

## Propagation of dasheen planting material of the genus *Alocasia* by *in vitro* culture

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### ABSTRACT

*Dasheen (Alocasia) is an option to produce animal food. However, at present it is not a commercial genus, so the availability of planting material is very limited. Biotechnology offers a rapid way of multiplying species of interest by in vitro culture. The objective of this work was to establish a complete protocol for the mass propagation of the cultivar 'verde picante' of the genus Alocasia by in vitro culture. Different experiments were developed to achieve efficient techniques for disinfection, establishment, types and concentrations of plant growth regulators, and the effects of the physical state of the culture medium in the multiplication and rooting phases. The multiplication in semi-automated culture systems and the conditions for the acclimatization phase were also evaluated. It was determined that disinfection should be performed with 3.0% sodium hypochlorite for 20 minutes. In the establishment and rooting phases, it is not necessary to use plant growth regulators, whereas in the multiplication phase 3.0 mg.L<sup>-1</sup> of 6-Benzylaminopurine (6-BAP) produced the best results. With the semi-solid culture medium, the best morphological growth characteristics of the explants were obtained after three subcultures. The multiplication coefficient when using temporary immersion systems was 12.6. A complete and efficient protocol was established for the in vitro multiplication of the cultivar 'verde picante', including the optimal conditions for its acclimatization. These results are of great importance to promote the development of this genus in Cuba.*

**Keywords:** *Dasheen, Alocasia, Micro propagation, Acclimatization.*

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### 1. INTRODUCTION

Among the rhizomes that people consume since a long time ago is Dasheen (*Alocasia*). Its leaves are eaten like vegetables in many Asian and African countries. The underground stem is appreciated for its starch and the aerial part is cooked once harvested. The skin is peeled, boiled in water, chopped and stewed with onions, herbs and tomatoes. For animals, it is common to include it for feeding fish, in the form of flour and mixed with sugar. It constitutes a very complete diet for chickens and pigs (Gómez-Alpízar, 2000).

The Research Institute on Tropical Ground Foods (INIVIT) in Cuba has a collection of *Alocasia* accessions, including those from the species *A. macrorrhiza* (L) Schott. Carbohydrates accumulate in the stem in the form of starch and in its leaves there is an important amount of protein, both characteristics make it a promising species for feeding pigs, birds and fishes. However, at

present this is not a commercial genus, so the availability of planting material is very limited, especially the cultivar 'verde picante', which has shown tremendous potential.

One of the alternatives to increase yields in this crop, and to enhance the production of high-quality planting material in the cultivars of interest, may be the application of biotechnological methods, which also have the advantages for obtaining new genotypes and in getting rid of pathogens (Archibald, 2005; Cabrera *et al.*, 2010). The objective of this research was to establish a methodology for the micro propagation of the cultivar 'verde picante' of the genus *Alocasia* by *in vitro* culture.

## 2. MATERIALS & METHODS

For the development of the experiments, the composition of the inorganic salts and vitamins proposed by Murashige and Skoog (1962) (MS) were used as the basal culture medium. Plants of Dasheen (*Alocasia*) cultivar 'verde picante' were selected from the germplasm bank that is conserved at INIVIT.

### Disinfection of the plant material

For the disinfection of the axillary buds to be established *in vitro*, three immersion times were used in 3% sodium hypochlorite (NaClO): 20, 25 and 30 minutes. At 30 days of culture the final result of the number of test tubes contaminated by bacteria and fungi was counted as well as the number of dead explants; both were expressed as percentage.

### Effects of the concentration of 6-Benzylaminopurine (6-BAP) in the establishment phase

In the establishment phase, the effects of 6-BAP in the culture medium were studied with concentrations of: 0; 0.1; 0.3; 0.5 and 0.7 mg.L<sup>-1</sup>.

Two evaluations were carried out at the ninth and eighteenth day after establishment to determine the number of those explants that presented morphological characteristics suitable to be passed to the multiplication phase.

### Types and concentrations of plant growth regulators in the multiplication phase

In order to stimulate axillary bud sprouting and explant multiplication, the effects of the types and concentrations of plant growth regulators in the culture medium was evaluated in this stage (Galvez *et al.*, 2013). The treatments were:

1. 1.0 mg.L<sup>-1</sup> of IAA + 3.0 mg.L<sup>-1</sup> of 6-BAP; Control (García *et al.*, 1999.)
2. 3.0 mg.L<sup>-1</sup> of 6-BAP
3. 4.0 mg.L<sup>-1</sup> of 6-BAP; Control (Galvez *et al.*, 2013).
4. 5.0 mg.L<sup>-1</sup> of 6-BAP
5. 1.0 mg.L<sup>-1</sup> of IAA + 6.0 mg.L<sup>-1</sup> of 6-BAP.

