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Synthesis of New Interspecific Triploid Hybrids from Natural AB Germplasm in Banana (*Musa* sp.)

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Abstract

The release of new sweet-acid banana cultivars resistant to black leaf streak, Sigatoka leaf spot and Fusarium wilt is important for domestic markets in tropical and subtropical countries. Common current breeding strategies consist of selecting tetraploid AAAB hybrids directly from crosses between AAB sweet-acid varieties pollinated with AA clones carrying disease-resistance genes. However, this crossing pathway is hampered by low gamete fertility and the rare occurrence of desired 2N gametes on the AAB female side (N= 3X = 33 chromosomes). We propose an alternative pathway which aims to create new triploid hybrids directly from AB landraces. Natural AB clones are sterile but their AABB tetraploid counterparts obtained by colchicine treatment are fertile. This gamete fertility was made profitable in crosses with AA and BB accessions to generate AAB and ABB hybrids. We present here agronomical results of various progenies involving an in vitro **synthesized tetraploid 'Kunnan' (AABB) and several AA and BB clones. These first results suggest the very high potential of this new strategy for the release of well-performing new hybrids combining higher productivity, disease resistance and better fruit quality. Hybrids with a high added-value, produced in this way, could be made available for evaluation within the ProMusa network.**

INTRODUCTION

The banana (*Musa* spp.), including sweet and cooking bananas, is the number one tropical fruit, with a global production exceeding 100 million tons in 2006. It is also a staple food for more than 400 million people (Loeillet, 2008). Banana cultivation for local consumption is based on a large number of cultivars adapted to different conditions of production as well as the varied uses and tastes of consumers. In this context, about 12 million metric tons of sweet-acid AAB and ABB bananas were grown in 2008 in the world (Lescot, 2010). India (4 million t/yr) and Brazil (3 million t/yr) are the two major producing countries of these types of cultivars. But, like Cavendish, these cultivars are susceptible to numerous diseases. For example, Fusarium wilt causes high yield losses on the popular but highly susceptible Silk banana ('Maçà', AAB) in Brazil and to a lower extent on the less susceptible 'Prata' (AAB) cultivars belonging to the Pome subgroup (de Matos et al., 2011). However, Prata cultivars are highly susceptible to leaf spot diseases and cannot produce satisfactory without recurrent applications of fungicides (Bakry et al., 2009). Therefore, there is an urgent need to release new disease-resistant interspecific varieties, that are tasty and are productive with less pesticides.

Ancestral bananas are diploid and fertile, but cultivated ones are mostly triploid, highly heterozygous and bearing low gametic fertility. Progenies are usually of small size and composed of a blend of plants of different ploidy levels, including aneuploid ones. In addition, a significant proportion of alleles deviates from Mendelian ratios in progenies (Hippolyte et al., 2010), so little knowledge on genetics and heredity becomes available.

The low fertility of cultivated bananas is a handicap for breeders. In triploid cultivars, the number of fruits per bunch varies from 100 to 250 and the number of ovules from 300 to 600 per fruits. In the absence of gametic sterility, the seed potential is theoretically estimated from 30,000 to 150,000 seeds/bunch. Consequently, it may be likely that in triploid bananas, reproductive barriers reduce the female fertility by approximately 99.9%. In diploids, the interspecific AB clones are almost sterile whereas AA varieties show a wide range of male and female fertility.

In spite of gametophytic sterility, notable progress has been made over the last 20 years in banana breeding. A common breeding strategy consists of selecting tetraploid AAAB hybrids directly from crosses between AAB sweet-acid cultivars pollinated with natural or improved AA clones carrying resistance genes. However, this crossing pathway is hampered by low gametic fertility and the rare occurrence of the desired 2N gametes at the AAB female side (N= 3X = 33 chromosomes).

We propose an alternative pathway which aims at creating improved triploid banana hybrids directly from diploid germplasm. Natural AB landraces are almost sterile and unsuitable for hybridization. But their AABB tetraploid counterparts, obtained through colchicine treatment, have been shown to be fertile when used in our breeding program (personal observation). This gamete fertility was made profitable in crosses with AA and BB accessions to generate AAB and ABB hybrids. We present here our first results of test-crosses on seed amounts and agronomical value of triploid progenies obtained from crosses involving an artificially synthesized tetraploid 'Kunnan' (AABB) and several AA and BB clones.

