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# Effect of Pectimorf® - A traditional growth regulator on the development and distribution in clones 'CMC-40' and 'Señorita' of Cassava (*Manihot esculenta* Crantz) stomata

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**ABSTRACT:**

The development of more efficient and sustainable technologies in the production of materials in '*in vitro*' cassava (*Manihot esculenta* Crantz), favor the improvement of seed quality and sanitation of the plant material. The purpose of the research is to evaluate the effectiveness of Pectimorf® (mixed oligo-galacturonide), it's safe and natural availability in Cuba. It is used as a possible complement or substitute for growth regulators traditionally used in the culture medium for the propagation of this crop *in vitro*. In this study, the results obtained indicate that, at least, under the experimental conditions, the Pectimorf®, altered patterns of development and distribution of stomata in the leaves of cassava plants, where the effect was most evident when the product is added to the culture medium. The new results contribute to the elucidation of the mechanisms of action of this substance.

**Keywords:**
Cassava, medium, plant anatomy, stoma, oligosaccharides.

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## INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a very versatile crop planted by small farmers in more than 100 countries (FAO, 2013). Therefore many clones are existing in this plant species (Alves *et al.,* 2011; Montero *et al*., 2011). It is either used for human consumption or dehydrated and stored for several years as a reserve food (Nassar *et al*., 2009). In recent years, the potential of this crop as an efficient source of raw material for the production of biofuels (Cortés *et al.,* 2010) is greatly increased.

The development of efficient and rapid methods of plant regeneration by culturing *in vitro* cassava tissue, either by somatic embryogenesis (Medina *et al*., 2003; Ochoa *et al.,* 2012) or organogenesis (Medero *et al.,* 2001; Mapayi *et al.,* 2013; Fan *et al.,* 2011) ensures the production of high quality plantlets basically needed for producers in the expansion of cultivation. However these techniques are still demanding about the composition of the culture medium, especially with regard to the use of growth regulators.

The introduction of bioactive substances produced domestically in the methodology of *in vitro* propagation of cassava (*Manihot esculenta*), could be a promising alternative to improve the economic efficiency of the process with the use of simple techniques and reliable domestic inputs. These bioactive products can be obtained with the brand name Pectimorf®, produced by the Department of Plant Physiology and Biochemistry, National Institute of Agricultural Sciences (INCA), Mayabeque, Cuba. This product is natural, harmless and obtained from citrus rind and is constituted by a mixture of oligo-galacturonide with high degree of polymerization between 7 and 16 (Cartaya *et al.,* 2011). The Pectimorf® is considered as a potent elicitor of plant defense (Hernández *et al*., 2010; Galletti *et al*., 2011). This product stimulates cell growth and differentiation of different plant species (Hernández *et al.,* 2009; Hernández *et al.,* 2010).

So far, the effect caused by this new substance is unknown in the *in vitro* propagation of cassava (*Manihot esculenta*). That is why, this study aims to histologically evaluate the effect of Pectimorf® on the two clones of cassava (*Manihot esculenta*) 'CMC-40' and 'señorita' *in vitro*, before and after acclimatization.

## MATERIALS AND METHODS

This work was done at the Laboratory of Biotechnology, Department of Genetics and Plant Breeding and the Department of Physiology and Biochemistry of the National Institute of Agricultural Sciences (INCA), located in the municipality of San Jose de Las Lajas, Mayabeque province.

### Plant Material

The two clones viz., 'CMC-40' and 'Senorita' were obtained from the Cuban bank of cassava germplasm, present at the Institute of Tropical Research Viandas (INIVIT), Santo Domingo, Villa Clara, which showed high productivity, short cycle (6-10 months ) and excellent cooking quality (INIVIT 2014). The latter is a prescribed amenity of 'Senorita' clone.

### General Procedure

The plants that had completed their growth phase *in vitro* were listed for acclimatization for about 30 days of age and 3 to 5 cm, in height. A total of eight treatments from the growth phase *in vitro* (Table I), as well as plants after 35 days of acclimatization from the control treatment and the Pectimorf® treatment were done systematically after propagation.

The epidermis of the abaxial leaf surface was scraped from the surface opposite to observe - allowing not only to obtain an image of distribution of stomata, but also to observe aspects of their structure. Leaf samples were always taken from the middle of the road and away from the edges.