The explants used came from the best variant of the establishment culture medium obtained in the previous experiment. These remained in establishment culture medium for 18 days. A total of 60 explants were used for each treatment. Two explants were placed per culture vessel according to the corresponding culture media in the semi-solid state.

Three evaluations were performed every 15 days until the 45<sup>th</sup> day of the experiment. The parameter analyzed was the multiplication coefficient (MC) when counting the total number of explants and dividing them by the initial number for each treatment.

### Effects of the physical state of the culture medium in the multiplication phase

The effects of the physical state of the culture medium on the multiplication coefficient during three subcultures was also evaluated. The treatments studied were:

1. S + S + S. Semi-solid culture medium during the three subcultures
2. L + S + L. Alternating culture media Liquid - semisolid - liquid
3. L + L + L. Liquid culture medium during the three subcultures
4. S + L + S. Alternating culture media semisolid – Liquid – semisolid

At 21 days, the subculture was carried out and the multiplication coefficient was determined by counting the total number of explants and dividing them by the initial number. This experiment had three replicates per treatment.

### **Multiplication in semi-automated culture systems**

In order to increase the multiplication coefficients according to previous experiments in other crops (Cabrera *et al.*, 2012), the effect of two semi-automated culture systems was evaluated: Temporary Immersion System (TIS) and Constant Immersion System (CIS) with aeration of the culture medium.

Fifty (50) explants and a volume of 1500 mL of multiplication culture media were placed in each system. For the temporary immersion system, a time of 14 minutes of immersion and frequency of immersion of every six hours was used (Santos *et al.*, 2011).

### **Effects of plant growth regulators and the physical state of the culture medium in the rooting phase**

In the rooting phase, the influence of the type and concentration of plant growth regulators and the physical state of the culture medium were evaluated. Four culture media were studied in the liquid and semi-solid state.

Treatments:

- 1) Without plant growth regulator
- 2) 0.5 mg.L<sup>-1</sup> IBA
- 3) 1.0 mg.L<sup>-1</sup> IBA (García *et al.*, 1999)
- 4) 1.5 mg.L<sup>-1</sup> IBA

Plants with roots, number of roots per plant, length of roots (cm) and height of plants (cm) were evaluated at 15 days.

### **Study of different substrates in the acclimatization phase**

In order to achieve optimum acclimatization of the plants obtained *in vitro*, five substrates were studied:

1. Soil 100%
2. Bagasse 100%
3. Soil 50% + bagasse 50%
4. Soil 90% + zeolite 10%
5. Bagasse 90% + zeolite 10%

Bagasse: Disposal of the industrial process of raw cane sugar; it contains a lot of Nitrogen, Calcium, Phosphorus and Organic Matter (Bhatt *et al.*, 2013).

Zeolite: Micro-porous mineral, which is a member of the aluminum silicate group. It facilitates greater stability of soil's organic materials, reducing the loss of organic matter for mineralization and improves its physical properties such as structure, moisture retention, aeration and porosity (Curi *et al.*, 2006).

Three evaluations related to the general development of the plants were carried out every 10 days, until the 30<sup>th</sup> day of the experiment. Plant height (cm), number of leaves and diameter of the stem (cm) were evaluated.

### **Effect of decapitation in the acclimatization phase**

To stimulate the development of the diameter of the stems, the effect of decapitation was evaluated. The evaluations were performed every 10 days until the 30<sup>th</sup> day of the experiment. The diameter (cm) of the plants was evaluated.

