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IN VITRO PLANTS RESPONSE OF YAM CLONE 'BLANCO DE GUINEA' TO PECTIMORF® USE

Respuesta de plantas *in vitro* de ñame clon 'Blanco de guinea' al uso del Pectimorf®

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ABSTRACT. The new growth bioregulators, as Pectimorf®, they have been used in the *in vitro* propagation of different cultivations. Nevertheless, the effect of this substance has not been evaluated in the yam *in vitro* propagation, therefore, this investigation had as objective to determine the influence of the Pectimorf® in the *in vitro* culture medium and its further effect in the plantlets acclimatization of yam (*Dioscorea rotundata* Poir) clone "Blanco de guinea". The MS culture medium to 75 % with different concentrations of Pectimorf® (3,0; 6,0; 9,0; 12,0; 15,0 mg L^{-1}) and a control treatment (without Pectimorf®) were used. The further residual effect of the best treatment (6,0 mg L^{-1}) was also evaluated in the plantlets acclimatization. To the 35 days it is selected 25 *in vitro* plants aleatorily to which were determined the following variables: roots number; greatest root length (cm), leaves number, bud *novo* number and shoot length (cm). After 45 days survival (%) in 70 plantlets is evaluated by treatment and it is selected in 40 plantlets by treatment to which were determined the following variables: buds number; shoot length (cm) and leaves number. The addition of Pectimorf® 6,0 mg L^{-1} in the *in vitro* culture medium was the most appropriate with a significant influence in the *in vitro* plants vegetative development and a favorable residual effect in the acclimatized plantlets of yam clone "Blanco of guinea".

Key words: growth bioregulators, *Dioscorea rotundata*, micropropagation, oligosaccharides

RESUMEN. Los nuevos biorreguladores del crecimiento, como el Pectimorf®, se han utilizado en la propagación *in vitro* de diferentes cultivos. No obstante, el efecto de esta sustancia no se ha evaluado en la propagación *in vitro* del ñame; por ello, esta investigación tuvo como objetivo determinar la influencia del Pectimorf® en el medio de cultivo *in vitro* y su posterior efecto en la aclimatización de las plántulas del ñame (*Dioscorea rotundata* Poir) clon "Blanco de guinea". Se utilizó el medio de cultivo MS al 75 %, que contenía distintas concentraciones de Pectimorf® (3,0; 6,0; 9,0; 12,0; 15,0 mg L^{-1}) y un tratamiento control (sin adición de Pectimorf®). También se evaluó el ulterior efecto residual del mejor tratamiento (6,0 mg L^{-1}) en la aclimatización de las plántulas. A los 35 días se tomaron aleatoriamente 25 plantas *in vitro* a las cuales se les determinaron las siguientes variables: número de raíces; longitud de la raíz de mayor tamaño (cm), número de hojas; número de nudos de novo y la longitud del vástago (cm). Al cabo de 45 días de aclimatización, se evaluó la supervivencia (%) en 70 plántulas por tratamiento, y se tomaron aleatoriamente 40 plántulas a las cuales se les determinaron las siguientes variables: número de brotes; longitud del tallo (cm) y número de hojas. La adición de Pectimorf® a razón de 6,0 mg L^{-1} en el medio de cultivo *in vitro* fue la concentración más adecuada con una influencia significativa en el desarrollo vegetativo de las plantas *in vitro* y un efecto residual favorable en las plántulas aclimatizadas de ñame clon "Blanco de guinea".

Palabras clave: biorregulador del crecimiento, *Dioscorea rotundata*, micropropagación, oligosacáridos

INTRODUCTION

The main disadvantages of yam production are the scarcity of healthy pest-free seeds and their planting in low-fertility soils, which lead to a loss of up to 90 % of the yields of this crop (1). The main goal of yam cultivation worldwide is to obtain good quality planting material (2).

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In Cuba the production of yams has declined in recent years. This has mainly been due to the fact that their cultivation has been limited to small producers and to the unavailability of good quality seed (3). On the other hand, the tubers, which constitute the useful part of the plant for food, also have to be used as planting plant material.

The clone of yam 'Blanco de guinea' (*Dioscorea rotundata* Poir) is characterized by its adaptability to the edaphoclimatic conditions of the main agricultural areas in the country. It has high nutritional value and it is one of the clones of greater acceptance by the population for its fresh consumption and in processed form.

