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In vitro germination and viability of pollen grains of banana diploids

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ABSTRACT - *The objective of this study was to evaluate the influence of pH on in vitro germination and pollen grain viability of banana diploids (AA) generated by the breeding program of Embrapa Mandioca e Fruticultura Tropical. The pollen grains were inoculated in culture medium containing 15% sucrose, 0.01% H_3BO_3, 0.01% KNO_3, 0.03% $Ca(NO_3)_2.4H_2O$, 0.02% $MgSO_4.7H_2O$, solidified with 0.8% agar and pH adjusted to 5.8 or 7.0. Pollen viability was evaluated by staining with 1% acetic carmine. The germination percentages of the genotypes 9187-01 (90.0%) and M-53 (89.7%) in pH 7.0 medium were highest, while the pollen tube length of genotype 9187-01 was approximately half the size (1.79mm) of genotype M-53 (3.84mm). The pollen viability of the genotypes evaluated was higher than 85%, even for the diploids with a low in vitro germination percentage.*

Key-words: *Musa acuminata*, diploids, genetic breeding, pollen tube, culture medium.

INTRODUCTION

Banana (*Musa* spp.) produces one of the worldwide most consumed fruits and is exploited in most tropical countries. It is mostly grown by small farmers and plays a considerable socioeconomic role in many countries, in view of the importance as foodstuff and source of foreign currency on the local and international market.

In a genetic breeding program, knowledge on pollen viability is one of the essential factors, particularly when one intends to use the technique of artificial hybridization, since the relation pollen - stigma depends on the grain viability, stigma receptivity and genetic interactions between pollen and stigma. In banana, studies on pollen grain *in vitro* germination can identify male gametes with high viability for hybridization programs (Krishnakumar et al. 1992).

Besides determining pollen fertility, *in vitro* germination is being used in cytogenetic, physiological and biochemical studies involving many plant species, in view of the speed and ease of the test. The understanding of the viability and capacity of pollen germination, aside from the pollen tube growth are fundamental for studies of reproductive biology and genetic breeding of some fruit plants, indicating the direction and underlying safe controlled hybridizations to generate new hybrids and/or increase the viability (Marcellán and Camadro 1996, Dane et al. 2004, Salles et al. 2006).

A low germination percentage and slow growth of the pollen tube can affect the plant seed formation.

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Buyukkartal (2003) stated that studies on the *in vitro* pollen germination and pollen tube growth are indispensable for fertilization and seed formation in the flowering plants and are, moreover, highly useful for explaining the absence of plant fertility.

Different culture media for the *in vitro* germination of pollen grains have been reported for a large number of species, with considerable variations within and among species (Pfahler et al. 1997). In most studies carbohydrates and germination-stimulating substances (micronutrients and hormones) are presented as the main components of the culture medium. Many organic and inorganic substances such as sucrose, boric acid (H_3BO_3), calcium nitrate [Ca (NO_3) 2.H_2O], potassium nitrate (KNO_3) and magnesium sulfate ($MgSO_4$.7H2O) exert an effect on *in vitro* pollen germination (Parton et al. 2002, Kopp et al. 2002, Moutinho et al. 2001, Bhattacharya and Mandal 2000, Silva et al. 1999, Galletta 1983). Furthermore, the factor pH should be taken into consideration, which also influences pollen germination (Pio et al. 2004).

To obtain reliable estimates of pollen viability, a culture medium must be available that makes the expression of its physiological capacity for pollen tube formation possible. Generally, fruit plants require only water and sucrose as source of pollen germination, whereas some species need a more complete medium, as in the case of the banana. The pollen grain of banana, even at an adequate physiological stage, requires a combination of substances for germination and pollen tube growth.

The maintenance of the osmotic equilibrium between the culture medium and grain content determines the cell integrity. Silva et al. (1999) stated that this equilibrium can be determined by the relation between the concentration of sucrose and of substances such as boric acid and calcium nitrate; an excess or deficiency of any of these components could result in the breaking of the pollen grains

In laboratory conditions, the viability can be quantified in media that favor the development of the pollen tube or by using dyes such as acetic carmine, propionic carmine, anilin blue, Alexander's stain, IKI (iodine + potassium iodide), FDA (fluorescein diacetate), NBT (p-nitroblue tetrazolium) and TTC (2,2,5 - triphenyltetrazolium chloride) (Bolat and Pirlak 1999, Wang et al. 2004).

