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Somatic Embryogenesis And Regeneration Of Plants In 'Blanco Criollo' Garlic (*Alliumsativum* L.) Cultivar

Diosdada Galvez Guerra[*], Sergio Juan Rodríguez Morales[1], Kenisleidy Domínguez Vázquez[1], Alay Jiménez Medina[1], Rafael Gómez Kosky[2], Laisyn Posada Pérez[3], Kolima Peña Calzada[4]and Bulen Kukurtcu[5]

1. Instituto de Investigaciones de Viandas Tropicales (INIVIT)
2. Estación Territorial de Investigaciones de la Caña de Azúcar (ETICA Centro Villa Clara)
3. Instituto de Biotecnología de las Plantas (IBP)
4. Universidad de Sancti Spíritus
5. Catalysis, S.L. Macarena, 14. 28016, Madrid, España

Abstract
In Cuba, garlic (Allium sativum L.) production has a low productivity, due to biotic and abiotic factors that affect the quality of the available seed. A methodology for the regeneration of plants from somatic embryogenesis is an alternative for seed production with good physiological quality and for its use in the genetic breeding of the crop. This methodology allows obtaining high multiplication coefficients in a shorter period of time than the conventional one, besides it lowers production costs, constituting an advantage over conventional in vitro propagation methods. The plant material disinfection protocol was adjusted to reduce the microbial contamination in the establishment. For in vitro establishment, the recommended culture medium was used with the addition of VIUSID Agro® to the culture medium in concentrations of 2.5 ml.L^{-1}. For the induction and multiplication of calluses, the most suitable culture medium was determined, which was constituted with salts and vitamins MS 50 %, sucrose 20 g.L^{-1}, 2,4-D 1 mg.L^{-1}, Kinetin 1 mg.L^{-1}. On the other hand, it was determined that with salts and vitamins MS 50 %, sucrose 100 g.L^{-1}, 2,4-D 0.5 mg.L^{-1}, a greater formation of somatic embryos was obtained. Maturity was possible with the culture medium, consisting of salts and vitamins MS 50 %, sucrose 12 g.L^{-1}, 2,4-D 0.75 mg.L^{-1}. Germination was achieved with the culture medium MS 50 %, sucrose 30 g.L^{-1}, 6 BAP 0.5 mg.L^{-1}. The formation of complete plants was achieved with the culture medium MS 100 %, thiamine 1 g.L^{-1}, sucrose 20 g.L^{-1}. The positive effect of VIUSID Agro®(2.5 ml.L^{-1}) was determined on in vitro bulbification and formation of cloves for the first time.

Keywords*: garlic, disinfection, embryogenesis, germination, maturity*

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I. Introduction

Garlic is an herbaceous plant, consisting of a subterranean bulb, formed by joined cloves at its base, around the true stem and covered by white or purple cataphylls, whose color varies depending on the variety and the planting site height (Castillo, 2004). The leaves are elongated, flat and sheathing, the flowers are pink or green. The false stem is soft, smooth and up to 40 cm long, from which aerial bulblets are born.

The root system consists of numerous simple, thin, sparsely branched adventitious roots which grow in the soil surface. The species of this genus have the same basic pattern of apical growth, both in the root and in the stem, and the branching frequency of the apical sprout varies depending on the species, cultivar and growing conditions. According to Eguillor (2010), China is the largest garlic producer, followed by India and the Republic of Korea. It is considered that the Asian continent produces about 80.7 % with a yield of 18.1 tons per hectare (ha), while Spain, France and Italy obtain 11.1 % of the harvested area and 6.4 % of world production, with the lowest yield of 6.2 t.ha^{-1}. In Latin America, Argentina has the largest planted area, with 15 600 ha and a yield of 9 t.ha^{-1}, followed by Brazil with 10 214 ha and a yield of 9 t.ha-1. However, Mexico and the United States have the highest yields (16.5 t.ha^{-1}), accounting for 2.5 % of world production, with only 1.6 % of the harvested area (Eguillor, 2010).

