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Original article

In vitro conservation of coffee-tree (*Coffea arabica* L.) by decreasing mineral salts in the culture medium

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ABSTRACT

The importance of the conservation of plant genetic resources is becoming higher at times when the consequences of global climate change are more acute. The coffee genus (*Coffea* spp) is not exempt from these risks, so it is researched some alternatives for its *ex situ* conservation. Specifically, the *in vitro* conservation methods known as 'slow growth' allow the medium-term preservation of plant material, by decreasing the growth rate and the frequency between subcultures. Studies are needed on the independent effect of variations in the concentration of essential macro and microelements in the medium-term preservation of coffee. The objective of the work was to determine the effect of the decrease of the mineral content of the culture medium in the response of *in vitro* coffee plants (*Coffea arabica* L.) preserved by a period between two and six months. The plants, previously obtained *in vitro*, were cultured in modified MS medium, with three treatments consisting in the reduction to 75, 50 and 25 % of their macro and microelements. The survival, the number of leaf pairs, the leaf abscission and the percentage of rooted plants were evaluated at two, four and six months. In the treatment of 50 % MS, the survival percentages varied between 85 and 100 %, and were obtained intermediate values of leaf pairs and leaf abscission, with respect to the rest of the treatments. In addition, the plants developed roots that allowed them to survive despite the lack of nutrients. With this treatment, which represents a saving of the half of the

mineral components of the culture medium, it is considered feasible to preserve the coffee plants for a period up to six months.

Key words: *in vitro* culture, *in vitro* plantlets, mineral nutrients, plant genetic resources, shoots

INTRODUCTION

Coffee (*Coffea* spp.) plays an important role in the economy worldwide, as it represents a major source of foreign income in approximately 80 producing countries [(1)]. It is estimated that 80% of the exported volume of coffee is currently produced in Latin American countries such as Brazil, Colombia and Costa Rica [(2)]. In Cuba, coffee is a priority in the agriculture sector, since from it a drink of habitual consumption is obtained by the population, and also constitutes an exportable item [(3)].

Although the most common way to produce coffee seedlings is the use of botanical seed, the loss of their germination during storage is a problem for the propagation and conservation of their genetic resources [(4)]. From this reality, the most widely used conservation method for coffee tree species collections are germplasm banks in field conditions. However, the threats to coffee plant genetic resources are numerous and their loss could cause significant genetic erosion to germplasm. One of the great difficulties of the ex situ collections in coffee fields is that they are often located in ecological conditions such as altitude, shading, relative humidity, which are not suitable for the survival of all plant material. Loss of plants as a result of aging, inappropriate cultivation methods, as well as pests and diseases, can cause genetic erosion, and the danger of climate change increases the urgency of conserving [(1)].

Therefore, other methods of *ex situ* conservation of coffee are studied that allow these barriers to be overcome, for example, by *in vitro* conservation, which constitutes one of the applications of *in vitro* culture. This is a valuable alternative for the germplasm preservation of botanical seed species of short viability such as coffee. It is also important for species of vegetative propagation or materials that need to be in a vegetative way propagated to maintain a particular genotype. *In vitro* conservation can be carried out through the methods of minimum growth and cryopreservation [(5)].

The minimum growth methods consist in reducing the growth rate of the plants, based on the decrease in cell division and metabolism, which is achieved by altering the optimal culture conditions. The composition of the culture medium can be varied, or the growth environment

can be modified (temperature, light intensity, oxygen availability) [6]. In this way, it is possible to conserve the plants in the short or medium term, which allows reduction in the frequency of subcultures and increase *in vitro* longevity of crops; as well as minimizing the risk of genetic changes [5,7].

Mineral nutrients are among the main components of plant tissue culture media [8], hence the cultivation of coffee has been carried out in different studies regarding the interaction between its concentration, the addition of growth regulators and osmotic regulators and temperature [9-11]. However, studies on the independent effect of variations in the concentration of these essential elements on the medium-term preservation of coffee are lacking.

