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# Genetic and resistance stability to Black Sigatoka disease during micropopagation of *Musa* CIEN BTA-03 somaclonal variant

(With 4 Tables & 3 Figures)

## *Estabilidad genética y de la resistencia a la enfermedad Black Sigatoka durante la micropropagación del variante somaclonal CIEN BTA-03 de Musa*

*(Con 4 Tablas y 3 Figuras)*

**Giménez[1] C, E de García[2], O Haddad [3]**

**Abstract.** Evaluation of clonal micropropagation and phenotype stability of elite somaclones are critical steps for development of new varieties. In the present work somaclon variant CIEN BTA-03 (resistant to Black Sigatoka), obtained through *in vitro* process from cultivar Williams (susceptible to Black Sigatoka), was micropropagated via apical shoot culture for five multiplication cycles in 0.5 mg/l of benzyl-aminopurine (BA). To verify the genetic stability of the progeny of this elite material, random amplified polymorphic DNA (RAPD) markers were used. A total of 5,292 monomorphic bands were obtained from the amplification of fifty six DNA samples (extracted from vitroplants randomly selected) with 10 different primer combinations. Non-polymorphic RAPD bands were found in this assay. Additionally, six plants of CIEN BTA-03 produced in the fifth cycle of micropropagation and the same number of plants from cultivars Pisang Mas,

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Williams and Yagambi Km5, were tested in the field to evaluate their response to Black Sigatoka disease. The clonally micropropagated CIEN BTA-03 plants preserved the Black Sigatoka resistance phenotype.

**Key words:** Micropropagation, genetic stability, RAPD, disease resistance, youngest leaf spotted (YLS).

**Resumen.** La evaluación de la micropropagación clonal y la estabilidad fenotípica de somaclones elites son pasos críticos para el desarrollo de nuevas variedades. En el presente trabajo el variante somaclonal CIEN BTA-03 (resistente a la Sigatoka negra), obtenido *in vitro* a partir del cultivar Williams (susceptible a la Sigatoka negra), fue micropropagado mediante el cultivo de ápices del vástago por cinco ciclos de multiplicación en medios con 0,5 mg/l de bencil aminopurina (BA). Para verificar la estabilidad genética de la progenie de ese material elite, se usaron como marcadores bandas de ADN polimórfico amplificado al azar (RAPD). Se obtuvieron un total de 5292 bandas monomórficas de la amplificación de cincuenta y seis muestras de ADN (extraídas de vitroplantas seleccionadas al azar), con la combinación de 10 diferentes iniciadores. No se observaron bandas polimórficas en este análisis. Adicionalmente, seis plantas de CIEN BTA-03 producidas en el quinto ciclo de micropropagación y el mismo número de plantas de los cultivares Pisang Mas, Williams y Yagambi Km5, se usaron en un ensayo de campo para evaluar sus respuestas al ataque de la Sigatoka negra. Las plantas de CIEN BTA-03 micropropagadas clonalmente preservaron el fenotipo de resistencia a la Sigatoka negra.

**Palabras clave:** Micropropagación, estabilidad genética, RAPD, Resistencia a enfermedades, hoja más joven con mancha (YLS).

## INTRODUCTION

The somaclonal variant CIEN BTA-03, resistant to Black Sigatoka, was obtained through adventitious shoot induction with 15 mg/l benzylaminopurine (BA) from *in vitro* plants of banana Williams, a cultivar susceptible to Sigatoka disease. This resistant somaclone was initially selected of the field with high pressure of Yellow Sigatoka disease (Trujillo & de García, 1996).

Several investigators have demonstrated morphological (Valerio et al., 2002), biochemical (Vidal & de García, 2000), and cytogenetic and genetics (Giménez et al., 2002) differences, between CIEN BTA-03 and its parental clone Williams. In a cluster analysis made by Giménez et al. (2002) using RAPD markers, the genetic differences between the somaclonal variant CIEN BTA-03 and its parental cultivar Williams were ratified. In addition a

comparison of characteristics of fruits from the somaclone CIEN BTA-03 and its parental clone Williams revealed that somaclone CIEN BTA-03 shows better sensory characteristics than its parental clone Williams (Unai et al., 2004). Moreover, agronomic analysis (Trujillo et al., 1998; Trujillo & García, 1996) showed that bunches of fruits from somaclone CIEN BTA-03 were heavier (average 35kg) than those from clone Williams (average 31kg).

