

## Contributions to knowledge of the functioning of national bioestimulators in plant biotechnology processes

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### ABSTRACT

In Cuba, biostimulators are being obtained, based on oligogalacturonides (Pectimorf) and brassinosteroids analogs (Biobras-6, Biobras-16 and MH-5) which were effective when applied in a single phase of the micropropagation processes for plant multiplication. However, the question remained if there could be an improvement when they were applied during the entire micropropagation process and if the effect would be sustained once transferring the plants to *ex vitro* conditions. In this work, the effectiveness of these biostimulators was evaluated as growth regulators, combined with auxins or cytokinins, in organogenesis and somatic embryogenesis of economically relevant crops, assessed through their biological activity, and the physiology and anatomy of plants. Regenerants showed genetic stability, the compounds been more effective than the auxin-cytokinin combinations. Pectimorf unprecedentedly influenced on hormones' concentration in banana plants during their acclimatization phase, and new methodologies were proposed for micropropagation of garlic, banana and plantain, *Spathiphyllum* sp. and *Citrus*, not inducing genetic variability, and a protocol for acclimatization of *Vriesea*. Moreover, pineapple plantlets were obtained of improved quality and uniformity, by combining immersion bioreactors with MH-5 supplementation of the culture medium. It was also corroborated in *Arabidopsis* sp. and sugarcane that Pectimorf and oligogalacturonides regulate vegetal growth. This research granted the 2015 Award of the Cuban National Academy of Sciences.

**Keywords:** *in vitro* culture, *ex vitro* culture, new methodologies, growth regulators, genetic variability, genetic stability, brassinosteroids analogs, oligogalacturonides

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### RESUMEN

**Aportes al conocimiento del funcionamiento de bioestimuladores nacionales en procesos de la Biotecnología Vegetal.** En Cuba se trabaja en la obtención de bioestimuladores a base de oligogalacturónidos (Pectimorf) y de análogos de brasinosteroides (Biobras-6, Biobras-16 y MH-5), que demostraron su efectividad en una u otra fase aislada de procesos biotecnológicos para la micropropagación de especies vegetales. Sin embargo, se desconocía su influencia al incluirlos en todas las fases de la micropropagación *in vitro* y su posterior efecto al transferir las vitroplantas a condiciones *ex vitro*. En este trabajo se evaluó la efectividad de estos bioestimuladores como reguladores del crecimiento, en combinación con auxinas o citoquininas, en los procesos de organogénesis y embriogénesis somática de especies vegetales de interés agrícola y en el modelo biológico *Arabidopsis* sp., según su actividad biológica en la morfogénesis, fisiología y anatomía de las plantas. Se comprobó la estabilidad genética de los regenerantes obtenidos, y dichos compuestos fueron más efectivos que la combinación de auxinas y citoquininas. De forma novedosa, el Pectimorf influyó en la concentración de hormonas en banano durante la fase de aclimatización y se propusieron nuevas metodologías para la propagación de ajo, plátano macho, *Spathiphyllum* sp. y cítricos, que no inducen variabilidad genética, así como un protocolo para la aclimatización de las *Vriesea*. Se obtuvieron plántulas de piña de mejor calidad y uniformidad en biorreactores de inmersión temporal combinados con el MH-5 en los medios de cultivo. Además, se comprobó en el modelo *Arabidopsis* sp. y en caña de azúcar que el Pectimorf y los oligogalacturónidos regulan el crecimiento vegetal. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2015.

**Palabras clave:** cultivo *in vitro*, cultivo *ex vitro*, nuevas metodologías, reguladores del crecimiento, variabilidad genética, estabilidad genética, análogos de brasinosteroides, oligogalacturónidos

## Introduction

The *in vitro* propagation techniques of vegetal species, like organogenesis and somatic embryogenesis, facilitate the large scale multiplication of plants, generate high biological quality seeds and promote the generation of genetically stable biological material. But such techniques depend on the use of artificial cultivation substrates and growth regulators, auxins and cytokinins, mostly.