Epidermal sheet sample was placed on a microscopic slide and one drop of toluidine blue was added and kept for a period of five minutes. Two washes

were done and a drop of glycerine was added and a cover slip was placed.

The samples were observed under an optical light microscope (Zeiss, MODEL; PAIS) and photographed with a camera (Motic) coupled thereto. Measurements and counts were performed on micrographs with the use of Image J program. Morphometric and linear measurement instruments were used for elucidating the lengths and breadths. For counting stomata and epidermal cells with a magnification of 400x and measurements of length and thickness of the guard cell 1 with a magnification of 1000x; six fields per plant were taken for a total of 60 fields per treatment.

For counting, a stoma is considered when two guard cells were present and in the case of epidermal cells, when they were 60% in the image area.

Stomatal Index (SI) was calculated using the formula suggested by Wilkinson (1979)

IE = (NE * 100) / (EC + NE)

Where, IE = Stomatal Index.

NE = Number of Stomata per field of view.

EC = Number of Epidermal Cells in the field of observation.

**Table 1. Origin of plantlets of cassava (*Manihot esculenta*) used in the experiments in the growth phase *in vitro***

| Treatments | NAA ($Mg.L^{-1}$) | Pectimorf® ($Mg.L^{-1}$) |
|---|---|---|
| 1(Control) | 0.01 | - |
| 2 (absolute control) | - | - |
| 3 | - | 05 |
| 4 | - | 10 |
| 5 | - | 15 |
| 6 | 0.01 | 05 |
| 7 | 0.01 | 10 |
| 8 | 0.01 | 15 |

The data obtained were statistically analysed using Analysis of Variance (ANOVA). Differences between means were elucidated by Duncan multiple Range test at 5% significant level.

## RESULTS AND DISCUSSION

In Figure I, you can see the stomata on the abaxial leaf surface of cassava (*Manihot esculenta*); thus proved parasitic or rubiaceous which accompany two adjoining cells that are arranged parallel or occlusive, that match the description made in other crops such as common bean plants (Sánchez *et al.,*1996). They are randomized and guard cells showed a kidney shape, as is the characteristic of the dicotyledonous plants (Taiz *et al.,* 2010). These structures were observed in both the abaxial and adaxial surface, which is considered as a anfiestomática species. It was observed in the higher frequency of stomata on the abaxial surface (ABA) with respect to the adaxial side (ADA); these results also match other cassava varieties (Ceballos *et al.,* 2002).

In Table 1, the results of histological analysis of cassava leaves at the end of micro propagation phase were given. In general, significant differences between the treatments were observed in both clones. In 'CMC-40', stomatal index on the abaxial surface at treatment-2 reached maximum (19.42), whereas the lowest value for this character is at the control treatment (9.85); no significant differences were seen on the treatment 8 (0.01 $mgL^{-1}$ NAA + 15 $mgL^{-1}$ of Pectimorf®), while at the adaxial surface, the maximum values were found in treatments where the product is used in the presence of NAA ( 6, 7 and 8); no significant difference were seen between them but with the rest, except six which did not differ from the lower value two. Treatment-3 was significantly different from the rest. It was shown that Pectimorf® increased stomatal index in both cultivars, which could infer photosynthetic activity and water status in the subsequent acclimatization plantlets.

In clone 'Señorita', stomatal index on the abaxial surface reached the highest value in treatments 2 and 7, which did not differ significantly from each other and

neither for the treatments 3, 5 and 6. The lower values for this character were found in 1, 4 and 8 due to the control treatment 10 $mgL^{-1}$ of Pectimorf® and the combination of 0.01 $mgL^{-1}$ NAA + 15 $mgL^{-1}$ Pectimorf® respectively, without significant differences between them. However, in the adaxial side treatments of 5, 6, 7 and 8; they showed no significant differences between them or with treatment 2. The lowest value was in treatment 4, which did not differ significantly with 1 and 3. Also in 'señorita' the application of Pectimorf® has produced increased stomatal index.

The increased stomatal index caused by the Pectimorf® when added to the culture medium surely have a positive impact on the development of plantlets in the acclimatization phase influencing photosynthetic activity and avoiding excessive perspiration. The results coincide with Altamura *et al.* (1998), who noted that the treatment with mixtures of biologically active oligo-galacturonide increase the formation of stomata on leaf explants snuff (*Nicotiana tabacum* L.) grown in culture media with the specific concentrations of auxins.