Tissue-culture instability of in vitro propagated plants is a common event in *Musa* genus (Vuylsteke et al., 1991). Some growth regulators as cytokinins and auxins can change the chromosome number of *Musa* spp. grown in vitro, which affect the nuclear DNA content of in vitro derived CIEN BTA-03 plants (Shepherd & Da Silva, 1996). The use of cytokinin BA in *Musa* multiplication has been proved to generate ascendant and descendant aneuploid cells (Sandoval et al., 1996; Shepherd & Dos Santos, 1996).

Genetic stability of in vitro derived CIEN BTA-03 plants is very important for scientists and commercial implications. Based on the priority of cloning this elite somaclonal variant, the objective of the present work was to evaluate the genetic stability of CIEN BTA-03 plants obtained through in vitro multiplication via apical shoot culture, and the stability of the resistance to Black Sigatoka phenotype, in those plants through five cycles of micropropagation.

## MATERIALS AND METHODS

**Plant material.** The original CIEN BTA-03 was micropropagated *in vitro* through five cycles, using a protocol established by Hardy and de García (1994), based on Vuylsteke report (1989). The shoot apexes of CIEN BTA-03 plants were cultured in Murashige and Skoog (1962) media with low concentration (0, 5 mg/l) of BA to avoid somaclonal variations in the micropropagated plants.

**Black Sigatoka resistance evaluation.** Six plants of CIEN BTA-03 from the fifth micropropagation cycle, and the same number of plants from *Musa* cultivars: Pisang Mas, Williams and Yangambi Km5, with known resistance levels to Black Sigatoka (Table 1), were used to evaluate the incidence of Black Sigatoka under field conditions.

**Table 1.** Cultivars of *Musa* used in the field to evaluate CIEN BTA-03 Black leaf streak resistance.

***Tabla 1.*** *Cultivares de Musa usados en el campo para evaluar la resistencia de las vetas negras de la hoja de CIEN BTA-03.*

| Cultivar | Subgroup | Black leaf streak response |
|---|---|---|
| Pisang Mas | *Musa* AA, Subgroup Sucrier | Highly susceptible |
| Williams | *Musa* AAA, Subgroup Cavendish | Susceptible |
| Yangambi km-5 | *Musa* AAA, Subgroup Ibota | Hypersensitive |

**Plot location.** Field experiments were performed in the experimental agricultural station called EXPERTA, Universidad Central de Venezuela, located in Maracay, Estado Aragua, central region of Venezuela, at 450 meters above sea level, with a dry tropical forest climate.

Plants 0.3-0.5 m height of the somaclone CIEN BTA-03, and those of the susceptible and resistant cultivars (Pisang Mas, Williams and Yangambi Km 5, table 1), were randomly planted in a block, with plant spacings of 2m x 2.5m, in a field with high pressure of the fungus *Mycosphaerella fijiensis* inoculum, which causes black Sigatoka on banana.

**Disease evaluation.** Leaves were scored for disease severity levels, using the Gauhl's modification (1990) of Stover's scale (1971). Percentage of leaf area affected by the black Sigatoka pathogen was expressed as weighted index disease grades (Orjeda, 1998). This information was recorded six months after planting, and at bunch emission time. Only upright leaves were recorded. The infection index (II) was calculated according the following equation:

$$II = \frac{\Sigma(n)b}{(N-1)\,T} \text{ X100}$$

Where n= the number of leaves in each grade, b= the disease grade, N= the number of Grades used in the scale (Gauhl, 1990), T= the total number of upright leaves scored (Orjeda, 1998).

**RAPD analysis of clonal population.** Nineteen *in vitro* plants of CIEN BTA-03 selected at random for each propagation cycle M1V1, M1V3, and M1V5 were used for DNA purification, according to Doyle and Doyle (1990), with minor modifications. A total population of 51 to 56 plants was amplified by PCR for RAPD analyses.