Unlike commercial clones belonging to the *D. alata* species, this cultivar of the *D. rotundata* species does not produce aerial bulbs so that between 35 and 40 % of the produced tubers must be preserved as "seed" for the next plantation (4).

The low multiplication rates of this clone through conventional propagation have led to the development of protocols for *in vitro* propagation and their introduction into biofactories in the country, which has allowed starting material to initiate quality seed production programs in this crop (5). Recently the field response of *in vitro* plants of *D. alata* at different planting times during the year was evaluated, which has contributed to the design of strategies for seed production in the country (6).

There are numerous investigations of a group of bioactive or growth-stimulating substances in Cuba and other countries, which can be used as partial or total substitutes for growth regulators commonly used in the culture media of micropropagation schemes in the field of Plant Biotechnology; within this group is Pectimorf®, which has been used to promote different processes in plants, both *in vitro* and *ex vitro* (7).

Pectimorf® is a natural and safe product, consisting of a mixture of biologically active oligosaccharides obtained from citrus pectin, the active ingredient of which is a mixture of α-1,4-oligogalacturonides with degree of polymerization (GP) between 9 and 16. It is considered a potent defense elicitor in plants and stimulates cell growth and differentiation of different plant species (8).

The clone of yam 'Blanco de guinea' (*D. rotundata*) represents one of the commercial clones of greater importance and demand by the producers of this crop, due to the excellent nutritional quality, culinary and of acceptability by the consumers.

There are protocols that use benzylaminopurine (BAP) alone or combined with naphthaleneacetic acid (ANA) in organogenic propagation *in vitro* from nodal segments in the *D.cayenensis-rotundata* and *D.alata* species, with a low efficiency (9). However, no research has been done on the effect of different concentrations of Pectimorf® on the culture medium on the vegetative development of the *in vitro* plants and the acclimatized seedlings in yam (*Dioscorea* spp.) and in particular on the species *D. Rotundata* clone 'Blanco de guinea'.

MATERIALS AND METHODS

The work was carried out at the Laboratory of the Plant Biotechnology Studies Center (CEBVEG) of the Faculty of Agricultural Sciences belonging to the University of Granma, Bayamo, Cuba.

Vegetal material

Uninodal segments of the yam 'Blanco de guinea' (*D. rotundata*) clone were used with a length of 12 to 15 mm, obtained from plants *in vitro* in the third subculture, from the Germplasm Bank of categorized seed of CEBVEG.

Characteristics of the clone 'Blanco de guinea'

It is characterized by presenting heart-curled leaves, green, whole, parallel veins, and opposite. cylindrical petioles, PILP (insertion limb petiole) and PIPT (insertion point petiole stalk) green, both young and adult leaves, cylindrical stems with green spines and wound in a clockwise direction. Cylindrical rhizomes, chestnut, rough skin, beige sub-epidermis, white mass, susceptible to anthracnose. It is necessary to cultivate it with tutors (10).

In vitro culture media and conditions

Basal culture medium composed of the salts of Murashige and Skoog (MS) at 75 % concentration (10), vitamins DM (11), sucrose 30 g L^{-1}, cysteine 10 mg L^{-1} and E agar (Biocen) 7,5 g L^{-1} (2). The pH of the culture medium was adjusted to 5,8 before autoclaving. The culture medium was distributed in 24x150 mm test tubes with 5 mL per tube. Finally, it was sterilized in a vertical autoclave (BK-75) at 121 °C temperature and 1,2 kgf cm^{-2} for 25 minutes.

The culture media were kept at rest three days prior to use, to detect any contamination thereof. Planting of the plant material in the culture vessels was carried out under a horizontal laminar flow cabinet. The culture conditions in the growth chambers of sunlight were: temperature, 25 ± 2 °C; relative humidity, 70-80 %; light intensity of 40 μE $m^{-2}s^{-1}$, and photoperiod duration of 12 light hours.

Influence of Pectimorf® on the "in vitro" culture medium of uninodal segments of yam clone 'Blanco de Guinea'

The purpose of the experiment was to determine the effect of different concentrations of Pectimorf® on the *in vitro* culture medium of uninodal segments of 'Blanco de guinea' clone yolk.