Dye tests are advantageous as indicators of pollen viability, for being quicker and easier than trials with *in vitro* pollen germination. However, different dye types may produce different results. On the other hand, the real quantity of viable pollen is only determined by the *in vitro* germination test (Bolat and Pirlak 1999), provided that the cultivation conditions are to permit an adequate expression of the physiological capacity of pollen tube formation.

Diploids of the genome group AA (*Musa acuminata*) are used in banana breeding for crosses with triploids (cultivars AAB) to generate AAAB tetraploids with satisfactory levels of fruit quality, yield and disease resistance (Silva et al. 2001). However, there is little information available on the pollen viability of this plant material.

The objective of this study was to test the effect of different pH levels on pollen grain germination *in vitro* and on pollen tube growth as well as determine the pollen viability of banana diploids (AA).

MATERIAL AND METHODS

In vitro germination of pollen grains

For the *in vitro* pollen germination, flowers of diploid hybrids (listed in Table 1) in anthesis were collected at 8:00 o'clock in the morning, from March to October 2005. The pollen grains of 10 anthers/genotype were removed with a scalpel and, without any process of disinfection, inoculated on Petri dishes (diameter 9 cm) containing 40 mL of culture medium, using a brush for a homogenous distribution of the material. The dishes were subdivided in quadrants, each one representing a replication with approximately 300-350 pollen grains, totaling 12 replications for each pH level (5.8 and 7.0).

Based on preliminary results of studies with *in vitro* germination of banana pollen, developed at the tissue culture laboratory of Embrapa Mandioca e Fruticultura Tropical, we decided to use only the culture medium that had obtained the best result in the pollen germination. It contained 15% sucrose, 0.01% boric acid, 0.01% potassium nitrate, 0.03% calcium nitrate and 0.02% magnesium sulphate and was solidified with 0.8% agar, pH adjusted to 5.8 and 7.0 and autoclaved at 121 ºC for 20 minutes.

After inoculation in the culture medium, the dishes were maintained at controlled temperature conditions (27±1 ºC) for 24 and 48 hours in the dark, before

Table 1. Pollen germination, pollen tube length and pollen grain viability of different genotypes of diploid banana (AA)