**DNA isolation.** Fresh leaves (100 mg) were ground with liquid nitrogen. The homogenate was mixed with 750 µl of extraction buffer: (2% cetyltrimethylammonium bromide [CTAB], 1.4M NaCl, 20mM ethylenediaminetetracetic acid [EDTA], 100 mM Tris-HCl pH= 8 and 1% (v/v) of ß-mercaptoethanol). The homogenate was incubated 45 minutes at 60 ºC, extracted with 750 µl chloroform: isoamyl alcohol (25:1; v:v), and centrifuged at 13,000 g for 5 minutes. The chloroform: isoamyl alcohol extraction was repeated twice and before the second extraction the aqueous phase was mixed with 50 µl of 10x CTAB in 0.7 M NaCl. DNA was precipitated by mixing the aqueous phase with one volume of cold isopropanol (- 20 ºC) during 30 minutes. Subsequently, the pellet was recovered by centrifugation at 13,000 g for 20 minutes. The DNA pellet was rinsed with 70% (v/v) ethanol and then with 95% (v/v) ethanol. The pellet was dried upside down until complete ethanol evaporation, and dissolved in 30 µl of distilled water. The total DNA was measured by using a spectrophotometer at 260, 280, and 320 nm, visually checked in 0.8% (w/v) agarose gel stained with ethidium bromide (0.5 µg m/l), and visualized under UV light at 315 nm.

**DNA amplifications.** Ten decameric primers from Operon Technologies INC, were used for DNA amplifications (Table 2). Amplification reactions were performed in a reaction mixture 25 µl of total volume with 50 ng of genomic DNA, 100 µM of each dNTP, 1 unit of recombinant Taq polymerase (Gibco BRL, Life Technologies TM), 2.5 µl of 10x reaction buffer ( 200 mM Tris-HCl pH: 8.4, 500 mM KCl), and 0.2 µM of decameric primer. For DNA amplification a MJ Research PTC 100 model thermal cycler was programmed for 35 cycles as follows: an initial denaturing step of 5 min. at 95 ºC and 35 cycles with touchdown denaturation temperature of 0.1 ºC by cycle, beginning with 94 ºC for 30 sec., 36 ºC for 30 sec., 72 ºC for 1 min, and finally 10 min. at 72 ºC .When the amplification was completed the samples were loaded and electrophoresed in a 1.5% (w/v) agarose gel on 1x TBE (Tris base-Boric acid-Na4EDTA) buffer, followed by staining with ethidium bromide.

**Table 2.** RAPD primer sequences used for DNA amplifications.

***Tabla 2.*** *Secuencias iniciadoras de RAPD usadas para amplificaciones de DNA.*

| Primers | Sequences |
|---|---|
| OPA-04 | AAT CGG GCT G |
| OPB-01 | GTT TCG CTC C |
| OPM-07 | CCG TGA CTC A |
| OPM-15 | GAC CTA CCA C |
| OPP-04 | GTG TCT CAG G |
| OPO-19 | GGT GCA CGT T |
| OPM-20 | AGG TCT TGG G |
| OPD-02 | GGA CCC AAC C |
| OPD-05 | CAT CCG TGC T |
| OPD-20 | ACC CGG TCA C |

## RESULTS AND DISCUSSION

**Evaluation of CIEN BTA-03 micropropagated plants for black Sigatoka resistance.** The Youngest Leaf Spotted (YLS) analysis during the vegetative phase (after six months of planting) showed that the disease symptoms developed earlier in the susceptible cultivars Pisang Mas (leaf number 9) and Williams (leaf number 10) than in the resistant cultivars CIEN BTA-03 (leaf number 13); Yamgambi Km5 did not show any symptoms (Table 3).

At bunch emission stage somaclonal variant CIEN BTA-03 and Yangambi Km5 did not show any disease symptom. At this stage, susceptible cultivars Williams and Pisang Mas presented disease symptoms at leaf number five (Table 3). These results indicate that the youngest leaf spotted

**Table 3.** Youngest leaf spotted at the vegetative phase (six month after planting) (YLSV) and at the flowering stage (YLSF). Infection index at the vegetative phase (IIV) and at flowering stage (IIF). Number of functional leaves at the flowering phase (NFLF). Mean of six plants per cultivar.

***Tabla 3.*** *Hoja más joven manchada en la fase vegetativa (seis meses después de la plantación) (YLSV) y en floración (YLSF). Índice de infección en la fase vegetativa (IIV) y en floración (IIF). Número de hojas funcionales en floración (NFLF). Los valores son promedios de 6 plantas por cultivar.*

| CULTIVAR | YLSV | YLSF | IIV | IIF | NFLF |
|---|---|---|---|---|---|
| Pisang Mas | 9a[1] | 5a | 12b | 38b | 4 |
| Williams | 10a | 5a | 25c | 40c | 4 |
| CIEN BTA -03 | 13b | (*)b | 5a | 0a | 10 |
| Yangambi km 5 | (*)c | (*)b | 0a | 0a | 12 |

[1]Different letters indicate significant differences among means according to Tukey´s multiple test at 5% probability. (*) No symptoms.