Garlic is affected by virus complexes that contaminate the seed and reduce its yield. Conci *et al*., 2007 state that practically all the seed of this crop is infested by a viral complex to a higher or lower degree. The disease symptoms are visible in mosaic form or yellow streaks on the leaves, whichcause decrease in the production of bulbs, which depends on the variety and the amount of virus present in the seed.

Somatic embryogenesis is an alternative for the propagation of plant material and an auxiliary tool for genetic breeding. For this reason, it is necessary to establish a method of regeneration of plants that facilitates the propagation of plants with genetic quality, which can be used as seed. Several studies Keles *et al*., 2011, Fereol *et al*., 2005 were dedicated to optimize the culture medium components and the selection of explants for the development of an adequate protocol, for the efficient regeneration of garlic plants. Different explant types (sprout, leaf, root, inflorescence, etc.) were used in these studies.

Taking into account that there is no a methodology for somatic embryogenesis and regeneration of plants in 'Blanco Criollo' cultivar, the present work was carried out with the objective of studying the stages since the material disinfection for the *in vitro* establishment, the entire embryogenic process until *in vitro* bulbification and formation of cloves that allow obtaining seeds with phytosanitary and sanitary quality to scale the results and establish field plantations.

II. Materials And Methods

The research was carried out at the Research Institute of Tropical Roots and Tuber Crops (INIVIT), where garlic meristems from 'Blanco Criollo' cultivar were used as plant material. For the development of the experiments, inorganic salts and vitamins proposed by Murashige and Skoog (1962) (MS) were used as basal culture medium. The pH of the culture mediums was adjusted to 5.7 with NaOH 0.5 $mol.L^{-1}$ and HCl 0.5 $mol.L^{-1}$ before autoclaving. The culture mediums and culture systems used in the experiments were steam sterilized in vertical autoclave (BK-75) at 121 ^{o}C and 1.20 $kg.cm^{-2}$.

Management influence of plant material disinfection

For disinfection of garlic bulbs to be established *in vitro*, four treatments were used.

Treatments:

1. Immersion in ethanol (70 %) + immersion in NaClO (3.0 %) for 20 min
2. Immersion in NaClO (3.0 %) for 20 minutes
3. Immersion in ethanol (70 %) + immersion in NaClO (3.0 %) for 25 minutes
4. Immersion in NaClO (3.0 %) for 25 minutes

After seven days of culture, the number of test tubes contaminated by bacteria and fungi was counted, as well as the number of dead explants, and the final result was expressed in percentage (%). This experiment had three replicates per treatment.

Effect of VIUSID Agro®on the development of explants for the *in vitro* establishment

With the objective of retaining the growth of leaves and increasing the thickening of the basal part, to take the meristem and place it in the culture medium of induction of calluses with embryogenic structures, the concentration influence of VIUSID Agro®in the culture medium in the establishment phase was evaluated.

Treatments:

Control. Salts and vitamins MS

1. Salts and vitamins MS + 0.5 $ml.L^{-1}$
2. Salts and vitamins MS + 1.0 $ml.L^{-1}$VIUSID Agro®
3. Salts and vitamins MS + 1.5 $ml.L^{-1}$VIUSID Agro®
4. Salts and vitamins MS + 2.0 $ml.L^{-1}$VIUSID Agro®
5. Salts and vitamins MS + 2.5 $ml.L^{-1}$VIUSID Agro®

The cloves were disinfected, according to the best variant obtained in the previous experiment. Fifty meristems were established *in vitro* per each treatment, five variants were studied in the culture medium. VIUSID Agro®was added to each test tube with 0.5 mL VIUSID Agro®. The number of explants that presented the appropriate morpho-physiological features was evaluated at 30 days, to extract the meristem and place it in the culture medium of callus induction. This experiment had three replicates per treatments.