### **Effect of sectioning the plants in the acclimatization phase**

Sprouting of the axillary buds was stimulated to obtain a greater number of plants using four variants:

1. Decapitated and without sectioning
2. Decapitated and split in half
3. Decapitated and divided into three parts
4. Decapitated and divided into four parts

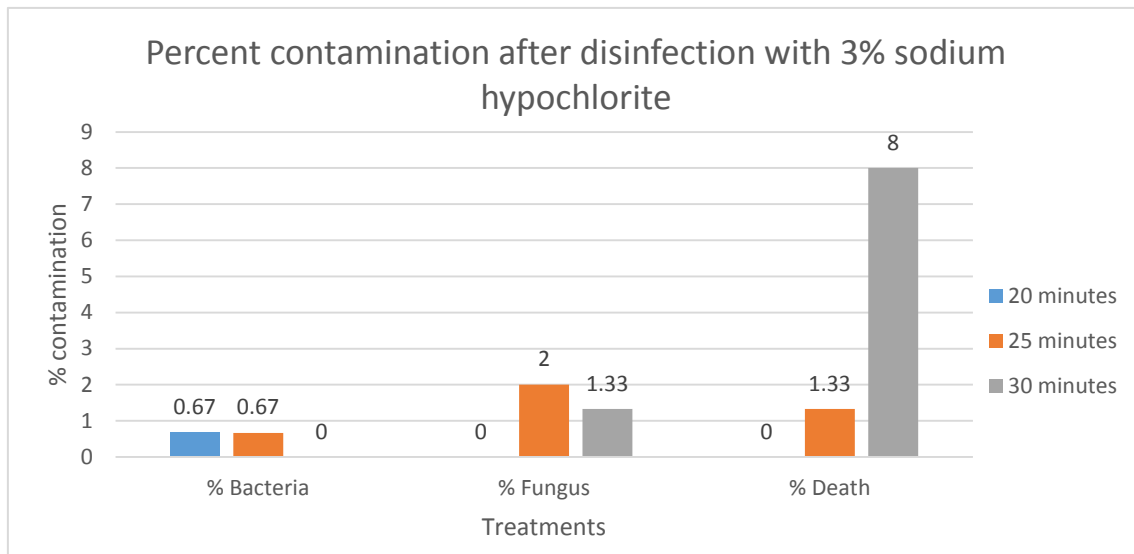
Four evaluations were performed every seven days, until the 28<sup>th</sup> day of the experiment. The number of shoots, number of suckers, number of leaves, height of plants (cm) and diameter (cm) were evaluated.

All statistical analyses of the research were performed using the SPSS software package version 15.0 for Windows®.

### 3. RESULTS & DISCUSSIONS

#### Disinfection of the plant material

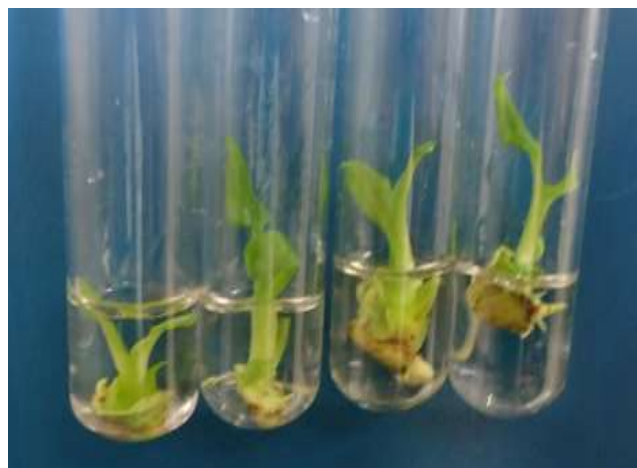
With the use of 3.0% sodium hypochlorite for the disinfection of the Dasheen corms for 20 minutes, the best results were achieved in terms of the establishment of axillary bud at 30 days of culture (Figure 1). An efficiency of the establishment of the shoots of the axillary buds of the dasheen cultivar 'verde picante' of 92.73% was achieved.



**Figure 1. Influence of sodium hypochlorite treatment at 3% on the disinfection of the plant material of Dasheen (*Alocasia*) cultivar 'verde picante' after 30 days of culture.**

When the explants were exposed for 30 minutes in the 3% sodium hypochlorite solution, 8% of the explants died. This result indicates that the explants exposed for an extended period of time led to irreversible tissue damage and the explants eventually died. The treatment with 20 minutes showed 0% deaths, 0% fungal contamination and only 0.67% bacterial contamination. Sodium hypochlorite has traditionally been used alone or in combination with other disinfectants in the disinfection of plant materials to be established *in vitro*, due to its high redox potential 1.36 eV (Calvo *et al.*, 2007). In each protocol for the *in vitro* establishment of plant species it is necessary to adjust the concentration and time of exposure to it. In general, concentrations ranging from 1.0 to 6.0% and times between 10 and 30 minutes have been used, depending on the morphological and phytosanitary characteristics of the plant material to be established (Vilchez *et al.*, 2011; Galvez *et al.*, 2013).