In fact, plant growth and development are highly regulated processes, in which the concerted action of several vegetal hormones (auxins, gibberellin, abscisic acid and ethylene) at precise concentrations suffices the physiological needs [1]. Among the growth regulators are also chemical substances, either natural or synthetic, displaying similar effects to that of hormones and influencing on plant growth from germination to senescence, the reason why they are frequently used for *in vitro* culture [2, 3].

Therefore, we focused on a number of vegetal species economically relevant in Cuba or abroad when treated with biostimulators. They were: garlic (*Allium sativum* L.), mandarin orange (*Citrus reshni* Hort. ex Tan.) and *Citrus macrophylla* Wester, plantain and banana (*Musa* spp.), sugarcane (*Saccharum* spp.) and ornamental plants (*Spathiphyllum* sp. and *Vriesea* sp.). Additionally, we also studied the *Arabidopsis thaliana* model species for the action of the compounds tested. Commonly, these species are propagated *in vitro* in biofactories, but they are exposed to several detrimental factors, such as: culture medium composition, the explant splitting technique, the low multiplication coefficient and high phenolization, all them influencing in production losses. Besides, chromosomal changes can occur during prolonged subculturing with low survival in acclimatization. Additionally, depending on the growth regulators used, phenotypic and genotypic effects can emerge, such as variegated plants [4, 5].

In Cuba, oligogalacturonides-based biostimulators have been obtained, as well as brassinosteroids analogs, which have demonstrated to be effective when tested alone in isolated phases of the biotechnological propagation processes of vegetal species (organogenesis or somatic embryogenesis). However, their systemic effect in the *in vitro* propagation of plants when present throughout the whole set of *in vitro* stages of the biotechnological processes was unknown, including their effect on vitroplants once transferred to *ex vitro* conditions.

At the same time, there was also interest to know if the treatment with those biostimulators (brassinosteroids-analogs and oligogalacturonides) could induce genetic variability on the obtained regenerants. In fact, the genetic stability of regenerants could be studied by techniques targeting cytogenetic [6], isoenzymatic [7] and molecular markers (*i.e.*; random amplified polymorphic DNA, RAPD)[8], in addition to traditional but very slow procedures based on morphological markers. Their combination seems to be efficacious to evaluate the genetic variability of crops, such as plantain and banana (*Musa* spp.) [8, 9], mandarin orange (*Citrus reshni* Hort. ex Tan.) [10], sweet potato (*Ipomoea batatas* L.) [11] and pineapple

(*Ananas comosus* [Lindley] Coppens and Leal) var. 'Bracteatus' [12], among others.

Therefore, in order to generate new knowledge on the integral characterization of the genetic stability of plants propagated *in vitro*, this work was aimed to evaluate the effectiveness of the use of brassinosteroids-analogs and oligogalacturonides of produced in Cuba as growth regulators for organogenesis and somatic embryogenesis of the abovementioned species. Moreover, their biological activity on the morphogenesis, physiology and anatomy of plants was also investigated, together with the genetic stability of the regenerants.

## Materials and methods

Assays were runned under aseptic, manipulation and sterilization standard conditions as required in tissue culture procedures. Culture were implemented in Murashige-Skoog (MS) basal salt medium [13], supplemented with 0.4 mg/L thiamine HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 2 mg/L glycine, 80 mg/L adenine sulphate, 100 mg/L myoinositol, 0.5 mg/L agar or gelrite, and 30 g/L sucrose. Additionally, a brassinosteroid analog formulation was added, containing the spirostane brassinosteroids analogues Biobras-6, Biobras-16 or MH-5, and a mixture of oligogalacturonides of polymerization degree 9-16 known as Pectimorf. pH was adjusted to 5.7-5.8 ± 0.1 and medium was sterilized in autoclave at 1.5 atm. and 121 °C for 15 min. Cultures were incubated at 22-27 °C, 25-35 µmol/m<sup>2</sup>/s and 16 h light photoperiod unless specified.

The material obtained under laboratory conditions was transferred into field conditions in plastic trays with 72 holes with a capacity for 47.61 cm<sup>3</sup> or in 125-cm<sup>3</sup> plastic pots, and following all the technical requirements as specified for plantlet acclimatization procedures [14-17].