For all these reasons, this paper aims to determine the effect of the decrease in the mineral content of the culture medium on the response of coffee plants (*Coffea arabica* L.) preserved in vitro for a period between two and six months.

MATERIALS AND METHODS

The work was carried out in the Biotechnology Laboratory of the Department of Genetics and Plant Improvement of the National Institute of Agricultural Sciences (INCA), located in San José de Las Lajas municipality, Mayabeque province.

In order to obtain *in vitro* coffee plants (*C. arabica*) seed disinfection was previously carried out by immersion in a solution of sodium hypochlorite (2.4% active chlorine) for 90 min. The seeds were washed three times with sterile distilled water and immersed in a 0.5% boric acid solution, with stirring in orbital strainer at 150 r.p.m. for 72 h. Zygotic embryos were extracted under sterile laminar flow conditions and seeded in Murashige-Skoog (MS) culture medium [12] with 30 g L^{-1} sucrose, 25 mg L^{-1} cysteine and 5 g L^{-1} agar. When the embryos germinated, when the seedlings had the first pair of true leaves, they were subcultured to MS culture medium with thiamine 5 mg L^{-1}, inositol 100 mg L^{-1}, sucrose 30 g L^{-1}, 6-benzyl-amino-purine (BAP) 0.5 mg L^{-1}, acid-acetaphthalene-acetic acid (ANA) 0.3 mg L^{-1}, gibberellic acid (AG3) 1 mg L^{-1} and agar 5 g L^{-1}. Once the plants had four pairs of leaves, finger pairs of leaves, used in the conservation study were taken as donors of the apical sprouts.

The subculture for in vitro preservation was performed in different variants of MS [12] culture medium supplemented with thiamine 5 mg L^{-1}, inositol 100 mg L^{-1}, cysteine 25 mg L^{-1},

sucrose 30 g L^{-1} and agar 5 g L^{-1}. In order to retard the growth of in vitro coffee plants, the variants of the culture medium consisted of a decrease in the concentration of the MS salts, so that T1 (control) was constituted by 100 % of the macro and microelements of MS; T2 for 75 %; T3 for 50 % and T4 for 25 %. For each variant, 20 apical shoots were sown and 3 repetitions were performed per treatment. As sowing containers, culture tubes (25/100 mm) with 20 mL of medium were used. They were placed in the growth room at a temperature of 23 ± 2 °C, under artificial lighting with a photoperiod of 16 light hours and light intensity of 18.75 µmol m^{-2} s^{-1} and relative humidity of 85 %.

From the two months of sowing and with this frequency until the six months, the evaluations of the survival percentage, the number of pairs of leaves, the foliar abscission (number of leaves cleaved per plant) and the percentage of plants were carried out With root formation. The variables expressed as a percentage were tested for proportions using the Statgraphics Plus 5.1 program, and the rest of the variables had a simple classification variance analysis, after checking compliance with their premises, and in case of differences between the means, a means comparison test (Duncan) was performed using the SPSS 10.0 program for Windows.

RESULTS AND DISCUSSION

After two months, 100 % plant survival was achieved in all treatments. Similarly, at four months the survival of the treatment plants with the highest concentrations of DM maintained this value, however, from this stage a decrease in the survival of the T3 and T4 treatment plants began, those with the lowest concentrations of the MS elements, obtaining significant differences between them and between them and the treatments T1 (control) and T2, as seen in Figure 1.

When analyzing the third evaluation, at six months, the survival of the T3 treatment plants decreased to 85 %, which is considered high for the growing conditions. At this stage, the survival of the T4 treatment plants decreased to 53.33 %, finding significant differences with the rest of the treatments (Figure 1). These results coincide with those reported by other authors [13] when performing *in vitro* conservation of Spanish carnation (*Dianthus caryophyllus* L.), which found significant differences in the survival of plants grown in medium with 25 % reduction of the salts of MS with respect to the rest of the treatments, after three months of cultivation.

