American Journal of Agricultural and Biological Sciences 2 (2): 43-48, 2007
ISSN 1557-4989

Generation of Five New *Musa* Hybrids With Resistance To Black Sigatoka and High Yield

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Abstract: The ability to identify genetic variation is indispensable to effective management and use of genetic resources. This work is the first approach concerning to generation and genetic differentiation of new *Musa* hybrid lines obtained in INIVIT (Cuba), crossing *Musa acuminata* male diploid (AA) x female triploid (ABB), genotypes selected by their resistant character to pests and diseases. From the obtained hybrids, only five were chosen because of their agronomic behavior and were genetically discriminated among them and also respect to the commercial clone FHIA-18 by AFLP polymorphism. Thanks to this prior genetic characterization *Musa* breeding programs could be consistently dinamised.

Key words: Banana, black sigatoka, hydric stress, AFLP profiles, *Musa*, silver staining, DNA extraction

INTRODUCTION

Bananas and plantains belonging to *Musa* spp. are the most important tropical fruit crops, which yield a world yearly production of 97 million tons[12]. The cultivated bananas and plantains are usually triploid and tetraploid, originating from interspecific hybrids of the two wild species *Musa balbisiana* (BB) and *M. acuminata* (AA)[27]. Both groups are the main components of the farming systems in the humid agro-ecological zones of the tropics, where their production represent an indispensable source of rural income. Many pests and diseases have significantly affected *Musa* cultivation they have spread dramatically during the past 30 years. As a consequence of these threats to *Musa* cultivation, there has been renewed interest in *Musa* breeding programs. Unfortunately, diseases caused by Panama disease, also known as Fusarium wilt, *(Fusarium oxysporium* Schlech f. sp. *Cubense)* [15], black sigatoka (*Mycosphaerella fijiensis* Morelet)[5] and dry climate affect culture and consequently reduce production rate. Although resistant character of clone 'FHIA-18' released by the (Fundación Hondureña de Investigaciones Agrícola)[9], is the most extensively commercial clone used, cultural attentions such as local defoliation and short cycles of production are currently applied to decrease the use of chemicals, but low benefits are obtained. Trying to provide a solution to these problems, INIVIT (Cuba) has been developing a *Musa* Breeding Program[26] since 1993. Nowadays the obtaining of new tetraploid hybrids is based on the breeding of recently selected genotypes from *Musa acuminata* diploid (AA) and a triploid (ABB)[24], which were not affected to the mentioned pests, diseases and stress conditions. From these progenies, five hybrid lines (H_1, H_5, H_9, H_{10}, H_{12}) were selected. After agronomic testing of the selected lines, isoenzyme and RAPD markers revealed strong differences between lines and original genotypes, but discrimination among lines was not possible to be defined[24].

Amplified fragment length polymorphism (AFLP) fingerprinting usually results in informative

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profiles due to the high number of genomic fragments that can be analyzed in a single assay. AFLP technique is being widely used for genetic diversity studies because it reveals significant polymorphism and is a reliable and robust genetic molecular-marker assay. AFLP technology has the potencial to produce dozens of mappable bands from a single reaction[10,11,32]. AFLP and related techniques has been used to discriminate between accessions in several plant species including banana, suggesting that AFLP technology has a high potential in contributing to the understanding of *Musa* genetic diversity and hybrids characterization[10, 11,14,22,29,30,33]. Owing to the great economic potential and the importance of the novel hybrids obtained, this study was designed with the aim of validate the genetic identity of the lines using AFLP markers. The main goals of this work are to certify: a) the existence of new hybrid lines and b) that the new hybrid lines are genetically different from 'FHIA-18'.