[1]*Letras diferentes indican diferencias significativas entre promedios de acuerdo a la prueba múltiple de Tukey a un nivel de probabilidad del 5%. (*) Sin síntomas.*

(YLS) appears earlier at the flowering than at the vegetative phase. At the vegetative phase, the YLS were the 9a and 10a leaves for susceptible cultivars. However, at flowering, the YLS was the 5a leaf for both susceptible cultivars. They also showed that the black Sigatoka disease developed faster in susceptible than in resistant cultivars. Escalant (2002), in the International *Musa* testing program (IMTP), reported an YLS in leaf 8 for the resistant cultivar Yangambi km5 plotted in Costa Rica, and in leaf 11 for plants of the same cultivar plotted in Tonga. These values differed from those reported in the present investigation. Escalant (2002) pointed out that disease development time (DDT) is not a reliable measure of resistance

because leaf symptoms can be affected by environmental differences and pathogen variations, among other factors.

**Disease severity.** Yangambi Km5 showed no symptoms either at 6 months after planting or at the bunch emission stage, which confirmed its resistance to black Sigatoka disease. The same pattern was observed with somaclon variant CIEN BTA-03. On the other hand, cultivars Pisang Mas and Williams showed infection indexes of 12% and 25%, respectively at the vegetative stage. At the flowering stage, infection values increased to 38 and 40% respectively (Table 3). These infection indexes are lower than those reported for the same clones in other locations (Escalant, 2002). Investigations are needed to determine whether the effect is due to management, soil fertility, climatic conditions, pathogen pressure or presence of aggressive strains of *M. fijiensis* (Escalant, 2002).

**Number of functional leaves at the flowering phase.** Resistant cultivars Yamgambi Km5 and CIEN BTA-03 had 12 and 10 (100%) active leaves, respectively, at the flowering phase. Meanwhile, susceptible cultivars Williams and Pisang Mas showed only four active leaves (Table 3). Four active leaves are not enough to produce good quality fruits. Daniels and Foster (2001) reported that for a good banana bunch fruits, the plant must have at least 10 leaves at flowering. Also, Molina and Castaño-Zapata (2003) found that fruit weight of cultivars with at least 11 functional leaves decreased more than half when the number of functional leaves at flowering was less than ten.

**RAPD analyses using DNA pool.** In order to reduce the number of PCR reactions, we tested the number of plants that could be mixed without losing polymorphism detection. DNAs from CIEN BTA-03 and Williams (AAA) parental materials were mixed using equimolar concentrations and different proportions of Williams: CIEN BTA-03 plants (Fig. 1). Three RAPD markers amplified with two *OPERON* primers (OPA-04 and OPB-01) were used to test the theoretical polymorphism detection level of this technique. These results showed that in a DNA mix of four plants, new or lost RAPD bands could be detected even if the change occurs in only one plant (Fig. 1). Based on these results we used two plants for RAPD evaluations pools of DNA. In some cases we perform individual amplifications.

**Verification of CIEN BTA-03 clonal propagated plants by RAPD markers.** Utility of RAPD as a mean of molecular analysis of *in vitro* regenerated plants has been well documented (Isabel et al., 1993; Munthali et al., 1998; De García & Giménez, 1999; Giménez et al., 2001; Latoo et al., 2006; Palombi et al., 2007). In Plantains (AAB),

Crouch et al. (1998) used RAPD assays for the analysis of two micropropagated populations, which were generated from single meristems of two cultivars.

**Fig 1.** RAPD markers identification in equimolar DNA pools.
1: 3 Williams + 1 CIEN BTA-03 2: 2 Williams + 2 CIEN BTA-03
3: 1 Williams + 3 CIEN BTA-03 4: 0 Williams + 4 CIEN BTA-03
5: 4 Williams + 0 CIEN BTA-03

***Fig 1.*** *Identificación de marcadores RAPD en cantidades equimolares de DNA.*
*1: 3 Williams + 1 CIEN BTA-03 2: 2 Williams + 2 CIEN BTA-03*
*3: 1 Williams + 3 CIEN BTA-03 4: 0 Williams + 4 CIEN BTA-03*
*5: 4 Williams + 0 CIEN BTA-03*