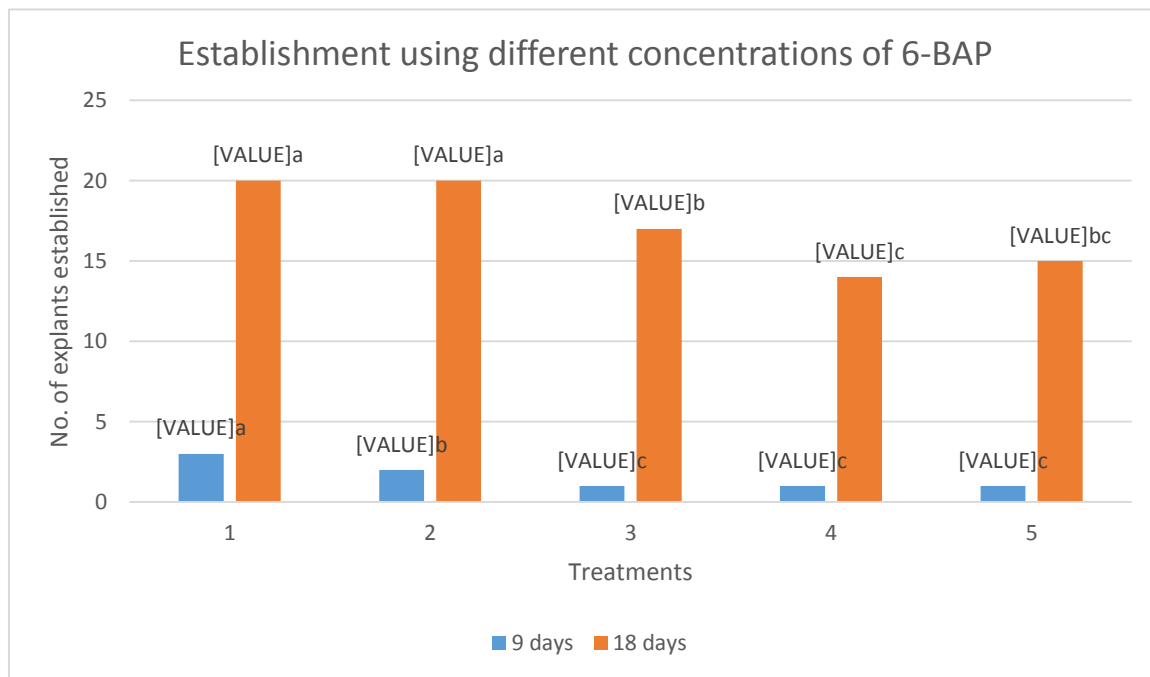
The results regarding disinfection of the axillary buds of dasheen cultivar 'verde picante' correspond to those described for the *in vitro* establishment of 'INIVIT MC 2001' (Galvez *et al.*, 2013). Sodium hypochlorite at 3.0% in a period of time ranging from 10 to 35 minutes for the disinfection of buds of the corms with an efficiency of 85.38% in the establishment phase. Shoots established can be seen in figure 2.



**Figure 2 Shoots in the establishment phase**

**Effects of the concentration of 6-Benzylaminopurine (6-BAP) in the establishment phase**

When analyzing the effect of 6-BAP concentration in the culture media (Figure 3), it was observed that there are no statistical differences between the treatments without the hormone and that with  $0.1 \text{ mg.L}^{-1}$ , which are treatments 1 and 2.



**Figure 3. Influence of 6-BAP concentration in the culture media in the establishment phase.**

**Legend:** 1. MS +  $0.1 \text{ mg.L}^{-1}$  6-BAP (Control); 2. MS; 3. MS +  $0.3 \text{ mg.L}^{-1}$  6-BAP; 4. MS +  $0.5 \text{ mg.L}^{-1}$  6-BAP; 5. MS +  $0.7 \text{ mg.L}^{-1}$  6-BAP. Means with different letters on the bars differ according to the nonparametric *Kruskal Wallis* test for  $p < 0.05$

In the case of treatments 1 and 2, eighteen days after establishment, 100% of explants with morphological characteristics were obtained to be passed to the multiplication culture medium in the semisolid state with significant differences with respect to the rest of the treatments.