Cytogenetic, molecular protein and DNA techniques (RAPD and AFLP) were applied to determine the possible genetic variability induced by the compounds used in the different vegetal species assayed.

In the case of determination and quantification of hormones in banana leaves, the endogenous levels of auxins (indole acetic acid, IAA), cytokinins (isopentenyladenine, iP; isopentenyladenosine, [9R] iP; zeatin (Z), zeatin riboside ([9R] Z) and abscisic acid, ABA) were advantageously determined in extracts of 1 g of leave tissue by using an immunoenzymatic method according to the procedures reported by Maldiney *et al.* [18]. The growth regulator compounds IAA, ABA and cytokinins were quantified by extracts analysis in HPLC conditions.

In ornamental plants, two bioassays were used, denominated Bioassay 1 and 2, respectively. In Bioassay 1, seeds from the genotypes wild-type (WT) and the transgenic lines 'Cde25' and 'Arath:WEE1' were cultured in medium containing MS salts supplemented with sucrose (30 g/L). The seeds were further treated with oligoxyloglucan (10 mg/L; OX); Pectimorf (P; 10 mg/L) and 5 mg/L Indolebutyric acid (IBA), and incubated in climatic chambers. In Bioassay 2, cells from leaves of *A. thaliana* L. strain L-MM1 ecotype 'Landsberg erecta' were cultured in MS medium (4.43 g/L) supplemented with sucrose (30 g/L), naphtalenic

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acid (0.5 mg/mL) and kinetin (0.05 mg/mL). Cultures were kept at 25 °C under a 12 h/8 h light/dark photoperiod and agitated in a rotational shaker at 100 rpm. Cells were diluted 10-times in fresh medium every 10 days. Aliquots of washed cells (100 mg of fresh weight/mL) were placed in glass vials and the culture medium was changed by the same volume of each oligogalacturonides solutions with a Pasteur pipette and agitated in a rotational shaker at 150 rpm.

For morphoagronomic evaluations, the *in vitro* results were validated under field conditions by determining yields and their components together with other agronomic variables.

Finally, all the experiments were run in triplicate for all the evaluated indicators. A completely random design was applied and data was processed by a one-way variance analysis test (ANOVA), and means were compared by the Tukey's test ( $p \leq 0.05$ ). Data was processed by using the package STATGRAPHICS Plus version 5.0 for Windows. Normal distribution (Kolmogorov-Smirnov's test) and variance homogeneity (Bartlett's test) were checked for all the data prior to statistical processing [19].

## Results and discussion

All the biostimulators improved the properties, behavior and resistance to stress at the given concentrations and the assayed stages. Their effects on each vegetal species assayed are presented in the following.

### Garlic (*Allium sativum* L.)

Biobras and Pectimorf can be applied to garlic as growth regulators in all the micropropagation stages. The combinations Biobras-6 (0.05 mg/L) + IAA (0.1 mg/L) and Pectimorf (1 mg/L) + IAA (0.5 mg/L) increased the *in vitro* establishment of caulinary apices and decreased in three or four days the permanence in the culture media as compared to the controls treated with IAA and 6-BAP. For obtaining multiple shoots, the best combinations were Biobras-6 (2 mg/L) + NAA (0.03 mg/L) and Pectimorf (10 mg/L) and IAA (0.5 mg/L). Survival and rooting of microbulbs increased by immersion in a solution containing Biobras-6 (1 mg/L) or Pectimorf (10 mg/L) for 15 min, followed by plantation in a loaded zeolite (Litonite, 25 %) substrate containing organic matter (decomposed filter cake, 75 %).

Results of the Amplified Fragment Length Polymorphism (AFLP) technique (primers EAAGG/MCGA, EACGG/MCAT, EACGG/MCTC, EACGG/MCGA and EAAGG/MTCT) showed 82 bands clearly resolved, all monomorphic, indicating no differences at molecular level among the plants obtained *in vitro* by treatment with either Biobras-6 or Pectimorf, as compared to control or parental plants from the field. Nevertheless, this molecular evidence seems not to be conclusive since AFLP markers target specific genomic sites, in spite of results being corroborated by agronomic studies under field conditions.