A completely randomized design was applied with 50 explants per treatment (three replicates in time), which consisted in the use of different concentrations of Pectimorf® (3,0, 6,0, 9,0, 12,0, 15, 0 mg L^{-1}) in 75 % DM culture medium, and a control treatment (without addition of Pectimorf®). At 35 days, 25 plants were randomly taken *in vitro* by treatment and the following variables were determined: number of roots; length of the largest root (cm), number of leaves per plant, number of nodes de novo per plant and length of stem (cm) per plant.

Residual effect of Pectimorf® during the acclimatization of the in vitro plants of 'Blanco de Guinea'

This experiment was carried out in order to determine the residual effect of Pectimorf® on the fundamental indicators of seedling development in the acclimatization phase.

As plant material, *in vitro* plants were used from the fifth subculture in 75 % DM culture medium with the Pectimorf® concentration most appropriate for its biological effect and saving of this bioregulator obtained in the *in vitro* phase (6 mg L^{-1}) and without addition of Pectimorf® (control).

A completely randomized design consisting of two treatments with 70 plants *in vitro* each (four replicates in time) was used, which were planted in 70-well polyurethane trays with 100 cm^3 capacity on a substrate composed of worm humus (50 %) + zeolite (50 %). One plant was placed per alveolus.

The trays were placed in protected culture house with a black zaran mesh (70 % reduction of sunlight). The photoperiod was 12 hours' light and the temperature was 33±2 °C. Irrigation was done by spraying with microjet and a daily frequency at full substrate capacity (90-95 % relative humidity). After 45 days, the survival rates in 70 and at 40 plants per treatment were evaluated. It was determined the following variables: number of outbreaks; length of major stem (cm) and number of leaves.

A simple classification variance analysis with Tukey's mean comparison test at 5 % probability of error was applied to evaluate the influence of Pectimorf® on the in vitro culture medium of uninodal segments of 'Blanco de guinea' clone yolk. To verify the normality of the data we used the Kolmogorov - Smirnov test and for the homogeneity of variances the Barttlet test. A Student's t-test was used to determine the residual effect of Pectimorf® during the acclimatization of the *in vitro* plants of the 'Blanco de guinea' clone.

All statistical analyzes were performed with the Statistica program for WINDOWS, version 10.0 (12).

RESULTS AND DISCUSSION

Efecto del Pectimorf® en el medio de cultivo "in vitro" de segmentos uninodales del ñame clon 'Blanco de guinea'

Effect of Pectimorf® on the *in vitro* culture medium of uninodal segments of yam clone 'Blanco de Guinea' The *in vitro* cultivation of yam in both *D. alata* and *D. rotundata* has as a characteristic that during the *in vitro* multiplication phase the plants rooted satisfactorily, so that it is considered an *in vitro* propagation phase that contemplates both step of multiplication as rooting without addition of growth regulators.

In analyzing the effect of the addition of Pectimorf® to the *in vitro* propagation medium of 'Banco de Guinea' yam (Table I), a significant increase in the vegetative development of plants *in vitro* (stem length, with the use of 6, 9 and 12 mg L^{-1}, which differ from the treatments composed of 3, 15 mg L^{-1} of Pectimorf® and control (without addition of Pectimorf®).

These results demonstrate an auxin/cytokinin effect of Pectimorf® from concentrations of 6 to 12 mg L^{-1}. The auxinic role exerts a greater cellular elongation, which is clearly evident in the significant increases of the vegetative indicators: stem length, longest root length and number of roots. Numerous studies corroborate that the Pectimorf® exerts an effect similar to that of the auxins, in the *in vitro* culture of plants (7).

When evaluating the effect of Pectimorf® on the cultivation of apexes of cassava plants *in vitro* (*Manihot esculenta* Crantz), clones `CMC-40' and` Señorita' (8), it was determined that the height of the plants *in vitro*, in The CMC-40 clone with 5 mg L^{-1} of Pectimorf® replacing ANA showed the highest value of height (2,88 cm), with no significant differences with the control (ANA and BAP), and treatments where Pectimorf® was added at 5 and 10 mg L^{-1} respectively, in the presence of ANA and BAP substitution.