Therefore in the establishment of the cultivar 'verde picante' it was shown that it is not necessary to use plant growth regulators. These results coincide with those obtained by Bhatt *et al.* (2013), who arrived at a similar conclusion during the micropropagation of five species of *Alocasia*.

However, according to Simpson (2010) usually at the apexes, the endogenous cytokinin is very low because the main site of synthesis is the roots. Therefore, exogenous addition in the establishment culture media is essential for the process of cell division, cytokinesis (cleavage of the cytoplasm to form two daughter cells). These results therefore demonstrate that each species or cultivar may respond differently to *in vitro* culture conditions, and in particular to the addition of plant growth regulators.

**Types and concentrations of plant growth regulators in the multiplication phase**

The types and concentrations of plant growth regulators in the culture medium influenced the multiplication coefficient of dasheen explants. When using culture media supplemented with  $3.0 \text{ mg.L}^{-1}$  of 6-BAP, with or without the addition of IAA, the best multiplication coefficient (3.03) were obtained at 21 days of culture (Figure 4), with significant differences to the rest of the treatments (Figure 5).

Increasing the concentrations of 6-BAP in the culture medium did not show a positive response in the number of shoots. Similar results were obtained by Bhatt *et al.* (2013) even though they used another growth regulator, (N-6-benzyladenine, BA), for which the best results were  $2.0 \text{ mg.L}^{-1}$  and higher concentrations ( $5-10 \text{ mg.L}^{-1}$ ) led to the formation of pale buds and delayed growth of five species of *Alocasia*. Bhatt *et al.* (2013) also reported the presence of alterations in the development of shoots of different species of *Alocasia* with high concentrations of BA. 6-BAP is a synthetic variant of the same group of cytokinins to which the BA belongs.



Figure 4. Dasheen explants in the multiplication phase

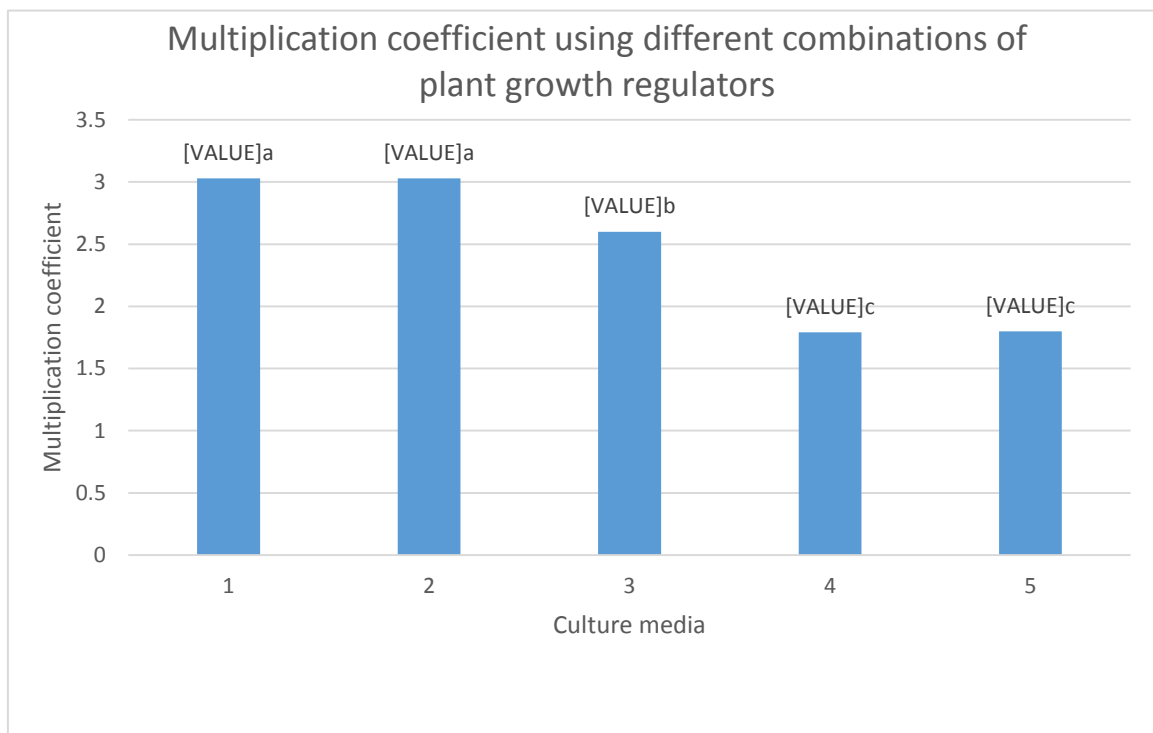


Figure 5. Influence of the types and concentrations of plant growth regulators on the multiplication coefficient of dasheen explants.