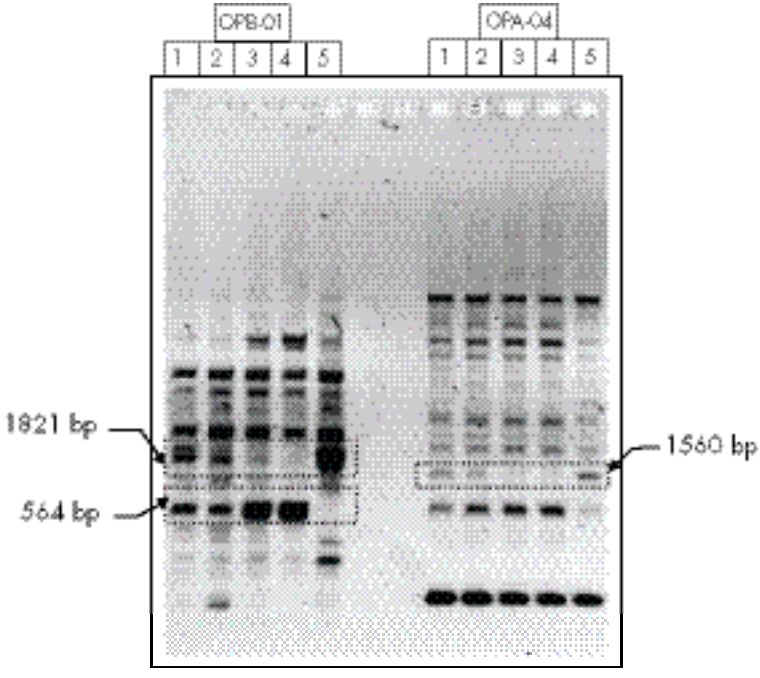
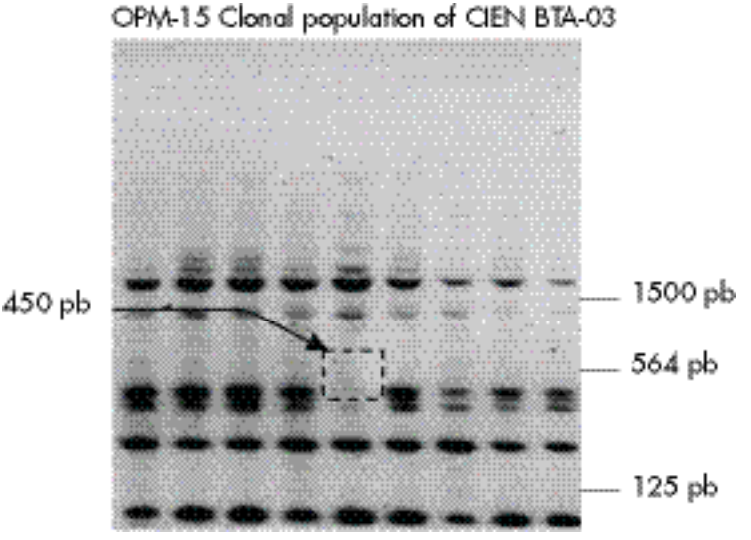


**Fig 2.** Amplifications in pools of 4 different plants DNA of CIEN BTA-03 clonal population. Black arrow shows an artifact.

***Fig 2.*** *Amplificaciones en las cantidades de DNA de cuatro plantas de la población clonal de CIEN BTA-03. La flecha negra muestra una situación engañosa.*




These populations were established in the field, morphologically characterized and subjected to intensive RAPD analysis. There was a clear correlation between the genotypic classification of individual plants and the tissue-cultured pedigree, suggesting that a substantial amount of the variation existed within the original cultured shoot apexes.

Plants (51 to 56) of the clonal population obtained from two multiplications cycles ($M_1V_1$ and $M_1V_5$), were amplified by PCR using DNA pools of two plants. The results showed some polymorphism at the DNA pool level but when we perform an individual verification of this polymorphism, we found that these loosing bands were PCR artifacts (Fig. 2).