MATERIALS AND METHODS

Plant Material

Plant material used in this study that was available at Bioversity's International *Musa* Germplasm Collection is referred to by its ITC accession number. The 'Kunnan' landrace (AB genome - ITC1034) was chosen because of its high popularity and consumption rate in India, where it may be eaten as fresh fruit or after processing (baby food). This clone was reported as resistant to nematodes (*Radopholus similis* and *Pratylenchus coffeae*) (Collingborn and Gowen, 1997; Moens et al., 2005). Moreover, although 'Kunnan' has been shown to harbor two integrated copies of banana streak virus (eBSV), the integrated sequences are strongly modified and are therefore supposed not to be infectious (Umber et al., 2011; Duroy et al., 2011). 'Kunnan' actually never expressed symptoms of streak disease in Guadeloupe (French West Indies).

However, 'Kunnan' has been shown to be susceptible to Sigatoka leaf spot in Guadeloupe. Alleles distribution as revealed by molecular studies using SSR markers indicates that the A genome of 'Kunnan' contains a high proportion of the *Musa acuminata* ssp. *malaccensis* genome whereas the B genome is close to the wild *Musa balbisiana* 'Lal Velchi' clone that originates from India (X. Perrier, pers. commun., 2011). Derived tetraploid clone 'Kunnan 4X' (doubled-diploid) was obtained by somatic doubling (Bakry et al., 2007) of the diploid accession.

Several diploid accessions were used in crosses with 'Kunnan 4X' in this study. These are 'Malaccensis', 'IDN110/AAcv Rose' and *Musa balbisiana*.

- *M. acuminata* ssp. *malaccensis* 'Malaccensis' (Internal CIRAD code II-04-001-001-505) is a wild type that originates from Malaysia. Like many wild diploids, it easily produces seeds and displays high pollen fertility.
- 'IDN110/AAcvRose' (AA genome - ITC0712): this dessert cultivar originates from Indonesia. This clone is placed within a set of clones coming from Indonesia and Thailand, called 'Khai' for simplicity purposes, for which the *M. acuminata* ssp. *malaccensis* genome contribution is high (Perrier et al., 2011). This accession produces some seeds (from 0 to 150 as female parent and over 1000 when used as male parent) in crosses and present medium pollen fertility. This accession is resistant to Sigatoka leaf spot and black leaf streak disease.
- *M. balbisiana*: several wild accessions of *M. balbisiana* present in the field genebank in Guadeloupe were used in crosses as female or male parent. Detailed results reported hereafter were obtained from crosses involving *M. balbisiana* 'Pisang Klutuk' (ITC1077) and *M. balbisiana* 'Cameroun' aka 'CMR' (ITC0246). In Guadeloupe, none of these accessions displays symptoms of Sigatoka leaf spot.

Methods

Crosses were made at the CIRAD research center in Guadeloupe. Hybridization was carried out according to Menendez and Shepherd (1975). At flowering of the female parent, the inflorescences were bagged with insect-proof airtight sheaths. Every day, the new hands of exposed female flowers were pollinated with pollen collected the same day. Pollinated bunches were harvested when the first ripe fruit turned yellow. Ripe fruits were deseeded finger by finger.

The embryos were rescued (Bakry, 2008) to obtain a high *in vitro* germination rate. Developed plantlets were thereafter transferred to the greenhouse and transplanted about 60 days later in the field. Ploidy level of hybrids was determined by flow cytometry (Dolezel and Bartos, 2005).

RESULTS

Seed Amounts

'Kunnan 4X' was tested in interploid crosses with a range of wild and cultivated diploids (see plant material). Table 1 displays the ranges of seeds set in crosses using the 'Kunnan 4X' genome, either as female or male parent.