Agronomic evaluations evidenced that Biobras-6 and Pectimorf increased production yields in 4.47 and 2.72 ton/ha, up to 12.42 and 11.67 ton/ha, respectively, as compared to control plants. From these results, a new micropropagation methodology was established for this garlic clone, not inducing genetic

variation and further validated for production purposes.

The results of using these biostimulators to promote growth in all the micropropagation phases of this garlic clone were all superior to those previously reported for equivalent traditional regulators [20-22], as well as for production yields [23, 24].

### Banana (*Musa* spp.) clone 'FHIA-18' (AAAB)

Biobras-6 and Pectimorf were also active as growth regulators in all the growth phases in banana (*Musa* spp.) clone 'FHIA-18' (AAAB), with an auxins-like effect (except for Pectimorf at the *in vitro* establishment phase with an effect similar to that of cytokinins) and reduced phenolization of explants (below 20 %). The treatment modified the anatomy of leaves without changes in genetic variability [25]. In fact, the interaction of these biostimulators with hormones improved plantlets survival during the acclimatization phase above 98 %, shortening in 15 days its duration [26, 27].

Results of histological analysis of banana plants leaves at the end of the acclimatization phase revealed that the use of Biobras-6 or Pectimorf in all the micropropagation phases neither modified the stomata density as reported for the species, nor its dimensions or that of epidermal cells, as shown in the table. Banana plants are amphistomatic, with a higher stomatal density in the abaxial surface as compared to the adaxial surface, at a 3:1 proportion. Furthermore, the dimensions of stomata and epidermal cells were higher in the abaxial surface. Similar results were reported by other groups in banana and plantain (*Musa* spp.) [28, 29]. Treatment with Pectimorf or Biobras-6 also increased thickening of the cuticle, the epidermis and the first layer of the palisade parenchyma, as compared to control plants (Table).

Particularly, Pectimorf treatment induced a higher endogenous level of total cytokinins as compared to the control, untreated plants (263.50 vs. 255.72 pmol/g of fresh weight), an unprecedented result. These aspects were properly validated at the Seed Biofactory in San José de las Lajas, increasing the number of shoots per explant and decreasing the stress of plantlets while transferring them to the acclimatization phase [30, 31] for a 97 % of survival from the 85.35 % obtained in control plants. Those results were properly introduced into the field at the agricultural Credit and Services Cooperative

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Table. Changes in morphological parameters of leaves of Banana (*Musa* spp.) clone 'FHIA-18' (AAAB) plants treated with Biobras-6 or Pectimorf in all the phases of micropropagation

Treated plants	Morphological parameters			
	Stomata		Epidermal cells	
	Density (per mm <sup>2</sup> )	Length (µm)	Length (µm)	Width (µm)
Adaxial surface	1.35-1.42	23.33-23.94	52.47-52.59	46.36-46.43
Abaxial surface	3.50-3.73	29.00-29.29	11.39-11.47	10.16-10.21
	Thickness (µm)			
Plants	Leaves surface	Cuticle	Epidermis	First layer of palisade parenchyma
Treated	Adaxial	1.05-1.06	4.16-4.17	17.25-17.29
	Abaxial	1.07-1.08	4.17-4.19	17.31-17.40
Control	Adaxial	0.91	4.08	17.04
	Abaxial	0.93	4.10	17.06

Nicomedes Corvo, in Mayabeque Province. The plants obtained showed a high genetic stability and were free of somaclonal variations. Moreover, there were no plants with deformations in the control treatment, but two plants were found variegated (1.42 %). Noteworthy, neither Biobras- nor Pectimorf-treated plants showed any irregular yellowish or greenish spots in leaves (variegation) or deformations. Previously, García [32] reported a lower number of variegated plants due to organogenesis (two plants) than in somatic embryogenesis (five), possibly due to a defect on photosynthesis pigment formation according to Zaffari *et al.* [33].

As reported by Turner *et al.* [34], water loss by transpiration is normally in the 98-99 % range. Therefore, Biobras-6 and Pectimorf increased the relative water content (RWC), contributing to decrease water loss by transpiration while simultaneously increasing water absorption. According to Mahouachi [35], stomata transpiration can reach up to 90 %, the other 10 % corresponding to cuticular transpiration. Therefore, cuticle thickness could influence on the transpiration level, contributing to reduce the stress in plants during its transfer from *in vitro* to *ex vitro* conditions.