MATERIALS AND METHODS

Plant materials *and DNA extraction*: The field experiment was conducted at Cuban Agricultural. The experimental plots were established in random block contained 100 plants (4 rows each). Fresh leaf tissues (150 - 500 mg) were taken from the following samples: five hybrid lines designed as H_1, H_5, H_9, H_{10}, H_{12}, male diploid progenitor SH 3362 (AA), female triploid progenitor Saba (ABB) and tetraploid 'FHIA-18' (AAAB). Fresh leaf tissues from mentioned samples were pulverized in liquid nitrogen. DNA was obtained and purified using CTAB method[28]. The DNA was collected after centrifugation at 8000 rpm for 10 min. The pellet was dried and re-suspended in a minimal volume of TE buffer and stored at 4 °C until processing.

Evaluation of morphoagronomic characters: After six months of having planted, different characters were evaluated fallowing the Descriptors for Banana (IPGRI/INIBAP/CIRAD, 1996): height of the plant, pseudostem perimeter, number of suckers and number of leaves at the flowering moment. Also during the yield were evaluated: weigh of bunches, number of hands, number of total fingers and long of fingers. The experiments were carries out in dry conditions (stress hydric), only favored for the rain. The other cultural attentions were carried out according to the Technical Instruction of Banana Cultivation[17].

Evaluation of Diseases: The experimental plots were located in an isolated area without chemical treatments for fungus. Preliminary study about performance to resistance of hybrids against black Sigatoka *(M. fijiensis*) was observed. The controls hybrid 'FHIA –18' (AAAB) resistant and clone 'Parecido al Rey' (AAA) susceptible were planted alternate plots between the hybrids. The methodology of Carlier *et al*.[5] for global evaluation of diseases in *Musa*, was applied during two cycle in field conditions.

Amplified Fragment Length Polymorphism Protocol: AFLP analysis was carried out according to described protocols[32]. We used 1.8 µL (100 – 250 ng) DNA double restriction-digested for 2 h at 37 °C with 0.625 units of EcoR I and Mse I in 6.25 µL buffer (10 mM tris-HCl, pH 7.5, 10 mM Mg-acetate and 50 M K-acetate) and heat inactivation for 15 min at 70 °C.

Genomic DNA fragments were ligated to E*co*R I and M*se* I adapters for 2 h at 22 °C. An aliquot of this ligation mix was diluted 1:10 with TE buffer and preamplified with adapter-derived primers having one additional (+1) nucleotide at the 3´ end. This reaction was performed 20 cycles with the following cycling profile: (i) 30 sec denaturation at 94 °C, (ii) 1 min annealing at 56 °C and (iii) 1 min extension at 72 °C. After 50-fold dilution in TE buffer, this stock was used for all further selective amplifications.

Diluted preamplified DNA stock was amplified by PCR in 5 µL reaction volume with 1.25 ng E*co*R I selective primer in combination with 7.5 ng of M*se* I selective primer and 5 µL / µL units *Taq* polymerase. For this selective amplification were used AFLP primers E*co*RI +3 (E- ACG) and M*se* I +3 (M-CTT), selected from a nine primers combination previously studied (data not showed). The E*co*R I primers were not radioactively labelled[4].

The cycling conditions were: a) 1 cycle at 94 °C for 30 sec, 65 °C for 30 sec and 72 °C for 60 sec; b) 13 cycles starting with an annealing temperature of 65 °C and lowered 0.7 °C every subsequent cycle; c) 23 cycles at 94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 60 sec.

Sequencing gels (6% polyacrylamide denaturalized, 7.5 % urea in TBE buffer) were prepared under standard conditions (GIBCO-BRL, Life Technologies). Long glass plates were pre-treated with Silane A-174 in order to bind the gels to them for easy handling. Gels were assembled in casts (0.4 mm thickness) employing the S_2 model sequencing apparatus (BioRad), which was run at 55 W for 2 h. The products were visualized by silver staining according to procedures described[2,7].