The length of stomata in 'CMC-40' (table – 2) showed significant differences between treatments. The maximum values were in 1 and 2 for the control medium and absolute control, which did not differ significantly between them. Treatment 2 and 3 did not differ significantly but differs with the addition of, Pectimorf® in the presence of NAA (treatments 6, 7 and 8). The lowest values were observed in the treatments 4 and 5 corresponding to the concentrations of 10 and 15 $mgL^{-1}$ respectively without NAA and Pectimorf®, with no significant differences between them. These results indicate that the modified length of stomatal cells, resulted in less conduction, minimized loss of water by evapo-transpiration and thus better survival of plants in the acclimatization phase.

In the clone 'Señorita', there were also significant differences between treatments (Table 2). Longest stomata were those that developed in treatments 1 and 7, which did not statistically differ from each other. The analysis also differed in treatments 6 and 8 corresponding to the media where Pectimorf® was employed in the presence of NAA. The lowest values for the length of stomata were present in the treatments 2 and 3, corresponding to the medium without the regulator 5 $mg.L^{-1}$ Pectimorf® (T3), with no significant differences between them. As in 'CMC-40' clone, results indicate that the product also decreased in stomatal cell length for providing less water wastage.

As the width of the guard cells in 'CMC-40' showed significant differences between the treatments (Table 2), the best treatment in 'Senorita' was 6 which differed significantly from the rest. The control treatment did not differ significantly from the treatments 7 and 8 corresponding to the media where Pectimorf® was used in the presence of NAA. Treatments 3, 4 and 5 did not differ between them and showed intermediate values for this character and corresponded to the media where the product as a substitute for NAA was added. The above table showed that the clone 'señorita' differences were significant but contrary to what happened in 'CMC-40', Control treatments, 5, 7 and 8 were higher without

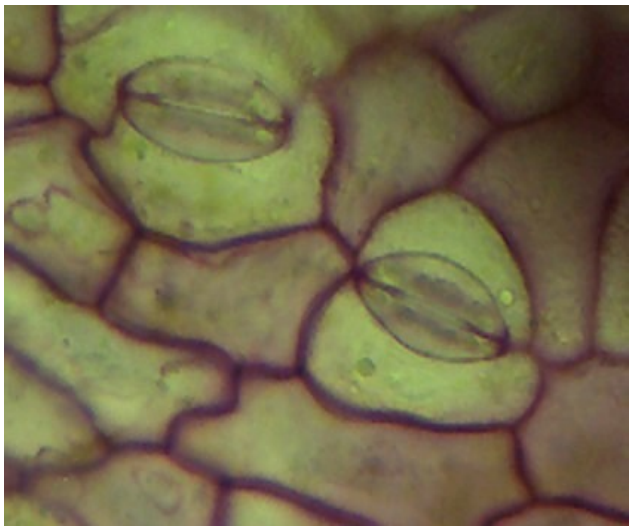

**Figure 1. Photomicrograph of the leaf epidermis of the abaxial surface of plantlets of cassava (*Manihot esculenta*). This Image is a representative for both clones. EC Epidermal Cells, Ca– Adjoining Cells, Co– Guard Cells and E-Stomata (1 000x).**

**Table 2. Influence of applying Pectimorf® on stomatal index, stomatal length and width of the stoma guard cells in the cassava (*Manihot esculenta*), clones 'CMC-40' and 'Señorita' at the end of micropropagation**