Formation of calluses with embryogenic structures

With the aim of inducing the formation of calluses with embryogenic structures from meristems from *in vitro* plants obtained at 30 days of culture, the culture medium MS (1962) was used at 50 % with two concentrations of (2,4-D) and Kinetin (0.5 and 1 $mg.L^{-1}$), added to each treatment independently. Twenty-one meristems were used per studied concentration. The number of explants that formed calluses with embryogenic structures was evaluated and the percentage that this represented was calculated at 8 weeks of culture. This experiment had three replicates per treatments.

Formation of somatic embryos

With the objective of achieving the differentiation of somatic embryos from embryogenic structures, MS (1962) culture medium was used, complemented with different sucrose concentrations 60, 80, 100 $g.L^{-1}$, plus the combination of 2,4-D (0.5, 1.0 and 1.5 $mg.L^{-1}$), added to each treatment independently. Twenty-one explants were used per studied concentration.The number of somatic embryos formed by explants, expressed in percentage and the length (mm) of the explants were quantified at 30 days of culture. This experiment had three replicates per treatments.

Maturity of somatic embryos

With the purpose of maturing the formed somatic embryos, the culture medium effect was determined, consisting of salts and vitamins MS (Murashige and Skoog, 1962), reduced to 50 %, with a combination of two sucrose concentrations (60, 120 $g.L^{-1}$) and three of 2,4-D (1.5, 0.75 and 0.3 $mg.L^{-1}$). This experiment had three replicates per treatments.

Germination of somatic embryos

With the aim of evaluating the maturity of somatic embryos, an experiment was carried out to determine the effect of three 6-BAP concentrations (0.2, 0.4 and 0.6 $mg.L^{-1}$) in culture medium, with 50 % salts and vitamins MS. This experiment had three replicates per treatments.

***In vitro* growth of plants**

With the objective of evaluating the development of complete plants, an experiment was carried out to determine the effect of three concentrations of thiamine (0.2, 0.4 and 0.6 mg/L^{-1}) in culture medium, with 50 % salts and vitamins MS and 20 g/L^{-1} sucrose. This experiment had three replicates per treatments.

Effect of VIUSID Agro® on *in vitro* bulbification in semi-automated culture system

With the purpose of achieving the formation of bulbs with cloves, the combination of 2ip, plus sucrose and VIUSID Agro® was evaluated in the semi-automated culture medium; two treatments were studied for the bulbification phase.

Treatments:

Control. Salts and vitamins MS +2.0 $mg.L^{-1}$ of 2ip + 90 g sucrose

Treatment 1. Salts and vitamins MS + 2.0 $mg.L^{-1}$ of 2ip + 90 g sucrose + 2.0 $ml.L^{-1}$VIUSID Agro®

Treatment 2. Salts and vitamins MS + 2.0 $mg.L^{-1}$ of 2ip + 90 g sucrose + 2.5 $ml.L^{-1}$VIUSID Agro®

Twenty explants and 1500 mL of bulbification culture medium were placed in each culture system. For the Temporary Immersion System (TIS), a time of 15 minutes of immersion and immersion frequency every three hours was used. This experiment had three replicates per each culture system. The bulbification coefficient was evaluated at 30 days of culture. VIUSID Agro® 0.5 mL was added to each flask every seven days until 21 days from the day of sowing. The explants used were from the best treatment of the experiment of growth of embryos. Twenty explants were used per treatment. Bulb formation and the occurrence of flowers were evaluated at 30 days of culture per each treatment. This experiment had three replicates per treatments.

III. Results And Discussion

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 exposure time to it. Generally, concentrations from 1.0 to 6.0 % and times between 10 and 30 minutes have been used, depending on the morphological and hygienic features of the plant material to be established (Vilchez *et al*., 2011, Galvez *et al*., 2013).

The results regarding with the garlic bulb disinfection coincide with those ones described for the *in vitro* establishment of taro INIVIT MC- 2001 (Galvez *et al*., 2013). These authors used sodium hypochlorite at 3.0 % in a time period that varied from 10 to 35 minutes, for the disinfection of sprouted buds from the corms, with an efficiency of 85.38 % in the establishment.