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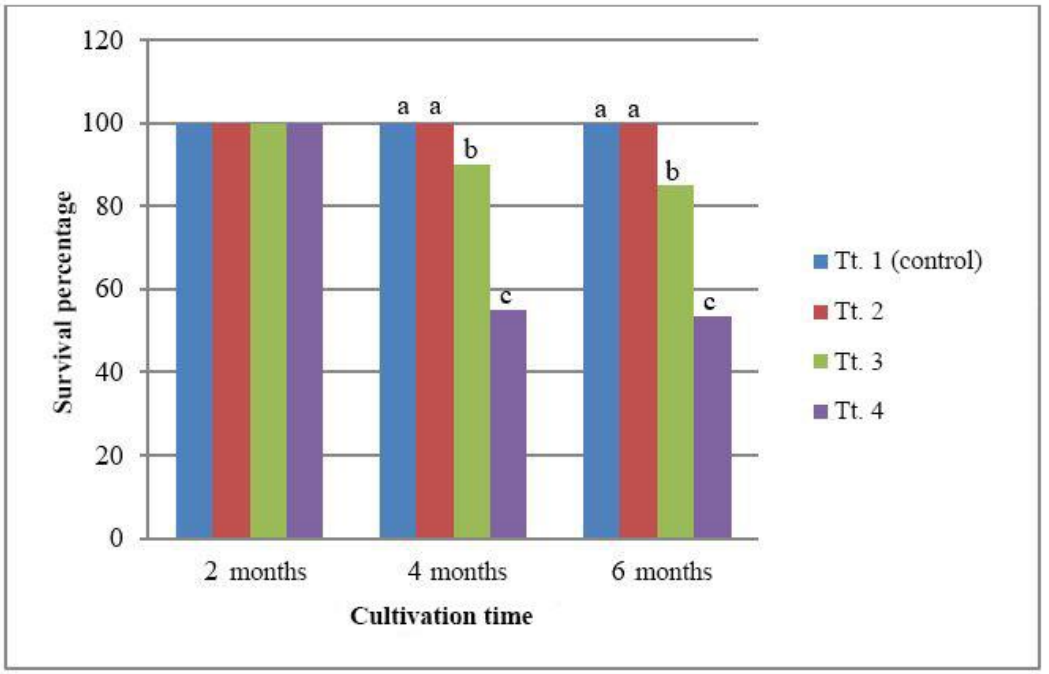
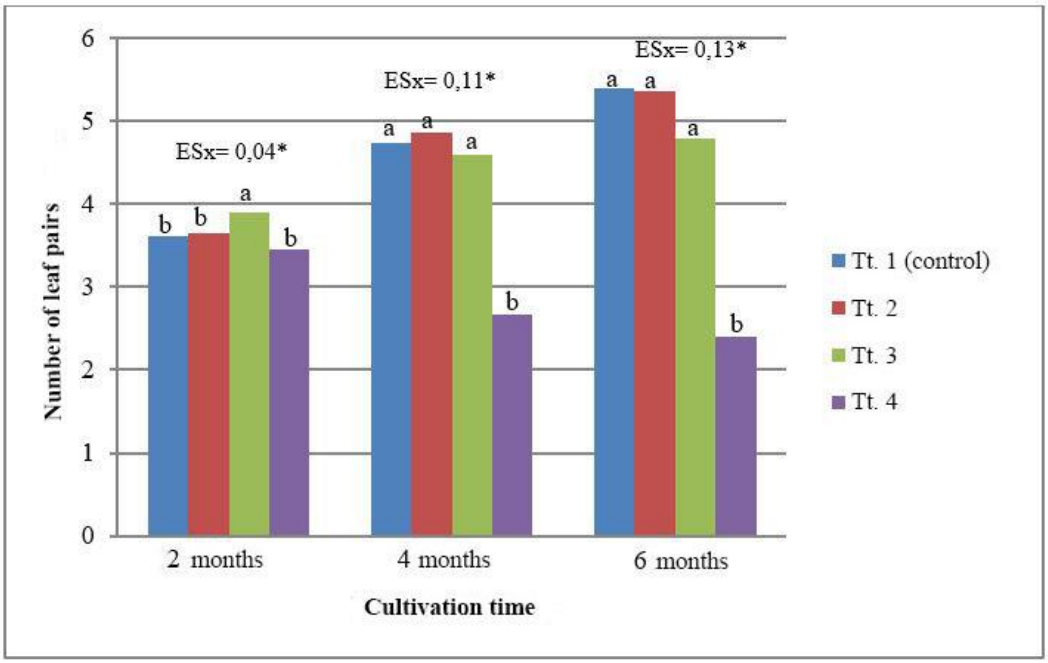