Means with different letters on the bars differ according to the nonparametric *Kruskall Wallis* test for  $p < 0.05$

Legend:

1- MS Salts and vitamins  $1.0 \text{ mg.L}^{-1}$  of IAA +  $3.0 \text{ mg.L}^{-1}$  of 6-BAP. 2- MS Salts and vitamins +  $3.0 \text{ mg.L}^{-1}$  of 6-BAP. 3- MS Salts and vitamins +  $4.0 \text{ mg.L}^{-1}$  of 6-BAP. 4- MS Salts and vitamins +  $5.0 \text{ mg.L}^{-1}$  of 6-BAP. 5- MS Salts and vitamins  $1.0 \text{ mg.L}^{-1}$  of IAA +  $6.0 \text{ mg.L}^{-1}$  of 6-BAP

The effect of the addition of 6-BAP on the multiplication of the shoots is explained by the fact that the shoot tips of dasheen have an endogenous concentration of auxins combined with that of exogenous 6-BAP added to the culture medium, hence they reach the adequate balance of auxins - cytokinins to stimulate growth and multiplication (George & Klerk, 2008).

**Effects of the physical state of the culture medium in the multiplication phase**

The management of the physical state of the culture medium during three subcultures influenced the multiplication coefficient in each subculture. When the culture medium was used in the semi-solid state in the three subcultures, the best morphological

growth of the dasheen explants was obtained. An average of 153 explants were obtained, with no significant differences with treatment four (148 explants), but with the other treatments (Table 1).

**Table 1. Effect of the physical state of the culture medium during three consecutive subcultures on the multiplication coefficient and the total number of dasheen explants.**

Treatments	Bacteria	Fungi	Initial Exp.	Final Exp.
1. S+S+S	0	2	30	153 a
2. L+S+L	4	0	30	58 b
3. L+L+L	6	0	30	45 b
4. S+L+S	0	3	30	148 a

Means with different letters within a column indicate significant differences according Kruskal-Wallis/Mann-Whitney test at  $p \leq 0.05$

**Legend:**

- 1. S + S + S. Semi-solid culture medium during the three subcultures
- 2. L + S + L. Alternating culture media Liquid - semisolid - liquid
- 3. L + L + L. Liquid culture medium during the three subcultures
- 4. S + L + S. Alternating culture media semisolid – Liquid – semisolid

The culture medium in the semi-solid state presents a higher matrix potential, decreases the rate of diffusion of molecules through the culture medium and regulates the availability of nutrients to the plant (Cabrera *et al.*, 2010).

Ruano (2015) in establishing *in vitro* *Alocasia cucullata* and *Alocasia longiloba -watsoniana-* from axillary buds, showed that there are no statistical differences between the explants established in liquid and semi-solid culture media, results that are similar to the research of Jo *et al.* (2008) who analyzed culture media of semi-solid and liquid state for the stages of establishment and multiplication of *Alocasia amazonica* in which they concluded that the physical state of the culture media does not influence the production of shoots.

**Multiplication in semi-automated culture systems**

In both semi-automated culture systems a renewal of the internal atmosphere of the culture vessel and a stimulation in the multiplication coefficient of the explants at 30 days of culture were achieved, with no significant differences between them: temporary immersion system (12.6 ) and constant immersion system with aeration to the culture medium (12.0) (Figure 6). The other parameters (plant height and diameter) didn't show any significant differences between them either. So in general, there wasn't any difference whether TIS or CIS was used.