Studies with 'FHIA-01', 'FHIA-02' and 'FHIA-03' hybrids showed cuticle thickness by the adaxial side in leaves ranging 2.38-2.81  $\mu\text{m}$  and epidermis thickness of 10.92-12.45  $\mu\text{m}$  [36]. Coincidentally, cuticle thickness determines the transpiration level. Schnurr *et al.* [37], found the gene LACS2 as mediating cuticle formation in *Arabidopsis*. Therefore, an equivalent gene could be mediating that function in the FHIA-18' banana clone (*Musa* spp.), inducing cuticle formation during the early development in response to stimulation with Biobras-6 or Pectimorf.

In respect to the acclimatization phase, it is the final phase of micropropagation protocols, determinant to guarantee the survival of plants. In this sense, foliar spray with Biobras-6 (0.1 mg/L) decreased the stress at high temperatures (34 °C), with a higher content of free proline in leaves (0.11-0.15  $\mu\text{g}/\text{mL}$ ), increased number of leaves, fresh weight and net photosynthesis rate (5-10.3  $\mu\text{mol CO}_2/\text{m}^2/\text{s}$ ). All these allowed reaching the 100 % of survival in treated plants, as compared to the control plants (97 %) [38].

Ultimately, Biobras-6 and Pectimorf increased culture yields (18.24 y 18.32 kg, respectively), as compared to control plants generated from asexual seeds (16.55 kg). They were consecutively applied in all the micropropagation phases *in vitro* and at acclimatization, been further validated under production conditions. Plants were free of phenotypic variations at the end of the second cultivation cycle (600 days). Hence, all these results were of high practical and theoretical relevance, since it was the very first demonstration of Biobras-6 and Pectimorf effects in the long-term without inducing plant changes under production conditions.

#### Plantain (*Musa* spp.) clone 'Sobrino' (AAB)

In Plantain (*Musa* spp.) clone 'Sobrino' (AAB), Biobras-6 (0.05 mg/L) and Pectimorf (5 mg/L) acted during the establishment phase, as auxin in substitution of the IAA (3 mg/L) and cytokinin for 6-BAP

(4 mg/L), favoring the survival of explants (higher than 90 %) [39].

The combination of Biobras-6 and 6-BAP in the multiplication phase increased the multiplication index (2.69 shoots per explant), and the Biobras-6 alone (0.05 mg/L) in substitution of IAA (1.3 mg/L) increased the vitroplant height (6.01 cm). The effect lasted for 76 days in the acclimatization phase, with values above those obtained with IAA for all the tested variables [40]. The results also supported the establishment of a new methodology for the *in vitro* propagation of this clone of plantain.

In *Arabidopsis*, brassinosteroids have shown a modulatory effect on the genes regulating the effects of auxin expression [41]. Therefore, it is expected to progressively be discovered new and more surprising effects with the advancement of the knowledge on their properties with the aid of more precise techniques.

#### Pineapple (*Ananas comosus* [L.] Merr.) cv. 'Cayena lisa'

In temporary immersion bioreactors, the propagation of Pineapple (*Ananas comosus* [L.] Merr.) cv. 'Cayena lisa' after treatment with the brassinosteroid analog MH-5 (0.1 mg/L) in the culture medium decreased losses due to low quality plants, with an increased uniformity among plants and a shorter time for reaching commercial-size fruits. The free-proline content was reduced in the vegetal tissue and, therefore, decreased the stress at the *ex vitro* acclimatization phase [42].

Pineapple is a Crassulacean Acid Metabolism (CAM) plant, supporting the temporary separation of  $\text{CO}_2$  fixation from its reduction. In the darkness, the phosphoenol pyruvate carboxylase catalyzes the fixation of  $\text{CO}_2$  and the formation of malate. Then, during the light period,  $\text{CO}_2$  is released from malate decarboxylation and assimilated back again through the reduction cycle of photosynthetic carbon. Nevertheless, González-Olmedo *et al.* [42] indicated that shoots grown on a BIT temporary immersion system were not true CAMs, since its metabolism behave as that of  $\text{C}_3$  plants.