Data analysis: Scanning of gels using Adobe Photoshop (8.0 educational version) program was preferred since it provides a computerized record of the

results and keeps the glass plates available. For diversity analysis, bands were scored as present (1) or absent (0) to form raw data matrix. Genetic diversity estimations were calculated as Jaccard Similarity Coefficient [21] and then the cluster UPGMA dendrogram was applied using Phylip Program ver. 3.5 [13].

RESULTS AND DISCUSSION

Evaluation of morphoagronomic characters: Morphoagronomic evaluation of five hybrids (Table 1) are shown obtained by means of the hybridization of the improved diploid SH 3362 (AA) x the triploid somaclon Saba (ABB). The hybrid ones obtained (H_1 and H_9) they registered optimum parameters in the different morphoagronomic characters evaluated, similar to those reached with the hybrid witness obtained by the FHIA, fundamentally as for its potential yield achieved under conditions of hydric stress. The other ones hybrid H_5, H_{10} and H_{12} they behaved for under in most of the evaluated parameters and fundamentally with the components of the yield (No. of hands, No. of fingers, long of the fingers and weight of bunches) (Table 1).

These values are undoubtedly in function of the behavior in front to black Sigatoka (*Mycospharella fijiensis* Morelet) and the deficiency caused by the dry condition, primordial factors to obtain high yields in this crop. For that reason it was included inside the program of improvement, the clones rustic "Burros" which have appropriate characteristics to resist the drought and resistance Sigatoka that sustains current INIVIT genetic improvement program.

Banana production areas with localized irrigation technology cover around 11, 000 ha (mainly Cavendish clones) and years of 35 ton/ha are achieved however, the cost of chemical to control black Sigatoka is more than USD 500/ ha. They are also some 20 000 ha of Cavendish banana located in the mountains intercropped, coffee mainly for local consumption.

At the end November 1996, the Island was hit be the hurricane Lily with affected seven Province and destroyed 76 % of the best banana plantations. Since the replanting process have been initiated in these areas used 'FHIA–01', 'FHIA– 18', 'SH-3436', and 'FHIA-03' hybrids, witch had been evaluated and multiplied since 1994.

Outstanding result have been obtained in Cuba through the introduction of promising materials, mainly from FHIA, thanks to the efforts of Drs. Phil Rowe and Franklin Rosales and more recently of INIBAP.

The introduction of these materials to the country began 1991 with 'FHIA-03' and 'FHIA-18' hybrids. Later 'FHIA-01'-V1, 'FHIA- 01',- 02,- 05,- 19,-21, - and - 22 were also introduced.

In august 1994, the first comparative experiments were established at "La Cuba" farm located in the Ciego de Avila province and in 1995 the first extension trials were conducted throughout the whole country; thus starting a massive multiplication program for 'FHIA-01'-V1, 'FHIA-03', 'FHIA-18', 'SH-3436' hybrids and more recently 'FHIA-02'.

All evaluated hybrids showed resistance to the black Sigatoka fungus. All materials reached flowering time with more than 11 functional leaves in the first cycle and 10 in the second cycle this situation allowed the more resistant clones ('FHIA-02', 'FHIA-03' and 'FHIA-18') to completed the vegetative cycle up harvest with more than 6 functional leaves (both cycle).

The most resistant hybrids ('FHIA-02', 'FHIA-03' and 'FHIA-18') showed incubation periods from 47 to 60 days and from 24 to 30 days during low and high inoculums concentration periods respectively. All hybrids show, in both cycles higher bunch weights and better yields per hectare than controls, more than 37 t/ha during both cycle[1].

Evaluation of black Sigatoka: The hybrids are show in front of the attack of black Sigatoka (*Mycosphaerella fijiensis* Morelet) (Table 2). The H_1 was able to arrive to the period of flowering with 11 functional leaves, with a similar behavior to the witness. This allowed the completed the vegetative cycle and to the crop arrive with more than 5 functional leaves. It was also determined that finally the most important parameter in resistance to this pathogen HMJM (Leaf but spotted youth) it was 8.7, very near at 9 obtained by the 'FHIA - 18' (AAAB) resistant.