Table I. Influence of the addition of Pectimorf® different concentrations on the vegetative development indicators of *in vitro* plants of 'Blanco de Guinea' clone at 35 days in medium of propagation culture

Concentration of Pectimorf® (mg L^{-1})	Length of stem (cm)	Length of root (cm)	Number of leaves	Number of new *nodes*	Number of roots
0	3,0 b	2,2 b	5,5 b	4,2 b	11,2 b
3	3,1 b	2,2 b	5,4 b	4 b	11,0 b
6	3,5 a	2,6 a	6,8 a	4,8 a	13,2 a
9	3,5 a	2,6 a	7,1 a	4,9 a	13,5 a
12	3,7 a	2,8 a	7,3 a	5,3 a	13,7 a
15	2,8 b	2 b	5,2 b	4,1 b	10,7 b
EE	0,12	0,07	0,11	0,13	0,17

Means with different letters differ significantly according to Tukey's test, p <0.05. EE, Standard Error

The results indicated that the product managed to compensate for the effect of auxin (ANA) in the absence of this in the medium; however, there was no marked (antagonistic) depressant effect when it was present.

However, (8) the highest values of height were obtained in the clone 'Missorita' where Pectimorf® was used in the presence of ANA and GA^3 at concentrations of 5 and 10 mg L^{-1}, which did not differ statistically between them and reached values above 2 cm in height. Both treatments did not differ from the control treatment, treatment with 6-BAP + 5 mg L^{-1} of Pectimorf® and the treatment where Pectimorf® was used in the presence of ANA at the concentration of 15 mg L^{-1}. In this case, the presence of ANA + Pectimorf® was necessary, where the product had a similar effect to the cytokinins as it replaced the absence of BAP and stimulated the growth of cassava apexes. This clone also required the presence of auxin and concentrations of 5 and 10 mg L^{-1} of Pectimorf®, which demonstrated that the endogenous content of auxins in this clone is lower, so its exogenous addition is necessary to achieve the appropriate balance for apex growth.

A similar response was reported in a similar range of use of Pectimorf® in *Nicotiana tabacum* and *Arabidopsis thaliana* (L.) Heynh (13); using 10 mg L^{-1} of Pectimorf® in the *in vitro* culture medium, where the best results were achieved in the root elongation.

Studies have demonstrated a synergistic auxinic effect of this substance with indole butyric acid (IBA) on rooting in papaya plants (*Carica papaya* L.), where the highest number of plants with roots as well as the highest percentage of survival was obtained in the treatment with 9 mg L^{-1} of Pectimorf® combined with 2 mg L^{-1} of IBA, in the absence of sucrose in the culture medium and using the zeolite as support (14).

The Pectimorf® cytokinin effect at concentrations of 6 to 12 mg L^{-1} was expressed in a higher cell division activity, which was evidenced in the significant increases in the vegetative indicators number of leaves, nodes de *novo* and roots of plants (Table I). In relation to this, when evaluating the *in vitro* propagation of segments of lily scales (15), similar results were obtained when verifying the stimulating effect of this mixture of oligogalacturonidoses in the concentration of 10 mg L^{-1} in the regeneration of plants with a greater number of bulbs, leaves and roots.

On the other hand, these results do not coincide with those obtained when evaluating the effect of Pectimorf® on the cultivation of apexes of cassava plants *in vitro* (*Manihot esculenta* Crantz) (8), for the number of leaves, in the clone 'CMC- 40', where they reached the highest values (5,5 leaves) when Pectimorf® was used in the concentration of 15 mg L^{-1}, in the presence of both regulators, without statistically differences to the treatment in the absence of ANA and 5 mg L^{-1} of Pectimorf®, but the rest of the treatments.

These results coincide with those obtained when incorporating Pectimorf® into the *in vitro* culture medium of cassava (*Manihot esculenta* L.) where the best results were obtained with 9 mg L^{-1} of Pectimorf® both in the rooting of the shoots *in vitro* of cassava as its subsequent acclimatization (16). However, in tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* (L.) Heynh, the best results were achieved using 10 mg L^{-1} of Pectimorf® in the *in vitro* culture medium (13).

The results obtained in the *in vitro* propagation phase indicate that the addition of Pectimorf® (6 mg L^{-1}) in the culture medium (Figure 1) is adequate for the vegetative development of the plants *in vitro* and in particular to increase the efficiency of *in vitro* micropropagation of yam clone 'Blanco de guinea'.

In *in vitro* culture, the Pectimorf® has been extensively studied, demonstrating its capacity as a substitute for traditional growth regulators, auxins and cytokinins, at different stages and in crops such as sugarcane, coffee, citrus, potato, tomato, tobacco, banana, rice, garlic, among others.