| Treat-ments | Stomatal Index | | Stomata Length (Microns) | Guard cells Width (Microns) | Stomatal Index | | Stomata Length (Microns) | Occlusive cell widths (Microns) |
|---|---|---|---|---|---|---|---|---|
| | CMC-40 | | | | Senorita | | | |
| | ABA | ADA | ABA | ABA | ABA | ADA | ABA | ABA |
| 1 | 09.85 $^{c}$ | 1.13 $^{c}$ | 109.41 $^{to}$ | 29.10 $^{b}$ | 12.02 $^{c}$ | 0.68 $^{cd}$ | 101.45 $^{to}$ | 27.86 $^{to}$ |
| 2 | 19.42 $^{to}$ | 1.45 $^{bc}$ | 99.47 $^{Ab}$ | 22.76 $^{d}$ | 20.04 $^{to}$ | 0.99 $^{abc}$ | 86.35 $^{d}$ | 18.22 $^{e}$ |
| 3 | 12.41 $^{b}$ | 0.41 $^{d}$ | 95.31 $^{bc}$ | 24.39 $^{cd}$ | 16.44 $^{Ab}$ | 0.88 $^{bcd}$ | 83.35 $^{d}$ | 23.68 $^{c}$ |
| 4 | 12.75 $^{b}$ | 0.64 $^{cd}$ | 78.57 $^{d}$ | 25.49 $^{c}$ | 14.23 $^{bc}$ | 0.14 $^{d}$ | 92.09 $^{c}$ | 19.84 $^{d}$ |
| 5 | 12.02 $^{b}$ | 1.08 $^{c}$ | 80.88 $^{d}$ | 24.38 $^{cd}$ | 17.51 $^{Ab}$ | 1.91 $^{to}$ | 91.59 c | 27.34 $^{Ab}$ |
| 6 | 12.14 $^{b}$ | 2.20 $^{Ab}$ | 83.43 $^{cd}$ | 33.33 $^{to}$ | 16.77 $^{Ab}$ | 1.72 $^{Ab}$ | 93.59 $^{bc}$ | 26.18 $^{b}$ |
| 7 | 12,92 $^{b}$ | 2.31 $^{to}$ | 89.40 $^{bcd}$ | 27.85 $^{b}$ | 18.80 $^{to}$ | 1.54 $^{abc}$ | 97,95 $^{Ab}$ | 27.74 $^{to}$ |
| 8 | 9.89 $^{c}$ | 2.96 $^{to}$ | 87.82 $^{bcd}$ | 29.06 $^{b}$ | 12.06 $^{c}$ | 1.35 $^{abc}$ | 95.45 $^{bc}$ | 27,59 $^{Ab}$ |
| 9 | 6.04 | 2.28 | 23.35 | 07.94 | 06.60 | 1.71 | 09.34 | 05.90 |

Legend: **ADA**: Adaxial (beam) **ABA**: Abaxial (underside) **NAA**: Naphthalene Acetic Acid

(**Treatment1**: Control Environment : 0.01mgL$^{-}$1 NAA, **Treatment 2:** Absolute Control : combination (without regulators), **Treatment 3:**5 mgL$^{-1}$ Pectimorf®, **Treatment 4:** mgL $^{-1}$ Pectimorf®, **Treatment 5:** 15 mgL$^{-1}$ Pectimorf®, **Treatment6 :** Combination (0.01 mgL$^{-1}$ NAA + 5 mgL$^{-1}$ Pectimorf®), **Treatment 7 :** Combination (0.01 mg L $^{-1}$ NAA + 10 mgL$^{-1}$ Pectimorf®)' **Treatment 8 :** Combination (0.01 mgL$^{-1}$ NAA + 15 mgL$^{-1}$ Pectimorf®))

Means with different letters differ statistically according to Duncan test $p \leq 0.05$. (* significant at $p < 0.1$; ** significant at $p < 0.01$; *** significant at $p < 0.001$)

significant differences between them, and for the control medium where 15 mgL$^{-1}$ of Pectimorf® was added in the absence/ presence of NAA and for treatment 6, it did not differ in the presence of 10 mgL$^{-1}$ of Pectimorf® in NAA. The lowest values of this character were for treatments 2, 3 and 4, which showed significant differences between them.

In both the cultivars, the lowest values to match Pectimorf® inclusion in the culture medium, resulted in reduced width of the guard cells; and this modification positively contributed in the acclimatization of plants.

If the value of size of stomata influenced by the length and width of the guard cells on the abaxial surface is taken into consideration, we could say that again on treatment 4 (10 mgL$^{-1}$ Pectimorf® as a substitute for NAA), under these culture conditions, caused changes in the structure of the guard cells, with smaller length and width compared to the control treatment. This could promote acclimatization stage in seedlings which achieved the conditions of *ex vitro,* therefore the potential to become stressed because of decrease in response to water deficit for getting transition to the new conditions of autotrophism.

Stomata morphology plays an important role in controlling water loss, which can be adapted to many plant species at varying environmental conditions (Hetherington *et al.,* 2003). The variation of the characteristics of stomata could play an important role in the process of acclimation of a species into that environment.