As a female parent, 'Kunnan 4x' produced over 200 seeds in a single cross when pollinated with *M. acuminata* ssp. *malaccensis* 'Malaccensis' which does not display any male fertility reduction. Lower results were obtained on average when crossing 'Kunnan 4X' with 'IDN110/cvRose', with seed sets varying from 0 to 150. This might result from some pollen abortion of 'IDN110/AAcvRose' due to its high structural heterozygosity. Nevertheless, this cross was fertile enough since 197 seeds were obtained in 7 crosses only.

Pollination of 'Kunnan 4X' with various sources of *M. balbisiana* pollen resulted in less seeds per bunch than when pollinated with *M. acuminata* ssp. *malaccensis* 'Malaccensis'. About 300 seeds were obtained in 11 crosses which can be regarded as satisfactory for breeding purposes.

As a male parent, 'Kunnan 4X' showed to be highly fertile when crossed with wild *M. acuminata* and *M. balbisiana* accessions. Seed sets showed to be regular with *M. acuminata* (100 to 500 seeds per bunch). They were less regular with the various *M. balbisiana* accessions tested in this study (0 to 300 seeds per bunch).

Some attempts of crosses involving the original 'Kunnan' (2X) accession were less successful. Almost no seed was obtained with the exception of 82 seeds from one cross between 'Kunnan' (2X) and *M. balbisiana* 'CMR'. In order to understand the dramatically reduced male fertility of 'Kunnan' (2X) as compared to its tetraploid counterpart, we evaluated their pollen viability using the Alexander protocol (Alexander, 1969). We observed a high frequency of dead and aborted pollen grains in 'Kunnan' (2X), while almost all grains being viable in 'Kunnan 4X' (Fig. 1). This observation may explain the fertility differences between the two clones when used as male parents.

Ploidy of Progeny

All hybrids produced from crosses involving 'Kunnan 4X' with a diploid were shown to be triploid plants, when verified using flow cytometry (Dolezel and Bartos, 2005).

Field Performance of Hybrids

We detail hereafter the first observations of some hybrids issued from crosses involving 'Kunnan 4X' as a male or female parent.

AAB Hybrids

1. *M. acuminata* ssp. *malaccensis* 'Malaccensis' (♀) x 'Kunnan 4X' (♂). A progeny of 38 hybrids was planted in the field and observed over various successive cycles. Out of 38 hybrids, 32 were parthenocarpic and seedless whereas the remaining 6 hybrids were non-parthenocarpic plants bearing seedless stunted fruits (Fig. 2). Parthenocarpic hybrids showed a wide range of bunch weights in the second and subsequent cycles, from 4 to 40 kg with an average of 18 kg. The most significant yield parameters of this progeny are summarized in Table 2. As for bunch weight, these parameters revealed high dispersion with variation coefficients ranging from 22 to 66%.

It is noteworthy that almost half of the progeny showed a bunch weight superior to both parents (Fig. 2). Same observations were noted for others characters. These results suggest that hybrid vigor in this cross is probably linked to a positive heterosis effect. In addition, all the hybrids were resistant to Sigatoka leaf spot under the field conditions of Guadeloupe, resistance being probably brought by the resistant *malaccensis* female parent. Resistance of these hybrids to nematodes has not yet been tested. Most of the hybrids had sweet-acid bananas with some variations in flavor and texture.

Some outstanding triploid hybrids were selected from this progeny. For example, hybrid '2006-22/III9' has a phenotype similar to Mysore clones (AAB genome, Mysore subgroup), with an average bunch weight of 28 kg and a taste similar to Silk bananas (AAB genome, Silk subgroup - Fig. **3**).

2. 'Kunnan 4X' (♀) x 'IDN110/AAcvRose' (♂). As a preliminary test, nine hybrids only were observed in the field, over several cycles. The results are similar to those obtained in the previous progeny but with some noticeable differences: all hybrids were parthenocarpic and bunch weights ranged from 23 to 42 kg in our conditions of Guadeloupe with an average of 31 kg which is 13 kg more than in the previous combination (Table 3). All these hybrids were shown to be resistant to Sigatoka leaf spot.

It is likely that Sigatoka leaf spot resistance observed in the hybrids was brought by the male 'IDN110/AAcvRose' parent in this cross.
Despite the small size of the progeny, one hybrid ('2005/25-L9') proved to be outstanding, producing a bunch of 52 kg in the first cycle, displaying 14 hands of well-formed and very tasty fruits resembling sweet-acid bananas.