The analysis of the effect on the different methods of culture and the photosynthesis photon flow (PPF) by Escalona *et al.* [43] revealed that the growth of shoots was partially independent from the photosynthesis process, since pineapple plants grown *in vitro* seemed to use more nutrients from the culture medium than those generated by the photosynthesis process. Therefore, this reinforces the view of increasing the photomixotrophic metabolism over the photoautotrophic for a successful result, by keeping high levels of PPF,  $\text{CO}_2$  and low sucrose concentrations [42].

In this study, it was shown that it is possible to reduce free proline content and its related stress by adding the MH-5 analog to the explant culture medium. This is in line with the first studies on the biological activity of brassinosteroids in plants, demonstrating that brassinosteroids effectiveness depended on its exogenous application, reducing the abiotic and biotic stress, including resistance to salinity, drought, extreme temperature changes and the attack by pathogens [44].

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## Ornamental plants

In *Spathiphyllum* sp., the combination of Pectimorf (10 mg/L) half the common concentration of 6-BAP (0.5 mg/L) increased 5-fold the number of shoots per plant. Moreover, Pectimorf alone or combined with the cytokinin stimulated root growth, with an effect analogous to that of auxins [45]. Such an effect was described previously for Pectimorf [46, 47], by combining it with 2,4-D to promote the formation and growth of calli in sugar cane and potato. Thus, a sustainable technique was established for the micropropagation of *Spathiphyllum* sp.

*Vriesea* is an economically relevant ornamental genus, due to the very attractive and colorful leaves of plants. That is why the increased survival rate obtained during the acclimatization phase (18-22 %) by spraying the MH-5 brassinosteroid analog (0.021- 0.216  $\mu\text{mol/L}$ ) is so important, which was accompanied by an increase in the number of roots and leaves and fresh weight [48]. Such effects could be related to the reduction of the free proline content which determines the anti-stress action displayed by this bioregulator [42], probably manifested as well in *Vriesea*.

Related to the previously mentioned effects of brassinosteroids, they are involved in cell elongation processes, mediated by gene expression and enzyme activity [49]. Therefore, the effects evidenced after the application of brassinosteroids result from specific cascades of biochemical events, which could be triggered directly from the plant cell genome or through signaling modulatory routes. Both types of pathways converge in a set of secondary mediators, which can act at extremely low concentrations [49, 50].

In summary, a new methodology was proposed for the acclimatization of these ornamental species, the procedures determinant for the success of the entire micropropagation process.

## Sugarcane (*Saccharum officinarum* L.) cv. 'Cuba 8751' and 'CP 52-43'

In sugarcane (*Saccharum officinarum* L. cv. 'Cuba 8751'), callus growth was stimulated by adding Pectimorf (10 mg/L), 2,4-D (3 mg/L), IAA (1 mg/L) and kinetin (0.1 mg/L) to the culture medium, for an adequate hormone balance. Shoot regeneration and size were higher with Pectimorf (1 mg/L) and kinetin (1 mg/L), the number of shoots per plant was the highest at 10 and 20 mg/L, respectively, while rooting increased at these last concentrations combined with IAA (1.3 mg/L). In the case of the 'CP 52-43' hybrid, Pectimorf (5 mg/L) combined with 2,4-D (1.5 mg/L) favored the number of embryos per gram of tissue when added to the culture medium, with increased homogeneity at the most advanced developmental phases [47].

Noteworthy, Pectimorf (5 mg/L) concentrates embryo formation, with predominance of its more advanced stages. In fact, 50 % of embryos were found at the scutellar-late developmental stage, 40 % in the early-scutellar stage and less than 10 % at the early globular stages. This means that more than 90 % of embryos were at the more advanced scutellar stage, with a marked reduction in the dispersion of this process.