The hybrids H_5, H_9, H_{10} and H_{12} had a similar behavior before the attack of fungus, with a number of functional leaves in the moment at the flowering between 8 and 10 arriving the crop with 3 and 3.8 functional leaves; valuing as fairly resistant. Finally the index leaf more spotted youth (HMJM) it was between 6.6 and 7.0 more down to the control.

The black Sigatoka is generally considered as the main one causing in the decrease of the yields for both cultivations[6,18,23]. The variation that exists in the reaction among of the spring bananas tetraploides suggests that the resistance to diseases could be regulated by. The bananas of the group AAB doesn't transit for the same situation that the banana plants and to weigh that in the decade of the 70 and until half-filled of that of the 80 decorous yields were obtained (17- 20 t/ha) in certain years, the presence of the black Sigatoka, the nematodes and other pest among other factors they caused a significant reduction in the yields to the point of this banana type practically disappeared of the market remaining alone in very limited areas of small producers.

Table 1: Morphoagronomic characteristics of hybrids and (FHIA- 18) as control in the field

Hybrids	**Height plants (m)**	**Pseudostem perimeter (cm)**	**No. of Suckes**	**Leaves at flowering moment**	**No. of Hands**	**No. of total Fingers**	**Long of Fingers (cm)**	**No. Finger 2nd hand**	**Weigh of bunches (kg)**
H_1	2.25	52.6	5	12.3	9.8	145	11	12.8	16.2
H_5	2.07	47.6	4	11.7	10.3	112	12	13.4	14.8
H_9	2.30	48.8	4	12.7	10.1	134	12	14.5	16.3
H_{10}	2.54	46.2	4	11.8	9.2	117	12	16.5	15
H_{12}	2.02	40	5	9.6	7	70	9.5	10	13
(FHIA-18)	2.26	46.7	3	11.1	9.1	140	15.7	14.2	16.6

Table 2: Performance of hybrids and control clones against Sigatoka

Hybrids	Flowering (Leaves No.)		Yield (Leaves No.)		HMJM*
	Total	Functional	Total	Functional	
H_1	12.33	11.0	6.2	5.2	8.7
H_5	11.25	10.1	5.4	3.4	6.6
H_9	12.0	10.8	5.6	3.8	7.5
H_{10}	9.66	8.2	4.3	3.0	6.8
H_{12}	10.5	9.0	4.8	3.0	7.0
FHIA-18	12.3	11.0	6.0	5.0	9.0

*****HMJM (Leaf more spotted youth). [5]

Table 3:Number of polymorphic AFLP bands obtained after the analysis of the five hybrid lines, their progenitors and the commercial hybrid 'FHIA -18'.

Variants	No. of bands	No. Polymorphic bands	Polymorphism rate (%)
S (AA)	29	17	58.6
SH (ABB)	27	17	68.9
H_1	31	19	61.3
H_5	21	9	42.8
H_9	36	22	61.1
H_{10}	33	20	60.0
H_{12}	34	23	67.6
FHIA-18	28	17	60.7
Total	239	144	60.25
Overall Mean	29.8	18	-

The introduction for the Instituto de Investigationes en Viandas Tropicales (INIVIT) of clones of the type Bluggoe (ABB) it compensated in certain measure the absence of the banana plantain being developed a *Musa* Breeding Program, using the traditional methods (hybridization, selection, etc) and auxiliary techniques with the purpose of to obtain in the smallest possible time, banana clones and plantain with characteristics required by producers and to increase the genetic variability prioritizing advanced diploids and clone of the group AAB.

Molina-Tirado and colleagues[19] carried out a study on the behavior of the hybrid ones of the FHIA ('FHIA-17', 01 and 21) in front of *Mycosphaerella fijiensis* obtaining as a result but five functional leaves in the moment of the crop in the 'FHIA-17' and -01 that which confirms the positive relationship among the number of functional leaves and the weight of the cluster, similar results were obtained in our work, that which will allow a later work of crossing with these hybrid one resistant to the diseases and with good agronomic results, that will facilitate the continuity of the future program of improvement in Cuba.