ABB Hybrids

1. 'Kunnan 4X' (♀) x *M. balbisiana* 'CMR' (♂). The test progeny was constituted of 15 plants: one was not parthenocarpic while the 14 other hybrids were, with bunch weights ranging from 14 to 35 kg (Table 3) and an average of 17.5 kg/bunch in the first cycle and 29.5 kg/bunch in the second cycle. It should be noted that a weight of 40 kg/bunch was recorded for one hybrid ('2008/12-I6') of this progeny in its third cycle. Some hybrids have the appearance of clones from the Awak subgroup (ABB genome, Fig. 3) and present a high hybrid vigor both for vegetative and reproductive parameters. The flesh of the fruits is sweet, with a white cream color and pleasant taste. These hybrids are resistant to Sigatoka leaf spot.

2. Kunnan 4X (♀) x *M. balbisiana* 'Pisang Klutuk' (♂). Eight hybrids were observed in the test progeny in the first and second cycle. As for the previous cross, most of the clones were parthenocarpic (7 out of 8) with bunch weights ranging from 24 to 42 kg (in the first cycle) and an average of 32 kg/bunch, suggesting a higher potential of the 'Pisang Klutuk' male parent in comparison to the 'CMR' male parent for this parameter (Table 3). At the vegetative level, hybrids derived from this cross are similar to those of the previous one. However, for bunch and fruit parameters, they look different: bunches are fairly compact and fruits are straight, erect and rounded. They are also resistant to Sigatoka leaf spot under the field conditions of Guadeloupe. The fruit quality of these hybrids does not recall any natural clone present in the collection of Guadeloupe and might be regarded as new plant material.

DISCUSSION

Breeding strategies developed at CIRAD aim at the development of triploid hybrids coming from crosses between diploid and doubled-diploid varieties (auto- or allotetraploid) obtained through chromosome doubling of diploid varieties. They result from the limitations of the triploid x diploid crosses (aka tetraploid strategy), hampered by the low gametic fertility of the triploid parents and its failure to produce fully adapted end products.

The triploid way searches to identify good specific combining abilities between diploids and doubled-diploids as donors of diplogametes. In addition, it aims to maximize heterosis in the triploid progenies. Genotypes (natural or improved diploid varieties) are selected according to the type of banana to be developed (cooking or dessert), their agronomic characteristics, their behavior with respect to diseases and pests, and their male and female fertility at diploid and tetraploid level. It is also based on an in-depth knowledge of the available genetic resources and the understanding of the relationships between ancestral and cultivated varieties, thus facilitating the definition of pools of parent lines according to the desired results.

At CIRAD, where this method has been prioritized for several years, progress has been made in the development of AAA dessert bananas. Several hybrids recently obtained have been released for large-scale evaluation to banana growers in the French West Indies and in the Caribbean. Moreover, some of them have been proposed for the next

International *Musa* Testing Program (IMTP) trials. Our preliminary results presented here validate the adaptation of this new triploid strategy for the development of AAB/ABB banana hybrids.

When the gametic fertility of the doubled AA diploids used in the development of AAA hybrids is rather unpredictable (some clones are fertile at both diploid and tetraploid levels, while others are completely sterile at the tetraploid level), all interspecific AB clones studied are sterile at the diploid level but have been shown to be systematically male and female fertile at the tetraploid level. The high fertility of the doubled AB leads to the production of large populations, in which it is possible to set up a true breeding program including an efficient selection process.

With all the crosses taken together, 70 hybrids have been field-tested, 61 of them involving a wild diploid, *M. acuminata* or *M. balbisiana*. It is noteworthy that most of these hybrids bear parthenocarpic (edible) fruits, despite their wild parentage. This result paves the way to the use of miscellaneous wild types. The diversity of the wild genepool should permit variation in the type of fruit, whether dessert or cooking, but also in terms of fruit sweetness or acidity and plant traits, like agronomic behavior and resistance to biotic and abiotic stresses.