Tt. 1 (control): 100 %; Tt. 2: 75 %; Tt 3: 50 %; Tt. 4: 25 %. For the same evaluation, common letters do not differ significantly (Duncan $p<0.05$)

Figure 1. Survival percentage of coffee plants (*C. arabica*) subcultured in conservation culture medium, with different concentrations of MS macro and microelements

At two months, the means for the number of leaf pairs of the T1 (control), T2 and T4 treatment plants did not differ statistically, however, the plants obtained in the T3 treatment reached a number of leaf pairs mayoral rest of the treatments (Figure 2). At four months it was the T4 treatment that differed from the rest, with an average of 2.67 pairs of leaves per plant, while in the other treatments the average varied between 4.60 and 4.87 pairs of leaves (T3 treatments and T2, respectively).

At six months, the tendency to decrease the number of leaf pairs of the T4 treatment plants continued, which is related to an increase in the number of cleaved leaves. In the rest of the treatments, at this stage the tendency was to an increase in the number of pairs of leaves. However, it is noteworthy that although the T3 treatment (50 % of the DM concentration) did not differ statistically from the treatments with the highest DM concentrations, in this case the value of the mean was lower and very close to that obtained in the second evaluation, showing a decrease in its growth rate (Figure 2).



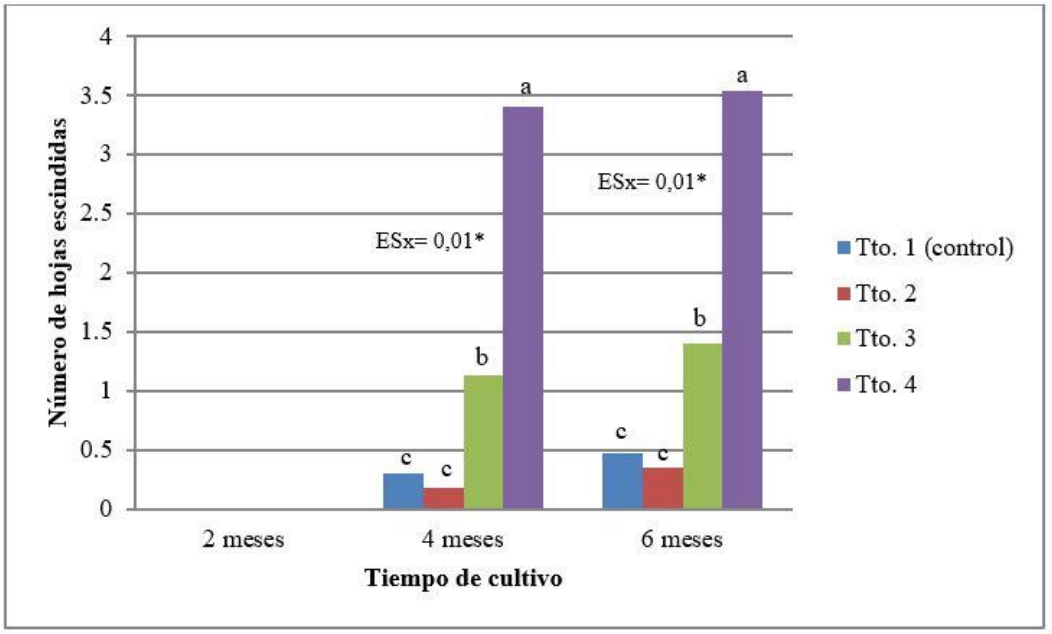
Tt. 1 (control): 100 %; Tt. 2: 75 %; Tt 3: 50 %; Tt. 4: 25 %. For the same evaluation, common letters do not differ significantly (Duncan $p<0.05$)