Genotypes	Pollen germination (%)		Pollen tube length (mm)		Viability %)
	pH 5.8	pH 7.0	pH 5.8	pH 7.0	
9187-01	81.75 ± 4.33Ba	90.00 ± 6.37Aa	1.91 ± 0.60Ac	1.79 ± 0.40Af	97.66a
M-53	80.08 ± 6.22Bb	89.66 ± 4.00Aa	3.19 ± 1.31Ba	3.84 ± 1.45Ab	98.33a
8987-01	42.03 ± 10.0Ac	33.33 ± 7.82Bb	3.09 ± 1.08Aa	2.19 ± 1.16Bd	87.33b
4154-08	21.00 ± 5.88Bd	30.08 ± 8.29Ab	3.19 ± 1.52Ba	4.25 ± 3.77Aa	93.00a
4285-02	13.94 ± 4.83Be	15.39 ± 5.59Ac	2.65 ± 1.03Ab	2.65 ± 1.07Ac	94.66a
7341-03	13.87 ± 2.59Ae	17.30 ± 3.26Ac	0.85 ± 1.38Ae	0.79 ± 0.94Ag	95.66a
87A79-01	10.75 ± 5.24Ae	12.07 ± 3.07Ad	2.16 ± 0.62Bc	2.47 ± 1.09Ac	96.66a
5854-03	9.55 ± 4.46Af	9.28 ± 3.75Ae	2.07 ± 0.87Ac	1.91 ± 1.00Ae	90.00b
SH3263	8.90 ± 2.66Af	12.70 ± 3.97Ad	1.12 ± 1.07Bd	1.52 ± 1.13Af	93.33a
5012-02	8.26 ± 2.55Af	8.17 ± 3.71Ae	2.14 ± 0.91Ac	1.90 ± 1.03Ae	92.33a
4279-06	7.63 ± 3.32Af	8.82 ± 2.88Ae	1.24 ± 1.18Ad	0.82 ± 1.03Bg	88.33b
9187-02	7.10 ± 2.19Bf	12.47 ± 3.28Ad	1.02 ± 0.83Bd	2.30 ± 0.92Ad	86.33b
86B79-12	6.15 ± 1.76Af	8.31 ± 4.80Ae	2.13 ± 1.07Ac	2.02 ± 0.99Ae	96.33a
86B79-10	4.62 ± 1.52Ag	8.67 ± 3.70Ae	0.57 ± 0.75Ae	0.74 ± 0.84Ag	93.00a
7341-01	3.82 ± 1.82Ag	7.96 ± 4.79Ae	0.76 ± 0.95Be	1.55 ± 0.97Af	92.33a
TH03-01	3.28 ± 2.39Ag	2.98 ± 1.12Af	1.36 ± 1.27Ad	0.67 ± 0.89Bg	88.00b
8694-15	3.26 ± 1.69Bg	11.53 ± 4.11Ad	0.55 ± 0.69Be	1.68 ± 0.84Af	92.00a
2803-01	3.00 ± 1.02Ag	4.46 ± 1.17Af	1.19 ± 0.66Bd	1.74 ± 0.86Af	93.33a
9179-03	2.33 ± 0.86Ag	6.69 ± 1.38Af	1.40 ± 0.79Bd	1.72 ± 0.84Af	97.00a
4279-13	1.81 ± 0.74Ag	3.87 ± 2.98Af	0.64 ± 0.80Ae	0.74 ± 0.81Ag	95.33a
Mean	16.4	19.59	1.62	1.81	93.05
C. V (%)	29.99		66.98		3.35

Means followed by the same capital letters in a row and small case letters in the column did not differ statistically from each other at a level of 5% probability by the test of Scott-Knott and the F test, respectively

counting the germinated pollen grains and measuring the pollen tube length, respectively, under a stereomicroscope (magnification 6x). The pollen tube length was measured with a micrometric slide and the data were transformed in millimeters, totaling 40 pollen tubes for each Petri dish.

The experimental design was completely randomized in 20 x 2 factorial scheme including 20 genotypes and two pH levels (5.8 and 7.0), with 12 replications. The data of *in vitro* pollen germination percentage were previously transformed to arcsen $(\sqrt{x/100})$ and processed by analysis of variance. The treatment means were evaluated, by the Scott-Knott grouping test, at a level of 5% probability, using software SAS (2000).

All grains of the dish were counted to evaluate the germination percentage *in vitro*. The experimental unit considered for the parameter pollen tube length was as follows: of the counted pollen tubes, 40 tubes from each one of three Petri dishes were randomly chosen for measurement (a total of 120 pollen tubes for each pH level and genotype under study). The pollen grains were considered germinated when the pollen tube length was equal to or longer than the diameter of the pollen grain itself.

Pollen viability

To evaluate pollen grain viability, the male inflorescence was protected with a polyethylene bag on the day before sampling, to avoid any contamination with pollen introduced by insects. On the next morning, a bract with the flowers in anthesis for each genotype was picked on the field. The pollen grains were excised from the anthers of the diploids tested *in vitro* and were stained on a glass slide with a drop of 1% carmine, covered with a coverslip, and observed under an optical microscope (10x lens).

The experimental design was completely randomized with three replications. One experimental unit consisted of one anther/genotype. To determine the pollen viability, three anthers were analyzed per genotype and 100 pollen grains/slide were counted. The percentage of pollen fertility was estimated based on

the proportion of the number of stained pollen grains (viable) by the unstained or with retracted cytoplasm (non-viable).