The statistical results based on the *non-parametric Kruskall Wallis´test* for $p<0.05$ (n=150), show that the averages Ranges with non-common letters in the same column differ among them (see figure 1), being the second treatment (3.33 %) the one that showed the best disinfection performance.

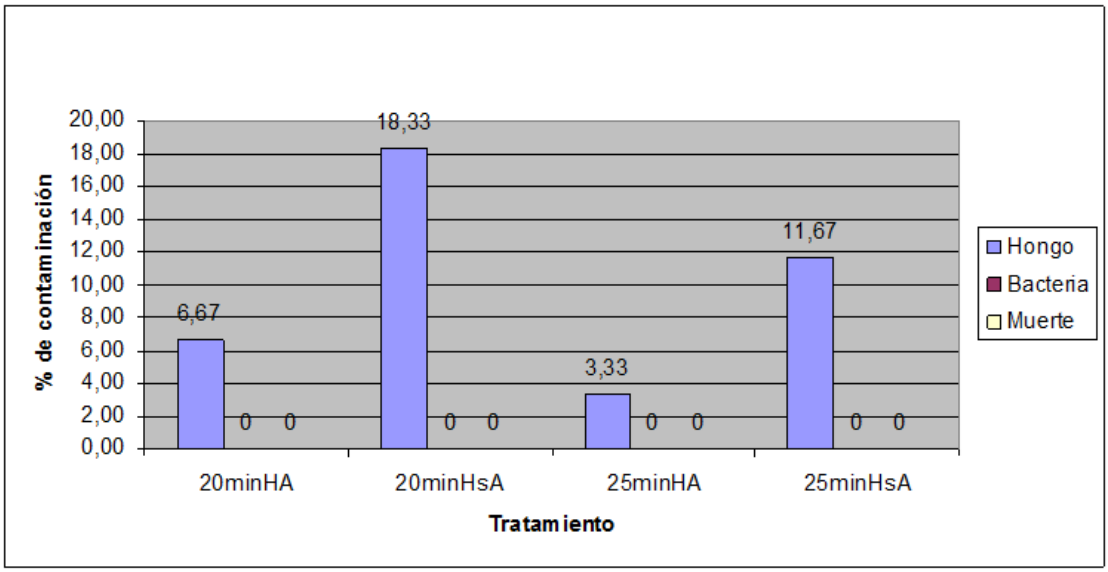

Figure 1. Influence of plant material disinfection management on the number of contaminated garlic explants at seven days of culture

Legend:
Control. Immersion in ethanol (70 %) + immersion in NaClO (3.0 %) for 20 minutes
Treatment 1. Immersion in NaClO (3.0 %) for 20 minutes
Treatment 2. Immersion in ethanol (70 %) + immersion in NaClO (3.0 %) for 25 minutes
Treatment 3. Immersion in NaClO (3.0 %) for 25 minutes
Figure 2 shows a high survival rate of garlic sprouts after being treated with disinfection.

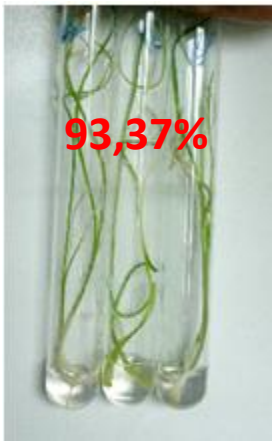

Figure 2. Garlic (*Allium sativum* L.) sprouts free of contamination in the establishment culture mediumat seven days

Effect of VIUSID Agro® on the development of explants for *in vitro* establishment

When analyzing the effect of VIUSID Agro® concentration in the culture medium (Table 1), it was observed that there were statistical differences among the studied treatments. The product addition in the culture medium determined the growth of the meristems during the establishment phase. When the culture medium was used, consisting of salts and vitamins MS + 2.5 ml.L^{-1} of VIUSID Agro®, it was achieved that 50 meristems at 18 days after establishment showed a good development. This allowed an excellent extraction of the meristems to place them in the culture medium for the induction of calluses in a semi-solid status, with significant differences, with respect to the rest of the treatments.