AFLP analysis: The quality and the concentration of DNA collected by the CETAB method was acceptable (80 – 250 ng/µL) and sufficient for obtain very clear banding profiles. A total of 239bands (data not showed) were detected located at 800 - 70 pb size, with overall mean (29.8 bands). From this 144 bands were polymorphic bands with overall mean (18 bands). In general 60.25 % polymorphism rate was obtained (Table 3).

The dendrogram showing bootstrap analysis (Figure. 1), based on Jaccard´s similitude coefficient, divide the genotypes into three differentiated groups. In the Group I, the hybrids H_5, H_9 and H_{12} are joined to the male diploid progenitor (AA), and also very similar to the tetraploid 'FHIA-18' (AAAB). Group II include the hybrid H_1 with the triploid female progenitor (ABB). The hybrid H_{10} builds group III, completely isolated from the rest of the hybrids, confirming the observations of the field morphoagronomic characteristics[24,25].

In the AFLPs generated (table. 3) an mean 18 bands were scored, this results are in agreement with the mean of 15,3 bands observed in *Triticum* characterization of genetic diversity[16]. The average of 60.25 % polymorphism rate obtained in this study is a similar polymorphism to observed in others research (44% and 79,9%) when cultivars of *Musa* were evaluated by AFLP marker[3,8].

Preliminary results over deferens to ADN level among hybrids, parents and control were obtained in this work. In this analysis it was evident the formation of group 2 where was isolate H_1 which was characterized as the best in all the hybrid ones by their biggest yield and their high resistance to Sigatoka, this it has been selected in the program to carry out other possible cross, apparently it possesses a similarity to the Saba clone as for other characters. While the H_{10} is alone located in the group 3 being this one of the worst for its low yields and differences in the evaluated agronomic parameters what influence to eliminate it.

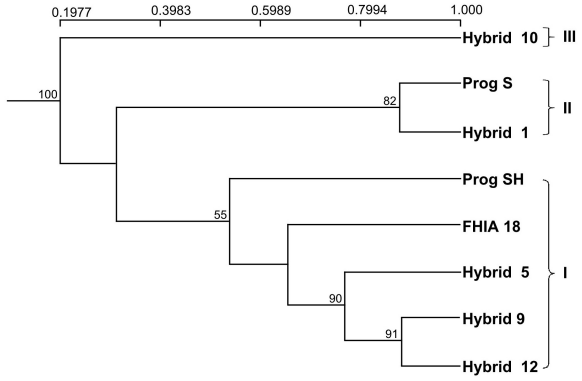

Fig. 1: Dendrogram showing genetic similarities and grouping between hybrids, parents and a commercial clone obtained using UPGMA cluster analysis. The groups are shown in Roman numerals (I, II, III). When greater than 50% the percentage of bootstrap subtrees corresponding to particular nodes is shown next to those nodes.

The application of this marker AFLP includes in the group 1 to H_5, H_9 and H_{12} next to the witness 'FHIA-18' and to the feminine progenitor (AA). The discrepancy among the differences opposing morfoagronomic among them and the little difference statistic detected with the marker AFLP suggests a narrow genetic base, that which could not be differentiated in this case [10,20]. These hybrids continued being studied, but they won't be of interest for next cross.

Band visualization with AFLP silver staining like other works[2,31] was easily got, showing the possibility of use this technique routinely in the laboratory for analysis the hybrids from the *Musa* breeding programs, in agreement with results obtained applying the procedures from Briard *et al.* [4] and Creste *et al.* [7].

ACKNOWLEDGMENTS

This work was supported by the INCO Project. Contract No. ICA4-CT – 2001 – 10063 (TIB plant).

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