Figure 2. Number of leaf pairs, of coffee plants (*C. arabica*) subcultured in conservation culture medium, with different concentrations of MS macro and microelements

Similar results have been obtained by performing in vitro conservation of nodal segments of four sweet potato genotypes (*Ipomoea batatas* (L.) Lam). In this case, when evaluating the height of the shoots sown in culture medium with concentrations of 100, 75 and 50 % DM and sucrose 30 g L^{-1}, no statistical differences were obtained between these treatments. Taking into account the survival percentages, it was feasible to preserve two of the sweet potato genotypes for a period of six months in the treatment of 50 % of the DM salts, while the rest was possible to keep it in this same treatment for a period up to nine months [(14)]. According to some authors, in *in vitro* conservation under conditions of minimum growth, excessive elongation of explants is not desirable because in addition to reducing the available space in the culture vessel, it also causes the depletion of nutrients from the medium and can cause death of plant material [(15)].

With respect to the abscission of the leaves, this variable began to have an impact after four months, when the T4 treatment plants were the most affected, losing an average of 3.40 leaves, which was significant with the T3 treatment plants. , which in turn lost an average of 1.13 leaves (Figure 3). The lowest values of foliar abscission were obtained in the T1

(control) and T2 treatment plants, in which the highest concentrations of the MS elements were used. At six months the number of fallen leaves on the plants of all treatments was increased, but the same significance groups were maintained. In this way, it is evident that the decrease of the mineral content in the culture media favors the retardation in the growth, due to variations that occur in the cellular metabolism. The increase in foliar abscission values indicates that as the concentration of mineral salts in the culture medium decreased, the deterioration and necrosis of the foliar tissue significantly increased [13].



Tt. 1 (control): 100 %; Tt. 2: 75 %; Tt 3: 50 % and Tt. 4: 25 %. For the same evaluation, common letters do not differ significantly (Duncan $p<0.05$)

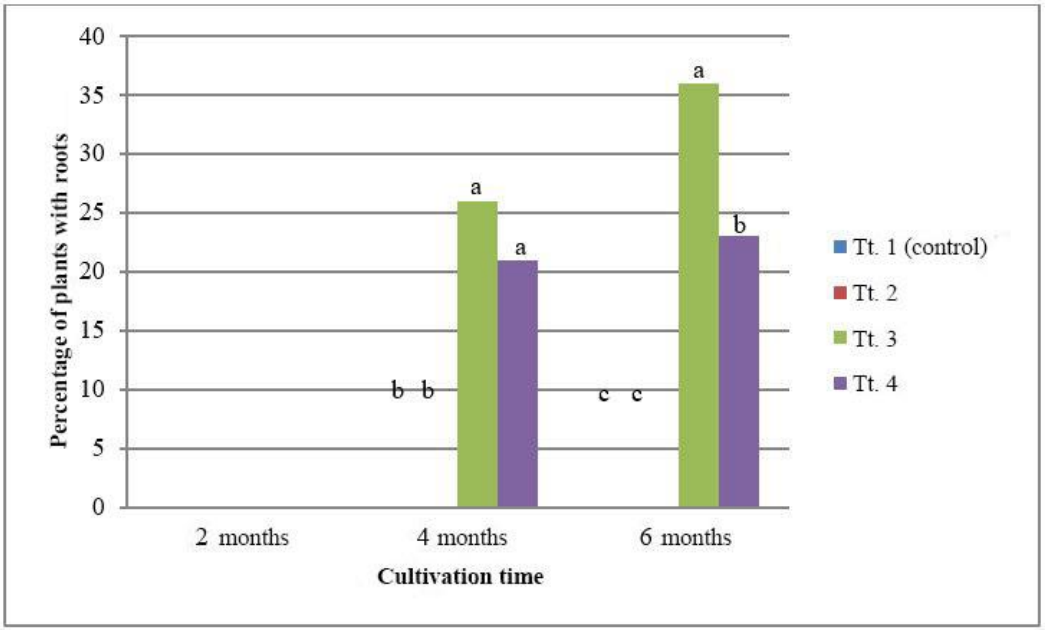
Figure 3. Number of cleaved leaves of coffee plants (*C. arabica*) subcultured in conservation culture medium, with different concentrations of MS macro and microelements