**Table 3. Pectimorf® influence on stomatal index, stomatal length and width of the stoma guard cells in the leaves of cassava (*Manihot esculenta*) clones'CMC-40' and 'Señorita', at 35 days of acclimatization**

| Treatments | Stomatal index | | Length of stomata (microns) | Width of guard cells (microns) | Stomatal index | | Length of stomata (microns) | Width of guard cells (microns) |
|---|---|---|---|---|---|---|---|---|
| | CMC-40 | | | | Senorita | | | |
| | ABA | ADA | ABA | ABA | ABA | ADA | ABA | ABA |
| **I** | 18.37 | 0.14 | 224.22 $^{b}$ | 64.65 | 16.70 | 0.35 | 227.56 $^{b}$ | 63.71 |
| **II** | 18.92 | 0.14 | 240.84 $^{to}$ | 64.85 | 17.38 | 0.32 | 238.51 $^{to}$ | 63.00 |
| Result | NS | NS | 0.11 *** | NS | NS | NS | 0.30 *** | NS |

**ADA:** Adaxial; **ABA:** Abaxial; **NAA:** Naphthalene Acética Acid

**1: Control** : *In vitro* plants from the control medium 0.01 mg.L$^{-1}$ NAA, **2** : *In vitro* Plants Provenientes medium with 10 mgL$^{-1}$ Pectimorf®. Means with different letters differ statistically according to Duncan test $p \leq 0.05$. (*significant at $p < 0.1$; ** significant at $p < 0.01$; *** significant at $p < 0.001$)

Similar results were shown in the cultivation of beans (*Phaseolus vulgaris*) and the effect of Pectimorf® on morphology and distribution of stomata was studied. Álvarez *et al.* (2012) reported that oligo-galacturonide mixture resulted in alterations in the density and size of stomatal guard cells and adaxial surface differences were observed in terms of length of the stoma.

The results of histological analysis in the leaves of cassava plantlets at the end of the acclimatization phase revealed that the use of 'Pectimorf®' modify some of the traits evaluated (Table 3). In both cultivars 'Señorita' and 'CMC-40', the stomatal was index showed no significant differences between treatments, however modified the size of the stomata since it altered the length of stomata with highly significant differences from the control. In the 'CMC.40', the clone stomatal average length was 240.84 microns and in 'Señorita' it was 238.51 microns; these plants were grown in the *in vitro* medium with 10 mgL$^{-1}$ Pectimorf®. The rest of the characters were not significantly different.

The size of the stomata is a key factor in the process of acclimatization, for having an inverse relationship between the size of the stoma and resistance to water stress (Aasaman *et al.,* 2001). Also authors have commented that the size of the stomata and stomata index appear to be the most sensitive to altered environmental conditions; avoiding excessive perspiration and allowing better adaptation of plants to conditions of greater water demand.

In this sense, the results obtained indicate that, at least under these conditions, the Pectimorf® probably changed the patterns of development and distribution of stomata in cassava plants. The effect was more evident when the product is added to the culture medium, which may be due to the controlled conditions and is a way of evading the effects of the substance in these conditions *in vitro*.

Also in *Mikania laevigata* ex Baker Shultz, it is said that a reduction of perspiration may be associated with high stomatal density, which is often observed in the conditions of more radiation or less water availability (Souza *et al.,* 2007). Álvarez *et al.* (2011) and Yin *et al.* (2006) have shown that oligo-galacturonide affect the growth and development of plant cells and organs and differentiation of stomata and pericycle cells.

The results indicate that the changes occurred in both the cultivars of cassava on addition of Pectimorf® in the *in vitro* phase, had no influence on stomatal index, but if the size of the guard cells are modified by the length of the stomata, the conditions transfer from *in vitro* to *ex vitro*. Castro *et al.* (2009) noted that the polar diameter or length of the stomata is directly related to the size of the stomata and that this could be varied in response to a hybrid deficit.

So far there are no literature about the possible role of Pectimorf® on the histology of cassava plants. Furthermore, these results constitute the basis for future research, where we can understand the possible effects of this substance in plant histology. The use of Pectimorf® helped reducing the stress of plants transferring from *in vitro* condition to *ex vitro* condition, which may be associated in including the amendments that produced this compound on leaf anatomy. However, there is a need to dwelve into the mechanisms of action of this oligo-galacturonide to determine the exact way with which it exerts its action and determine whether the product may or may not induce genetic variability on the materials spread.

## CONCLUSIONS

The Pectimorf® incorporation resulted increased stomatal index and size of stomata in the *in vitro* growth phase and acclimatization of plantlets of the cassava (*Manihot esculenta*), clones 'CMC-40' and 'Señorita'.

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