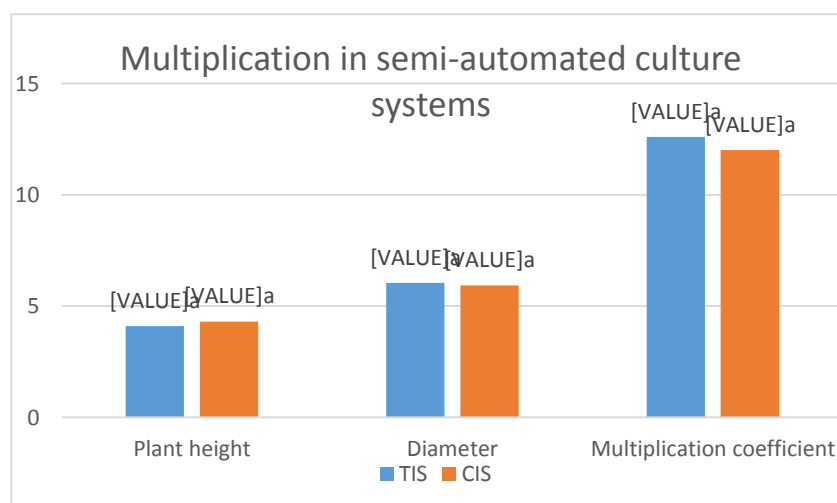


Figure 6. Effect of semi-automated culture systems on the multiplication coefficient of dasheen explants at 30 days of culture

Legend: Temporary immersion system (TIS), Constant immersion system (CIS) with aeration to the culture medium.



The culture conditions that were created in TIS and CIS allowed a better growth and multiplication of dasheen explants. According to Escalona (2006) and Cabrera *et al.* (2012), in the semi-automated culture systems, factors that allow a continuous or intermittent contact of the culture medium with the plant material are co-related, which makes possible a more efficient contribution of nutritive elements and a periodic renewal of the internal atmosphere of the culture vessel.

With the use of CIS, it was corroborated by Kozai *et al.* (2005) and Cabrera *et al.* (2011) who proposed the application of pre-moist air to achieve a renewal of the internal atmosphere as a way to achieve mixotrophic growth and increase the multiplication and growth rate of plant materials grown *in vitro*.

### Effects of plant growth regulators and the physical state of the culture medium in the rooting phase

When studying the rooting phase, it was observed that there were no differences between the treatments studied in terms of the parameters evaluated (Table 2). Therefore, it is evident that the use of plant growth regulators is not necessary to achieve good rooting of the plants and the physical state of the culture medium does not influence the development of the rooting system *in vitro*.

**Table 2. Effect of IBA concentration and the physical state of the culture medium on rooting**

IBA (mg.L <sup>-1</sup> )	Physical state of the culture medium	Plants with roots	Number of roots	Length of the roots (cm)	Height of the plant (cm)
1. 0.0	Solid	22 a	6	3.30 a	4.5 a
	Liquid	18 a	6	3.60 a	5.0 a
2. 0.5	Solid	22 a	5	3.72 a	4.7 a
	Liquid	20 a	6	3.64 a	4.5 a
3. 1.0	Solid	22 a	7	3.52 a	5.1 a
	Liquid	20 a	7	3.90 a	4.7 a
4. 1.5	Solid	22 a	6	3.34 a	4.7 a
	Liquid	20 a	6	3.42 a	4.8 a

Means with different letters within a column indicate significant differences according Kruskal-Wallis/Mann-Whitney test at  $p \leq 0.05$

This result coincides with a study by Bhatt *et al.* (2013) with five species of *Alocasia*, although they used another plant growth regulator. They found that only at low concentrations of BA (N6-benzyladenine) or in the absence of this regulator, further root formation was possible, however, they concluded that in the absence of the plant growth regulator the roots were longer.

On the other hand, Chan and Chong (2010) established a protocol for the micropropagation of *Alocasia longiloba* 'Watsoniana', where they obtained roots of 2 cm in length when 0.5 mg.L<sup>-1</sup> IBA was used, which are lower values than those obtained in this research.

### Study of different substrate in the acclimatization phase

When studying the response of plants obtained *in vitro* in five substrates, there wasn't a clear trend indicating that any of the substrate was superior (Table 3). The results obtained showed that any of the substrates used could acclimatized the plants produced successfully. So, it would depend on the availability of these substrates at the time. Similar results were obtained by Bhatt *et al.*, (2013) in five species of *Alocasia*, who successfully used a substrate composed of soil/organic matter (1:1).