From the four months, in the plants of the treatments T3 and T4 the formation of roots was observed (Figure 4), possibly as a result of the shortage of nutrients that influenced the plants to emit roots that facilitated the obtaining of substances that were they sold out first in the environment closest to the explant. Root formation percentages were 26 % in the T3 treatment and 21 % in the T4 treatment in the second evaluation, and 36 and 23 % respectively, in the third evaluation.

In an investigation with the objective of preserving germplasm of *C. arabica* by cultivating shoots obtained from apical meristems of plants in vitro, the modification of the culture

medium has been carried out by adding the 6-benzyldenine growth regulators and Zeatin at different concentrations. When coffee meristems were grown in Murashige-Skoog (MS) medium supplemented with 6-benzyladenine (BA) or zeatin at concentrations of 5 or 10 µM, multiple outbreaks were obtained, while at lower levels of these hormones (0.1 or 1 µM), single shoots were obtained. Root regeneration was only possible by growing differentiated shoots in a 50 % MS medium, free of sucrose and supplemented with 1µM indole-butyric acid (AIB), which facilitated the preservation of plants [(9)].

It is interesting to note that in the present study, using a single subculture in the middle of 50 % of the macro and microelements of MS and in the absence of growth regulators, it was possible to achieve both the growth retardation of coffee plants, and their emission of roots, which simplifies the composition and elaboration of the culture medium, as well as the conservation process.



Tt. 1 (control): 100 %; Tt. 2: 75 %; Tt 3: 50 %; Tt. 4: 25 %. For the same evaluation, common letters do not differ significantly (Duncan $p<0.05$)

Figure 4. Percentage of coffee plants (*C. arabica*) that formed roots when subcultured in conservation culture medium, with different concentrations of the macro and microelements of MS

In spite of the response of the emission of roots, the plants of the T4 treatment, which was the one consisting of the lowest concentration of the nutrients of DM, did not present high percentages of survival at six months, since apparently the lack of macro and microelements

exceeded their nutritional requirements. In addition, for this stage, this treatment was in which greater means of foliar abscission and lower means of leaf formation were obtained (Figure 5). However, at two months these plants retain acceptable values for all the variables evaluated, so it is considered that for this period it is feasible to use the treatment of 25 % of the macro and microelements of MS, also taking into account that this results the treatment that allows greater savings of components of the culture medium.