Figure 1. Significant vegetative development of the *in vitro* plants of "Blanco de Guinea" clone in the culture medium with Pectimorf® at 6 mg L^{-1} (Left, Treatment 2) in relation to the control without Pectimorf® (Right, Treatment 6)

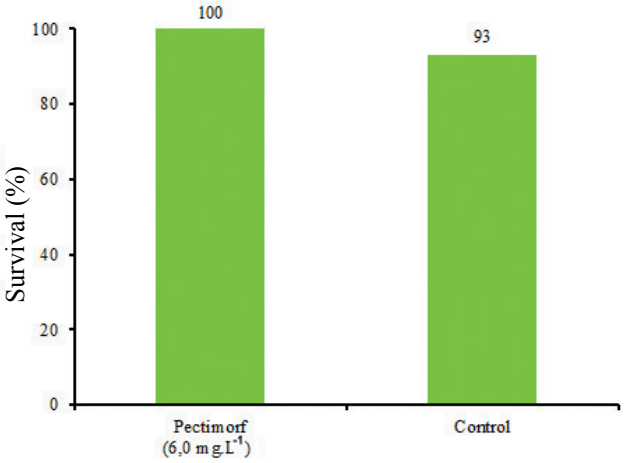
Benefits to the crop have also been observed, such as the promotion of rooting, the increase of shoots, as well as beneficial results in the stage of ex vitro adaptation (7).

Pectimorf® promotes the development of roots in plants at concentrations between 5 and 20 mg L^{-1}, which has been demonstrated in different cultures (7).

The results obtained in this research in *D. rotundata* "Blanco de Guinea" clone with the best concentrations of Pectimorf® (6 to 12 mg L^{-1}) at 35 days of culture, when compared with those obtained by other authors (9) on micropropagation from nodal segments of *D.cayenensis-rotundata*, Kratsi clone in the DM medium with the growth regulators ANA/BAP (0,5-0,5 mg L^{-1}) at 60 days of culture ((Although almost double the time) (5), are similar for the number of new (5) knots, higher for the number of leaves (5) and roots (3.9) and slightly lower for the length of the stem (4,55). This evidences the marked auxin / cytokininic effect of Pectimorf® in the in vitro propagation of yam (*D. rotundata*) Guinean white clone from 6 to 12 mg L^{-1} in the 75 % MS medium and its potential as Substitute for the growth regulators traditionally employed in the propagation of this crop.

Residual effect of Pectimorf® during the acclimatization of the *in vitro* plants of the 'Blanco de Guinea' clone

The results on the effect of the most appropriate Pectimorf® concentration (6 mg L^{-1}) obtained in the *in vitro* culture medium on the survival of the 'Blanco de Guinea' clone yam seedlings are presented in Figure 2. 45 days of acclimatization in a substrate composed of worm humus (50 %) + zeolite (50 %) As can be seen, there was a significant increase (7 %) in the survival of the plants in the treatment with 6 mg L^{-1} of Pectimorf®, which could be attributed to a residual effect of this bioregulator due to a greater accumulation of reserve substances in the *in vitro* phase, which propitiates a greater resistance and tolerance to the stress conditions during this phase of acclimatization, recover faster and present better conditions for final field transplantation.



Means with different letters differ significantly according to the student t test for p <0,05 (t=8,4, df=2, p=0,000000)

Figure 2. Residual effect of the addition of 6 mg L^{-1} Pectimorf® in the *in vitro* culture medium on the survival of "Blanco de Guinea" yam clones at 45 days of acclimatization in a substrate composed of humus worm: zeolite (1: 1)

Similar results have been achieved in the Pectimorf® influence evaluation on the acclimatization of in vitro banana plants (Musa spp.) Of the FHIA 18 clone during the immersion of plant roots in vitro for 15 minutes before planting and foliar spraying with oligogalacturonide at different concentrations (1, 5 and 10 mg L^{-1}); 15 days after planting, plant survival increased by approximately 8 % with respect to control[A].

[A] Izquierdo, H. I. Empleo de nuevas sustancias como reguladores del crecimiento en la micropropagación del banano (*Musa* spp.) clon «FHIA-18» (AAAB). Tesis de Doctorado, Instituto Nacional de Ciencias Agrícolas, 2013, Mayabeque, Cuba, 102 p.