Table 1.VIUSID Agro®influence on the development of explants for the *in vitro* establishment

Treatments	Plant Height (cm)	Bulb Perimeter (cm)
Control	11.13 a	0.135 c
Treatment 1	6.75 b	0.150 b

Treatment 2	5.63 b	0.175 b
Treatment 3	4.90 c	0.187 b
Treatment 4	2.45 d	1.080 a
Treatment 5	2.18 d	1.120 a

Average ranges with non-common letters differ in the same column, according to Kruskal-Wallis/ Mann-Whitney´s non parametric test for $p<0.05$ (n=150).

Legend:
Control. Salts and vitamins MS
Treatment 1. Salts and vitamins MS + 0.5 $ml.L^{-1}$VIUSID Agro®
Treatment 2. Salts and vitamins MS + 1.0 $ml.L^{-1}$VIUSID Agro®
Treatment 3. Salts and vitamins MS + 1.5 $ml.L^{-1}$VIUSID Agro®
Treatment 4. Salts and vitamins MS + 2.0 $ml.L^{-1}$VIUSID Agro®
Treatment 5. Salts and vitamins MS + 2.5 $ml.L^{-1}$VIUSID Agro®
At 18 days of culture, 100 % of the established explants in the culture medium, composed of salts and vitamins MS with 2.5 $ml.L^{-1}$ of VIUSID Agro®, showed a green coloration, typical of the clone, retention of the growth of leaves and a good bulb thickening (Figure 3).

Figure 3. Morphological features of garlic (*Allium sativum* L.) explants at 30 days of culture

In the other variants of culture mediums with different concentrations of VIUSID Agro®, it was necessary to wait until 30 days of cultivation. This was the maximum time of development of the establishment experiment, to achieve the highest number of apices per ready treatments to extract the meristem, in the case of the control, there was no a positive response.

For the establishment of *in vitro* garlic cultivation the use of auxin is not necessary, since its development is fast, but not very favorable due to the presence of elongation of the leaves and a very weak bulb. With the use of VIUSID Agro® a positive response was obtained, because the elongation of leaves and bulb thickening were retained (Figure 3).

Simpson (2006) refers that usually in the apices, endogenous cytokinin is very low, because the roots are the main synthesis site, so the exogenous addition of it in the establishment culture mediums is essential for the process of cell division. In studies on the influence of hormones on DNA synthesis and cell division, it was observed that even when auxins were necessary for mitosis and DNA synthesis, mitosis and cytokinesis only occurred when there were adequate levels of cytokinins because of the influence they could do on the metabolism of nucleic acids and proteins (Jackson, 2005).

Influence of the type and concentration of growth regulators in the culture medium for the formation of calluses with embryogenic structures

The highest percentage of calluses with embryogenic structures (95.2 %) was obtained at eight weeks in the MS culture medium, complemented with 1.0 $mg.L^{-1}$ of 2,4-D in combination with 1.0 $mg.L^{-1}$ of Kinetin (Figure 4), with significant differences with the rest of the treatments used. With lower concentration of 2,4-D and Kinetin, the percentage of calluses with embryogenic structures decreased to (39.2 %).

Table 2. Combination influence of 2,4-D and Kinetin on the formation of embryogenic calluses in 'Blanco Criollo' garlic cultivar at eight weeks of culture

Treatments	2-4D (mg)	Kinetin (mg)	Formation of Calluses (%)	Callus diameter (cm)	Color
1	0.5	0.5	39.2 c	3.3 c	Cream
2	1	0.5	66.6 b	4.6 b	Cream
3	0.5	1.0	42.8 c	4.3 b	Cream
4	1	1.0	95.2 a	6.9 a	Dark yellow

Figure 4. Callus formation in 'Blanco Criollo' garlic cultivar at eight weeks of culture

Combination influence of sucrose and 2,4-D in the culture medium for the formation of somatic embryos
When comparing the combination of sucrose and 2,4-D in the culture medium for the formation of somatic embryos, the best results were achieved in the treatment with 100 $g.L^{-1}$sucrose, combined with 1 $mg.L^{-1}$ of 2,4-D (Table 3).