A common feature within the progenies evaluated is the positive heterosis effect observed. For most characters, mean values observed are significantly superior than the average values of the parents, and in some cases exceed the value of the best parent.

Thus, within a limited number of hybrids, it has been possible to select some outstanding hybrids from each cross: the AAB '2006-22/III9' from the cross with *malaccensis*, with a Mysore morphology; the sweet-acid AAB banana hybrid '2005/25-L9' issued from 'IDN110/AAcvRose' crosses; the Pisang Awak-like '2008/12-I6' selected within *balbisiana* 'CMR' progeny; and ABB hybrids with heavy bunches from the cross involving 'Pisang Klutuk'.

All the hybrids produced have shown resistance to Sigatoka leaf spot under field conditions in Guadeloupe suggesting that the resistance is transmitted in a dominant way by the diploid resistant parent. The size of the progenies studied is however not sufficient to establish that resistance is in a homozygous state in the parents. As it could be expected from the eBSV pattern of 'Kunnan', none of the hybrids displayed symptoms of streak disease in the field. Selected hybrids have not yet been tested against black leaf streak disease (BLSD), as the disease is absent in Guadeloupe. In the future, they will be characterized according to their reaction to BLSD under controlled conditions at CIRAD in Montpellier, as well as to their behavior when infested with nematodes. Resistance to Fusarium wilt, race 1 and tropical race 4, has not been tested, but considering their pedigree, the ABB hybrids might be regarded as good alternatives to Pisang Awak clones or other ABB natural clones in traditional cropping systems where banana production is constrained by this pathogen. Besides pest and disease resistance, these promising hybrids will need further physiological and physicochemical characterization to better define their fruit quality, food value, acceptability by farmers and consumers, and their potential for transport, conservation and ripening, etc. The ProMusa network might be an ideal context to facilitate this work.

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Tables

Table 1. Amounts of seeds/bunch (S/B) produced in interploid crosses with 'Kunnan 4X'.

		Kunnan 4X			
		female parent		male parent	
		NR	S/B	NR	S/B
Other parent	*M. a. malaccensis*	1	200	3	100 -> 500
	IDN110/cv Rose	7	0 -> 150	2	0
	M. balbisiana	9	15 -> 70	11	0 -> 300

NR: number of crosses

Table 2. Yield parameters of *Musa acuminata* ssp. *malaccensis* 'Malaccensis' x 'Kunnan 4X' cross.

	HT	PG	BW	NH	NF	FL	FD
Mean	390	68	18	11	182	100	30
min	210	33	2	4	7	50	15
max	600	100	40	18	349	160	41
CV (%)	23	25	66	32	48	24	22

HT: plant height (cm) – PG: pseudostem girth at height 100 cm (cm) – BW: Bunch weight (kg) – NH: hands – NF: number of fruits of the bunch – FL: medium external fruit on third hand length (mm) – FD: medium external fruit on third hand diameter (mm)

Table 3. Yield parameters of crosses with 'Kunnan 4X' as female parent.

	HT	PG	BW	NH	NF	FL	FD
Male parent: 'IDN110/AAcvRose'							
Mean	398	66	26	11	182	127	38
min	245	35	6	5	16	80	29
max	480	88	44	16	282	160	52
CV(%)	17	20	39	23	41	16	13
Male parent: 'Pisang Klutuk'							
Mean	417	78	32	10	168	126	42
min	334	57	17	7	106	105	32
max	480	100	57	15	282	145	47
CV (%)	13	18	43	25	38	13	13
Male parent: *Musa balbisiana* 'CMR'							
Mean	490	86	29	12	187	112	43
min	273	48	6	6	61	90	34
max	640	100	44	17	341	140	60
CV (%)	17	16	31	26	36	13	16

HT: plant height (cm) – PG: pseudostem girth at height 100 cm (cm) – BW: Bunch weight (kg) – NH: hands – NF: number of fruits of the bunch – FL: medium external fruit on third hand length (mm) – FD: medium external fruit on third hand diameter (mm)