The effect results of the most appropriate Pectimorf® concentration (6 mg L^{-1}) obtained in the *in vitro* culture medium on the number of shoots, stem length and number of leaves during their acclimatization is shown in Table II. A substrate composed of 1: 1 of worm humus and zeolite. In all cases, a significant influence of Pectimorf® on all indicators evaluated was observed, indicating the potent action of this substance in the later vegetative development of the seedlings during acclimatization and adaptation to natural *ex vitro* conditions.

In this sense, in the FHIA 18 clone banana crop, results similar to the influence of the Pectimorf® addition in the DM medium on plant height, number of leaves and stem thickness were obtained. Acclimatization, where they observed a significant increase of these parameters and a decrease of the abiotic stress induced by the techniques of *in vitro* culture (less content of proline foliar) with addition of Pectimorf® in the culture medium of rooting from 5 mg L^{-1} and a favorable effect at this stage of micropropagation.

In general, Pectimorf® 3 mg L^{-1} (lowest) and 15 mg L^{-1} (highest) concentrations were found to have the lowest significant values in the morphological indicators evaluated. This could be given for the lower concentration at which the adequate hormonal balance was not reached, to induce the significant increase of the vegetative development processes of the plants in vitro, so that a greater exogenous addition of Pectimorf® was necessary from 6 mg L^{-1}. This confirms that generally the response of Pectimorf® as a bioregulator in the *in vitro* culture of many plants is achieved from 5 mg L^{-1}, whereas the highest concentration (15 mg L^{-1}) did not have a favorable effect, which indicates that its addition exceeds the optimum hormonal balance of the explant, resulting in such an inappropriate concentration to stimulate the propagation of *D. rotundata*, clone 'Blanco de Guinea' (7) *in vitro* plants.

Finally, the results of this research showed a significant and more appropriate biological effect with the addition of 6 mg L^{-1} of Pectimorf® in the *in vitro* culture medium of the 'Blanco de Guinea' clone yam both in the micropropagation phase (Figure 3) and in the acclimatization phase of the seedlings (Figure 4), which can be attributed to their marked bioregulatory effect on the vegetative development of plants under *in vitro* and *ex vitro* conditions.

Figure 3. Vegetative development of *in vitro* plants of "Blanco de Guinea" at 35 days in the micropropagation phase, from 75 % culture medium with 6 mg L^{-1} of Pectimorf® (left) and the control treatment (right)

Figure 4. Yam seedlings "Blanco de Guinea" at 45 days in the acclimatization phase, from the 75 % culture medium with 6 mg L^{-1} of Pectimorf® (left) and the control treatment (right)

Table II. Residual effect of the addition of 6 mg L^{-1} Pectimorf® in the *in vitro* culture medium on the vegetative development indicators of "Blanco de Guinea" yam seedlings at 45 days of acclimatization in a substrate composed of humus worm: zeolite (1: 1)

Concentration of Pectimorf® (mg L^{-1})	Number of sprouts	Length of stem (cm)	Number of leaves
0	3,0 b	3 b	5,6 b
6	3,5 a	5 a	6,8 a
t	3,98	6,79	14,14

Means with different letters differ significantly according to the student t test for $p<0.05$ (df=23, p=0.000000)

Similar results were achieved by other investigators (17), who demonstrated that Pectimorf® at 10 mg L^{-1} concentration accelerate and increase the *in vitro* somatic embryogenesis process of *Citrus macrophylla* Wester and they were also used in different biotechnological processes as: Growth of cassava seedlings (*Manihot esculenta* C.) during their acclimatization phase (18).

Recently it has also been stated that the response of this mixture of oligogalacturonides (7, 8), whose effect is similar to that of auxins or cytokinins, may be mainly due to the hormonal balance of the explant and the composition of growth regulators in the medium of cultivation; in addition, regulate, among other processes, the interaction between auxins, cytokinins, gibberellins and ethylene, which validates Pectimorf® as a promising alternative in Cuban vegetable biotechnology.

CONCLUSION

The results obtained demonstrate the potential of Pectimorf® as a promoter of morphogenetic processes in plant species; it is evidenced that the incorporation of 6 mg L^{-1} of Pectimorf® into the *in vitro* culture medium of the yam (*D. rotundata*) "Blanco de Guinea" clone has a significant effect on the vegetative development of *in vitro* plants and a favorable residual influence in the acclimatized seedlings.

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