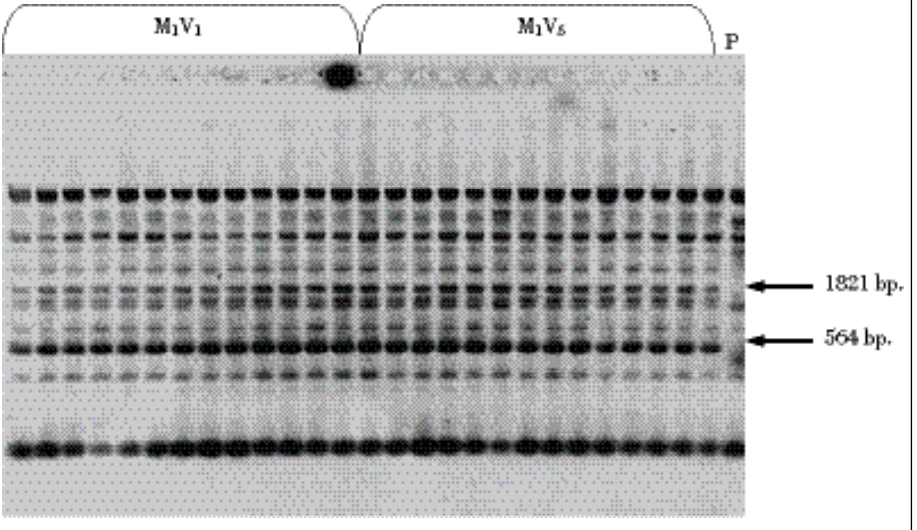
Ninety one different RAPD bands were amplified with 10 decameric primers in a population of 56 micropropagated plants ($M_1V_1$ and $M_1V_5$).

No polymorphism was detected in the 4,971 RAPD bands analyzed (Fig. 3 and Table 4).

The monomorphic banding pattern in micropropagated plants suggested no genomic alterations in the clonal CIEN BTA-03 population; we estimated that the theoretical polymorphism of this population would be less than 0.02% (1/4,971 x100). In oil palm, Rival et al. (1997) amplified 8,900 RAPD bands and did not find polymorphism in a population of plants propagated *in vitro*. However, Munthali et al. (1998) detected two polymorphisms in 4,557 RAPD bands amplified in a population of 120 sugar beet plants.

**Fig 3.** CIEN BTA-03 micropropagated plants of different multiplication cycles, M1V1 (24 plants) and M1V5 (27 plants), amplified with OPB-01 primer. P= Parental Williams (AAA).

***Fig 3.*** *Plantas de CIEN BTA-03 micropropagadas de diferentes ciclos de multiplicación, M1V1 (24 plantas) y M1V5 (27 plantas), amplificadas con el iniciador OPB-01. P= Williams parental (AAA).*




Somaclonal variation is an effective tool in the improvement of horticultural traits of banana. This method has advantages and disadvantages.

**Table 4.** Evaluation of the genetic integrity of CIEN BTA-03 clonal population by RAPD markers.

***Tabla 4.*** *Evaluación de la integridad genética de la población clonal de CIEN BTA-03 por indicadores RAPD.*

| | OPA-04 | OPB-01 | OPP-04 | OPO-19 | OPM-07 | OPM-15 | OPM-20 | OPD-02 | OPD-05 | OPD-20 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 51 | 51 | 56 | 56 | 56 | 52 | 56 | 56 | 56 | 52 | TOTAL |
| B | 5 | 8 | 10 | 12 | 11 | 6 | 12 | 10 | 8 | 9 | 91 |
| C | 255 | 408 | 560 | 672 | 616 | 312 | 672 | 560 | 448 | 468 | 4971 |

**A**= number of amplified plants
**B** = number of bands amplified by primer reaction in each plant.
**C**= A x B= theoretical number of amplified bands in pools of DNA.

***A****= número de plantas amplificadas por cada iniciador.*
***B*** *= número de bandas amplificadas por la reacción del iniciador en cada planta.*
***C****= A x B= número teórico de bandas amplificadas en cantidades de DNA.*

One of the disadvantages is the fact that somaclonal variation is a random process and thus it is non- directional; besides, it is a non predictable phenomenon and can not be controlled. The main advantage of somaclonal variation is that the selection procedure of the useful somaclonal variants is simple and straight- forward; this is especially true for vegetatively propagated crops such as banana (Tang, 2005).

In conclusion, a clonally micropropagated population was obtained after in vitro culture of shoot apexes of somaclone CIEN BTA-03 during five multiplication cycles. The monomorphic banding pattern in micropropagated plants suggested no genomic alterations in this clonal CIEN BTA-03 population. The evaluation of the CIEN BTA-03 plants at the field showed that this clonally micropropagated population, after five multiplication cycles, exhibited infection indexes similar to those of the resistant reference cultivar Yangambi Km5.

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