**Table 3. Effect of the type of substrate in the acclimatization phase**

Substrates	10 Days		20 Days			30 Days		
	No. Leave	Height	No. leaves	height	diameter	No. leaves	height	diameter
Soil 100%	3.4 a	4.50 b	4.1 a	5.95 a	2.85 c	4.2 b	7.84 b	3.06 b
Bagasse 100%	2.7 d	4.31 c	3.3 b	5.01 c	3.14 b	4.4 b	7.90 b	3.48 ab
Soil:bagasse (1:1)	3.8 a	4.80 a	4.0 a	6.09 a	3.43 a	4.9 a	8.54 a	3.76 a
Soil:Zeolite (9:1)	3.3 c	4.68 b	3.9 a	5.28 b	3.23 b	4.1 b	7.20 c	3.54 a



Bagasse:Zeolite (9:1)	3.7 b	4.53 b	3.7 a	5.33 b	3.12 b	4.0 b	7.97 b	3.55 a
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Means with different letters within a column indicate significant differences according Kruskal-Wallis/Mann-Whitney test at  $p \leq 0.05$

**Effect of decapitation in the acclimatization phase**

In order to achieve a greater robustness of the plants prior to their transplantation to field conditions, plants were decapitated when they reached a height of approximately 8-10 cm (Figure 7).



**Figure 7 Decapitated plants of the *Alocasia* 'verde picante' cultivar produced *in vitro* when they reached a height of 8-10 cm**

It was observed that the decapitation stimulated the increase of the diameter of the pseudo stem of the plants in all the treatments (Table 4), but the best results were observed when using the bagasse substrate or the soil/bagasse (1:1). This can be explained by the amount of nutrients, especially nitrogen that is incorporated in bagasse, which stimulates the vegetative development of plants.

**Table 4. Effect of decapitation on the diameter of the pseudo stem in plants produced *in vitro* of the cultivar 'verde picante' of *Alocasia* during the acclimatization phase.**

Substrate	Diameter of the pseudo-stem (cm)		
	Evaluation 1	Evaluation 2	Evaluation 3
1	2.68	3.92	4.89 b
2	2.58	4.33	5.24 a
3	2.58	4.23	5.13 a
4	2.21	3.62	4.56 c
5	2.51	4.22	5.04 ab

Means with different letters within a column indicate significant differences according Kruskal-Wallis/Mann-Whitney test at  $p \leq 0.05$

Legend:

Substrates: 1. Soil 100% 2. Bagasse 100% 3. Soil 50% + Bagasse 50% 4. Soil 90% + Zeolite 10% 5. Bagasse 90% + Zeolite 10%.

Although it was not possible to find references to similar studies in literature consulted, it is evident that the decapitation induces a significant thickening of the pseudo stem of the plants produced *in vitro*, which is favorable for its survival in field conditions.

**Effect of sectioning the plants in the acclimatization phase**

After concluding the experiment of decapitation, the longitudinal sectioning of the plants obtained to increase the multiplication coefficient in the acclimatization phase was carried out. The best results were obtained by sectioning in three parts (Table 5), because the sprout starts first and after 28 days in the post-sectioning acclimatization phase, plants with an optimal height and thickness are obtained to be transplanted to the field with a coefficient of 1:3 for each initial plant.

Table 5. Effect of sectioning of the plants in the acclimatization phase.

Treatments	7Days		14 Days		21 Days			28 Days		
	# of shoots	# of shoots	# of suckers	# of leaf	# of shoots	# of suckers	# of leaf	Height	diameter	# of leaf
Decapitated & without sectioning	0 c	0.8 a	0 c	0.1 c	1.0 a	2.20 b	1.00 c	3.6 b	2.26 b	2.00 a
Decapitated & sectioned in half	0.1 b	0.1 b	0.1 b	0.15 b	0.5 b	0.95 c	1.25 b	2.86 c	2.50 ab	2.10 a
Decapitated & sectioned in three	1.0 a	1.0 a	0.6 a	0.66 a	1.0 a	2.47 a	1.76 a	4.05 a	2.40 a	2.16 a
Decapitated & sectioned in four	1.0 a	1.0 a	0.6 a	0.2 b	1.0 a	0.40 a	2.00 a	3.34 b	2.10 b	2.00 a

Means with different letters within a column indicate significant differences according Kruskal-Wallis/Mann-Whitney test at  $p \leq 0.05$

The results allowed to establish an efficient methodology for the *in vitro* multiplication of the cultivar 'verde picante' of the genus *Alocasia*, from the disinfection of the explants to the handling of the plants produced in the acclimatization phase, which is very important because it guarantees an effective way to obtain quality planting material in the effort to develop this crop and enhance its use in animal feed.

#### 4. CONCLUSION

A complete efficient *in vitro* micro propagation protocol for the cultivar 'verde picante' of the genus *Alocasia* was established. This protocol encompasses techniques from the disinfection of the initial explants to obtaining vigorous plants in the acclimatization phase ready to be planted in field conditions.

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