Table 3. Culture medium influence on the formation of somatic embryos in 'Blanco Criollo' garlic cultivar at 30 days of culture

Treatments	Sucrose (g)	2,4-D ($mg.L^{-1}$)	Formation of Embryos (%)	Length of Somatic Embryos(mm)
1	60	1	3.2 c	2 c
2	60	3	0.0 d	0 d
3	60	5	0.0 d	0 d
4	80	1	12.3 b	5 b
5	80	3	0.0 d	0 d
6	80	5	0.0 d	0 d
7	100	1	59.2 a	8 a
8	100	3	0.0 d	0 d
9	100	5	0.0 d	0 d

Figure 5. Combination effect of MS culture medium, complemented with 100 $g.L^{-1}$ sucrose, plus 1.0 $mg.L^{-1}$ 2,4-D and on the formation of somatic embryos, obtained in 'Blanco Criollo' garlic cultivar at four weeks

Combination influence of sucrose and 2,4-D in the culture medium for the maturity of somatic embryos

At 30 days after the embryos were incubated in different culture mediums for their maturity, the highest number of 76 u was obtained in the culture medium with the salts reduced to 50 %, plus the combination of 12 g of sucrose and 0.75 of 2,4-D, with differences from the other treatments (Table 4).

Table 4. Culture medium influence on the maturity of somatic embryos in 'Blanco Criollo' garlic cultivar at 30 days of culture

Treatments	Sucrose (g)	2,4-D (mg)	Mature Somatic Embryos(U)
1	60	1.5	0 d
2	60	0.75	0 d
3	60	0.3	0 d
4	120	1.5	29 b
5	120	0.75	76 a
6	120	0.3	12 c

Figure 6. Maturity of somatic embryos in 'Blanco Criollo' garlic cultivar at 30 days of culture

Concentration influence of 6-BAP in the culture medium for the germination of somatic embryos

The concentration of 6-BAP in the culture medium influenced the germination of the embryos, when the culture medium was used constituted by salts and vitamins MS at 50 %, plus 0.6 $mg.L^{-1}$ of 6-BAP, a greater number of germination was achieved. At 30 days, 100 % of them presented the morpho-physiological features, which allowed them to pass to culture medium for the formation of plants in semi-solid status, with significant differences, with respect to the rest of the treatments (Figure 7).

Table 5. Culture medium influence on the germination of somatic embryos in 'Blanco Criollo' garlic cultivar at 30 days of culture

Treatments	6 BAP (mg)	Germinated Somatic Embryos(U)	Length of Embryos(cm)
1	0.2	23 c	0.9 a
2	0.4	39 b	1.1 a
3	0.6	68 a	1.4 a
4	0.0	0 d	0 b

Figure 7. Combination effect of MS culture medium, complemented with 0.6 $mg.L^{-1}$ of 6-BAP on the germination of somatic embryos, obtained from 'Blanco Criollo' garlic cultivar at four weeks

Thiamine concentration influence in the culture medium for the formation of complete plants

When analyzing the Thiamine effect in the culture medium for the formation of plants (Table 6), it was observed that there was no difference in the formation of plants, but there was a difference in plant length and coloration. It was achieved that out of 21 *in vitro* plants, established in the culture medium, consisting of 50 % salts and vitamins MS, 20 $g.L^{-1}$ sucrose and 0.6 $mg.L^{-1}$ thiamine, all presented adequate morphophysiological features, which allowed them to pass to bulbification culture medium in liquid status, with significant differences, with respect to the rest of the treatments (Figure 8).