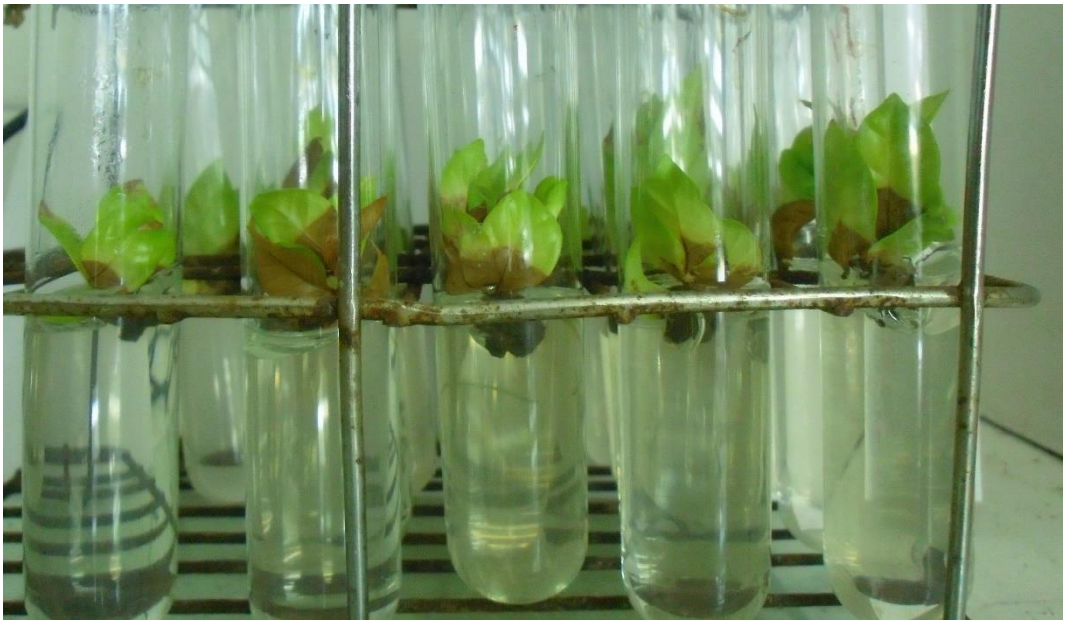


Figure 5. *In vitro* coffee plants (*C. arabica*) subcultured for six months in conservation culture medium, with a 25 % decrease in the macro and microelements of MS (T4 treatment). Foliar wilt is observed, as evidence that generally precedes its abscission

On the other hand, at six months, the T3 treatment plants managed to survive in a high percentage (85 %) and had lower leaf formation values than the T1 (control) and T2 treatments and intermediate leaf loss values between these two treatments and T4. Additionally, a significant number of these plants formed roots, which possibly give them greater chances of survival, due to greater efficiency in the absorption of nutrients, as well as a better support in the culture medium.

In this way, it is considered that the treatment of the macro and microelements reduction of MS to 50 %, is the most suitable for the conservation of *in vitro* coffee plants for six months (Figure 6). The observed with this concentration of MS elements, coincides with that carried out by other authors [(10)] when carrying out the conservation of coffee germplasm (*C. arabica*)

by encapsulating apical buds. In this case, the explants were encapsulated in 5 % sodium alginate and preserved at three temperatures in different variants of culture media. The one that allowed the growth to be reduced for a longer period of time (12 months) was the culture medium containing the 50 % DM salts, with 1 % sucrose and 10 mg L^{-1} abscic acid (ABA), at a temperature of 20 °C.

Figure 6. *In vitro* coffee plants (*C. arabica*) subcultured for six months in conservation culture medium with a 50 % decrease in the macro and microelements of MS (T3 treatment)

The reduction of the levels of macro and microelements of MS in the culture medium is one of the most common strategies for the in vitro conservation of plants [(14)]. The conservation of coffee sprouts by this method is a simple and accessible to laboratories with limited resources, which can facilitate the short and medium term preservation of germplasm and even allow reagent savings during the development of culture medium, as well as time due to the decrease in the frequency of subcultures.

CONCLUSIONS

It is feasible to carry out the conservation of coffee plants in vitro for a period of two and up to six months, with the macro and microelement reduction treatments of MS at 25 and 50 %, respectively.

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