trays containing the following substrates: coconut fiber (CF); 2/3 coconut fiber + 1/3 vermiculite (2/3 CF + 1/3 V); Plantmax forest® (P); and 2/3 Plantmax Forest® + 1/3 vermiculite (2/3 P + 1/3 V). Stimulate® phytoregulator (0.0, 0.5, 1.0, 1.5 and 2.0 mL L^{-1}) was sprayed onto the embryos with a hand sprayer, which had a capacity of 500 mL, after the transfer of embryos to the cotyledonary substrate and every 20 days, totaling four applications during the trial period. Irrigation was performed by means of micro-sprinklers, keeping the relative humidity at 85 ± 5%.

Statistical analysis

The experimental design used in the experiments 1 and 2 were completely randomized in a factorial design with four replications, and each experimental unit consisted of 25 cotyledonary embryos. At 60 days, the experiments were evaluated by the conversion percentage of cotyledonary embryos into plantlets, which was characterized by at least two pairs of leaves. The significance difference among treatments was done using the F test at 5% probability and the mean separation among treatments were realized by the Scott-Knott test.

Experiment 3: seedling growth on different substrates and concentrations of Osmocote®

The seedlings used in the experiment were derived from cotyledon embryos acclimatized in a greenhouse for a period of 60 days in polyethylene trays (128 cells) with Plantmax Forest® substrate. The substrates used in the experiment were as follows: Plantmax Forest® (P); and 2/3 Plantmax Forest® + 1/3 vermiculite (2/3 P + 1/3 V). The concentrations of slow release fertilizer (Osmocote®) used were 0.0, 2.72, 5.45, 8.17 and 10.90 g L^{-1}, which corresponded to 0, 150, 300, 450 and 600 g of fertilizer per bag of 55 L^{-1} commercial substrate, respectively. The slow release fertilizer contained 15-10-10 + micronutrients with 15.0% N, 10.0% P_2O_5, 10.0% K_2O, 3.5% Ca, 1.5% Mg, 3.0%S, 0.02% B, 0.05% Cu, 0.5% Fe, 0.1% Mn, 0.004% Mo and 0.05% Zn. The experiment was conducted with 50% shade created with a "shading" screen in a greenhouse located in the city of Muzambinho-MG. The containers were black and had a conical shape with 8 internally perforated longitudinal grooves at the lower end, and they had a 120 mL volumetric capacity. The containers were previously sterilized with 0.4% sodium hypochlorite. The tubes were placed on supports made from wire (3.5 mm in diameter) with a quadratic mesh (1 1/2 wide and 1.20 m). The screen was set at a 1 m height from the ground surface using an iron structure built on eucalyptus posts used for packaging the tubes. Irrigation was performed with micro-sprinklers twice daily to avoid water deficits.

Statistical analysis

The experimental design was completely randomized with a 2x5 factorial design (two substrates and five concentrations of slow release fertilizer) with four replications, and each experimental unit consisted of five plants. Evaluations were performed 180 days after the experiment when the plants were at the stage of field planting (average of four pairs of leaves). The following characteristics were evaluated: plant height (AP, cm), stem diameter at the height of the neck (DC, mm), number of pairs of true leaves (NPF), leaf area (LA,

cm^2), fresh weight (g) and fresh weight of roots (g), dry weight (g) and dry weight of roots (g) and nutrient content in the leaves. Data were statistically analyzed using the F test at 5% probability, and the means separation was realized using the Scott-Knott test.

Conclusions

A higher conversion percentage of cotyledonary embryos into seedlings were obtained using medium cotyledon embryos (1.51 to 2.50 cm) grown in Plantmax Forest® substrate. Cotyledonary embryos acclimated in a substrate composed of vermiculite and Plantmax Forest® had higher seedling growth rates, with concentrations ranging from 0 to 2.0 mL L^{-1}. Stimulate® and the Osmocote® slow release fertilizer (15-10-10 + micronutrients) at a dose of 10.9 g L^{-1} provided better quality coffee seedlings and had a positive effect on the agronomic and nutritional characteristics of coffee seedlings derived from somatic embryogenesis.

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