Figures

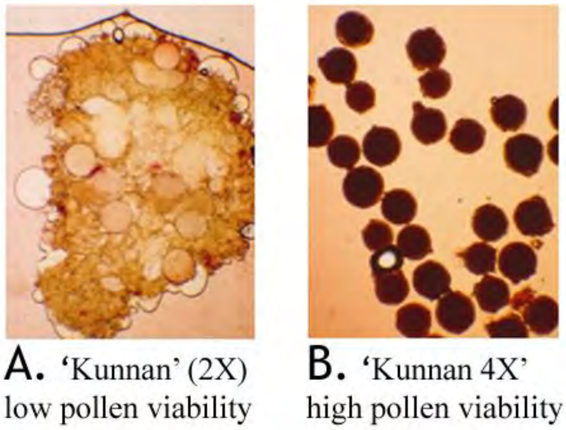

Fig. 1. Comparison of pollen viability in 'Kunnan' (2X) and 'Kunnan 4X' (4X). Scales: Fig 1A. 1cm/280µ - Fig 1B. 1cm/330µ

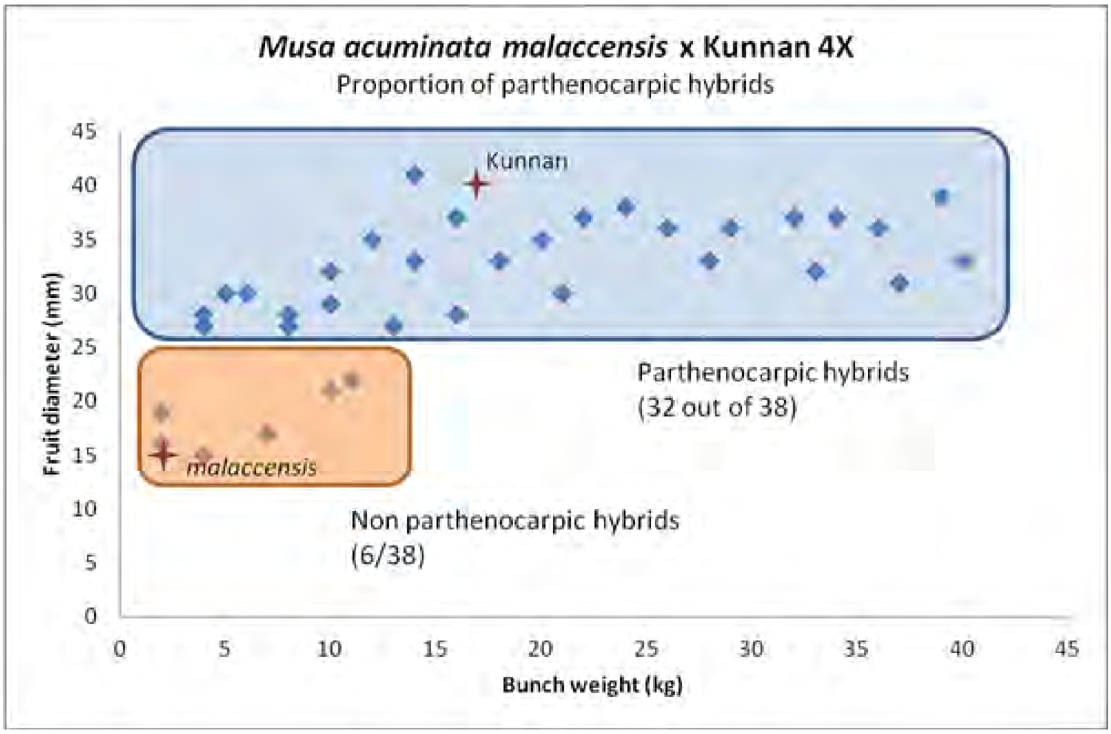

Fig. 2. Distribution of bunch weight and fruit diameter in the progeny from *Musa acuminata* ssp. *malaccensis* x 'Kunnan 4X'. Non-parthenocarpic hybrids have a fruit diameter < 25mm at harvest time. Parents are represented by the red stars.

A. AAB hybrid
'Malaccensis' x 'Kunnan 4X'

B. ABB hybrid
'Kunnan 4X' x 'CMR'

Fig. 3. Bunches from hybrids obtained through *Musa acuminata* x *balbisiana* interspecific crosses.