## Citrus (*Citrus* spp.) rootstocks

Notably, Pectimorf shortens in 90 days the time needed to obtain plantlets by somatic embryogenesis in rootstocks of mandarin orange 'Cleopatra' and *Citrus macrophylla*, while brassinosteroids analogs could be used to maintain the embryogenic line for a shorter period (180 days) [51, 52]. This is advantageous for fast multiplication of cultivars in situations of natural disasters or for procedures of genetic improvement, preservation, genetic engineering and germoplasm exchange. It was corroborated that there was no genetic variability in plants obtained by replacing traditional growth regulators with Pectimorf [10]. In fact, it was reported by Héctor *et al.* [40] that Pectimorf and brassinosteroids analogs stimulated plantlet formation in *Musa* spp.

The use of morphological characteristics together with cytogenetic and molecular analyses (protein and DNA characterization techniques) during somatic embryogenesis in 'Cleopatra' mandarin orange and *Citrus macrophylla* Wester could be advantageous for the generation and characterization of vegetal structures. It could be also considered for propagation of these species, since Pectimorf and brassinosteroids demonstrated to regulate growth when added to the culture medium at all the growth stages during somatic embryogenesis of these rootstocks. In fact, Pectimorf and brassinosteroids analogs can be differentially combined, the first one for somatic embryogenesis and the analogs for maintaining the embryogenic line for a shorter period. Their joint use, without any other traditional growth regulator, is an alternative to save production costs. Based on our results, a new integrated methodology was proposed for the complete somatic embryogenesis of 'Cleopatra' mandarin orange and *Citrus macrophylla* Wester rootstocks in Cuba, also including the characterization of the genetic stability of the vegetal material obtained by using karyotype analysis, genetic-biochemical and DNA molecular markers. Additionally, two new retrotransposons (GenBank® Accession Nos., AY841150 and AA986991, *Citrus reshni* TY1 copia-like retrotransposon reverse transcriptase mRNA; 2004).

## Characterization of the effects of oligogalacturonides in the biological model of *A. thaliana* L.

The results obtained in Bioassay 1 corroborated previous reports of the regulatory effect of oligogalacturonides on vegetal growth, since genotypes WT and transgenic cell lines 'Cde25' and 'Arath:WEE1' showed elongation in the primary root and repressed elongation of lateral roots in response to the treatment with either oligoxyloglucan, Pectimorf or IBA. Only the oligosaccharins (*i.e.*, oligoxyloglucan and Pectimorf) were able to induce a positive effect in the elongation of the root meristem and the mitotic index, significantly higher than the other treatments applied in the three genotypes assayed, what suggested the shortening of the cell cycle [53].

In Bioassay 2, using the ecotype 'Landsberg erecta', only the oligogalacturonides with polymerization degree above nine recognized the membrane receptor WAK1, which mediates the signal transduction into the cell and form the 'egg box'. In this regard,

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Spiro *et al.* [54] reported that the terminal reduction of oligogalactouronides reduced its biological activity, as compared to the unmodified oligogalactouronides. In this work, it was demonstrated the capacity of oligogalactouronides with modified reductor extremes to induce early defensive responses in cell suspensions of *A. thaliana*, in comparison with unmodified oligogalactouronides. In general, oligogalactouronides decreased the efflux of  $K^+$  and improved the cellular viability in cell suspensions [55, 56].

## Conclusions

It was successfully demonstrated that the vegetal growth biostimulators (Pectimorf, Biobras-6, Biobras-16 and MH-5) can be used as growth regulators when added into the culture medium for different biotechnological processes in the species assayed, including the gold standard *A. thaliana* model in vegetal biology. Overall, the use of these growth biostimulators increased yield, quality and survival of the plants obtained and decreased the in vitro permanence of explants in the acclimatization phase of culture. They also decreased the stress by transference from in vitro to ex vitro culture conditions.

In summary, the results of these two bioassays in the *A. thaliana* model increased the knowledge on the function of these biostimulators in vegetal biotechnology. Moreover, new technologies were proposed and implemented for the different assayed species, all of them economically relevant, since they do not induce genetic variability and promote an adequate acclimatization. Some of them were validated as part of production

processes, what increase their practical value, to be further complemented with well documented economical estimations. Additionally, these results are readily introduced in educational programs at the Faculty of Biology, in the University of Havana, and in some foreign educational institutions in Mexico, Belgium and Spain. Such educational practices have also contributed to imbricate theoretical and practical knowledge.

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