Table 6. Thiamine influence in the culture medium for the formation of plants in 'Blanco Criollo' garlic cultivar at 21 days of culture

Treatments	Thiamine (mg)	Formation of Plantas (U)	Length (cm)	Color
1	1.00	21 a	5.3 a	Green
2	0.75	21 a	1.2 b	Yellowish
3	0.30	21 a	1.6 b	Yellowish
4	0	0 b	0 c	0

Figure 8. Garlic (*Allium sativum* L.) plants developed in the culture medium, consisting of 50 % salts and vitamins MS, sucrose 20 $g.L^{-1}$, Thiamine 0.6 $mg.L^{-1}$ at 21 days of culture

Effect of VIUSID Agro® in semi-automated culture system (TIS) on *in vitro* garlic (*Allium sativum* L.) bulbification

The semi-automated culture system influenced the bulbification coefficient, since a regeneration of the internal atmosphere of the culture flask was achieved, the highest bulbification coefficient was obtained at 30 days of culture, with the use of the culture medium, constituted by salts and vitamins MS, with 2.0 $mg.L^{-1}$ of 2ip, 90 g of sucrose, 2.5 $ml.L^{-1}$ of VIUSID Agro®, without significant differences with treatment 1, but with the control (Table 7).

Table 7. VIUSID Agro® effect on *in vitro* bulbification of garlic (*Allium sativum* L.) in semi-automated cultivation systems (TIS)

Treatments	Plant Diameter(cm)	Number of Bulbs	Numberof Flowers
Control	5.50±0.24 b	1.00±0.02 b	0.00 b
Treatment 1	14.10±0.65 a	12.00±0.50 a	0.7±1.02 a
Treatment 2	13.60±0.69 a	13.80±0.43 a	0.9±1.12 a

Means with non-common letters differ, according to Tukey's test for $p<0.05$

Legend:
Control. Salts and vitamins MS, + 2.0 $ml.L^{-1}$ of 2ip + 90 g sucrose
Treatment 1. Salts and vitamins MS + 2.0 $ml.L^{-1}$ of 2ip + 90 g sucrose + 2.0 $ml.L^{-1}$VIUSID Agro®
Salts and vitamins MS + 2.0 $ml.L^{-1}$ of 2ip + 90 g sucrose + 2.5 $ml.L^{-1}$VIUSID Agro®
In the case of bulbification, a possible explanation may be the effect of amino acids in the plant, such as arginine, whose function is to stimulate the growth of roots, being one of the main amino acids of the rhizosphere, besides the aspartic acid, which is involved in almost all metabolic processes of the plant and one of them in garlic is the bulbification (Figures 9 and 10).

Figure 9. Bulbification of *in vitro* garlic plants in a temporary immersion system at 30 days of culture (Control)

Figure 10. Bulbification of *in vitro* garlic plants in a temporary immersion system with the use of VIUSID Agro® at 30 days of culture

Taking into account the previous results, studies were carried out where it was determined to use an immersion frequency of 15 minutes every 3 hours, for 5 L flasks, a density of 20 sprouts, a volume of 100 mL of culture medium per sprout, with the addition of 2.5 ml.L^{-1}VIUSID Agro® and a culture time of 30 days. With these cultivation parameters, the best response of the plant material and the highest bulbification coefficient (13.80) were obtained. This allowed obtaining a greater number of bulbs with the appropriate morphological features, as *in vitro* seeds, which can be taken to production directly.

In the TIS with the use of VIUSID Agro®, the presence of flowering was also observed, which does not occur without the use of this stimulant.

The cultivation conditions created in the TIS allow the best growth of garlic explants. According to Escalona (2006) and Cabrera *et al*. (2012), semi-automated cultivation systems enable a more efficient supply of nutrients and a periodic regeneration of the internal atmosphere of the culture flask.

IV. Conclusions

- A new methodology was created for somatic embryogenesis and regeneration of plants in 'Blanco Criollo' garlic (*Allium sativum* L.) cultivar.
- VIUSID Agro® is an effective stimulant for the *in vitro* development of bulbs and formation of cloves in garlic (*Allium sativum* L.).

RECOMMENDATIONS

To extend the research to new garlic cultivars with the introduction of VIUSID Agro® as a stimulant in the use of semi-automated cultivation systems (TIS).

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