The values expressing the number of viable pollen grains were transformed to arcsen $(\sqrt{x/100})$ prior to statistical analysis. The means were tested by the clustering method of Scott-Knott at 5% probability, using software SAS (2000).

RESULTS AND DISCUSSION

Among the improved diploids, different groups were formed by the Scott-Knott test (at 5% probability), in a comparison of the response of the genotypes evaluated at the pH levels tested for germination percentage as well as pollen tube length; there was also an interaction between these two factors.

There were significant differences in the response to the pH level. In the culture medium adjusted to pH 7.0, the mean pollen germination was higher (19.6%), as well as the pollen tube length greater (1.81 mm) than at pH 5.8 (16.4% and 1.62 mm, respectively) (Table 1). The germination percentage of diploid 9187-01 (90.0%) was highest when pollen was cultured in pH 7.0 medium (Figure 1a), although there was no statistical difference to diploid M-53 (89.7%). However, in terms of pollen tube length, genotype 9187-01 was approximately half the size (1.79 mm) of M-53 (3.84 mm). Genotype 8987-01 was the only one with a better germination at pH 5.8 than at pH 7.0, yet the values were almost half of those of the diploids 9187-01 and M-53 while pollen tube growth at pH 7.0 was intermediate (Figure 1b). The lowest germination percentage as well as the shortest pollen tube length were observed in genotype 4279-13 (1.81% and 0.64 mm, respectively) at pH 5.8. Despite the evaluations had been performed 24 hours after inoculation in the culture medium, the pollen grains of some genotypes began to germinate one hour after inoculation in the culture medium, growing pollen tubes to a length of approximately four times the diameter of the pollen grain of banana (0.3 mm).

There are a number of factors that influence *in vitro* pollen germination. One can cite the botanic species, nutritional state of the plants, the culture medium, temperature and incubation period, the sampling time, flower development stage at pollen sampling, photoperiod, sampling method, application of pesticides and insecticides to the plants, aside from the pollen storage conditions (Stanley and Linskens 1974, Neves et al. 1997).

Burke et al. (2004) tested the effect of pH in the range of 6-8 in steps of 0.5 on *in vitro* germination of cotton pollen, as well as the elongation of the pollen tube and stated no differences between the tested pH values. On the other hand, Salles et al. (2006) verified differences between the pH levels in the range from 3.5 to 6.5 in three citrus varieties, as well as an interaction between varieties and pH levels, influencing *in vitro* pollen germination. The importance of the determination of the ideal pH in the physiological processes that involve pollen grains is linked to the resulting higher germination percentage, which increases the chances of fertilization (Salles et al. 2006), aside from influencing the nutrient availability, plant regulators and the degree of agar solidification (Pasqual et al. 2002).

Studies have indicated differences in the *in vitro* pollen grain germination as a result of the complex interaction between morphology and physiology of the pollen grain and components of the medium (Gwata et al. 2003). Kakani et al. (2005) claimed that the differences observed in the germination *in vitro* and pollen tube growth of 12 cotton cultivars (*Gossypium hirsutum*) were a reflex of the variability in the cultivars. Similarly, Frazon et al. (2005) stated that there are differences between species and between cultivars within the species in terms of the conditions required of a medium for *in vitro* pollen germination. The variation in the growth rate of the pollen tube in angiosperms is extremely high (Baker and Baker 1979). Still, there is little information available on pollen tube growth in banana. Here, of pollen tube grew, independently of the pH, to a mean length of 0.65 - 3.72 mm, after 48 hours incubation. The pollen grains of many species, when grown in adequate culture medium at satisfactory sucrose concentrations, are re-hydrated and are then able to germinate during a period of 2-24 hours, at an elongation rate of less than 1 μm s^{-1}, and stop growing thereafter (Stone et al. 2004).

In Angiosperms, the energy required for pollen grain germination and formation of the cell wall components and callose is supplied by the nutrient reserves of the pollen grain itself (Baker and Baker 1979). These reserves are important for the regulation of the sucrose concentration used for *in vitro* germination. The sugar in the culture medium ought to provide an osmotic balance between the pollen and the germination

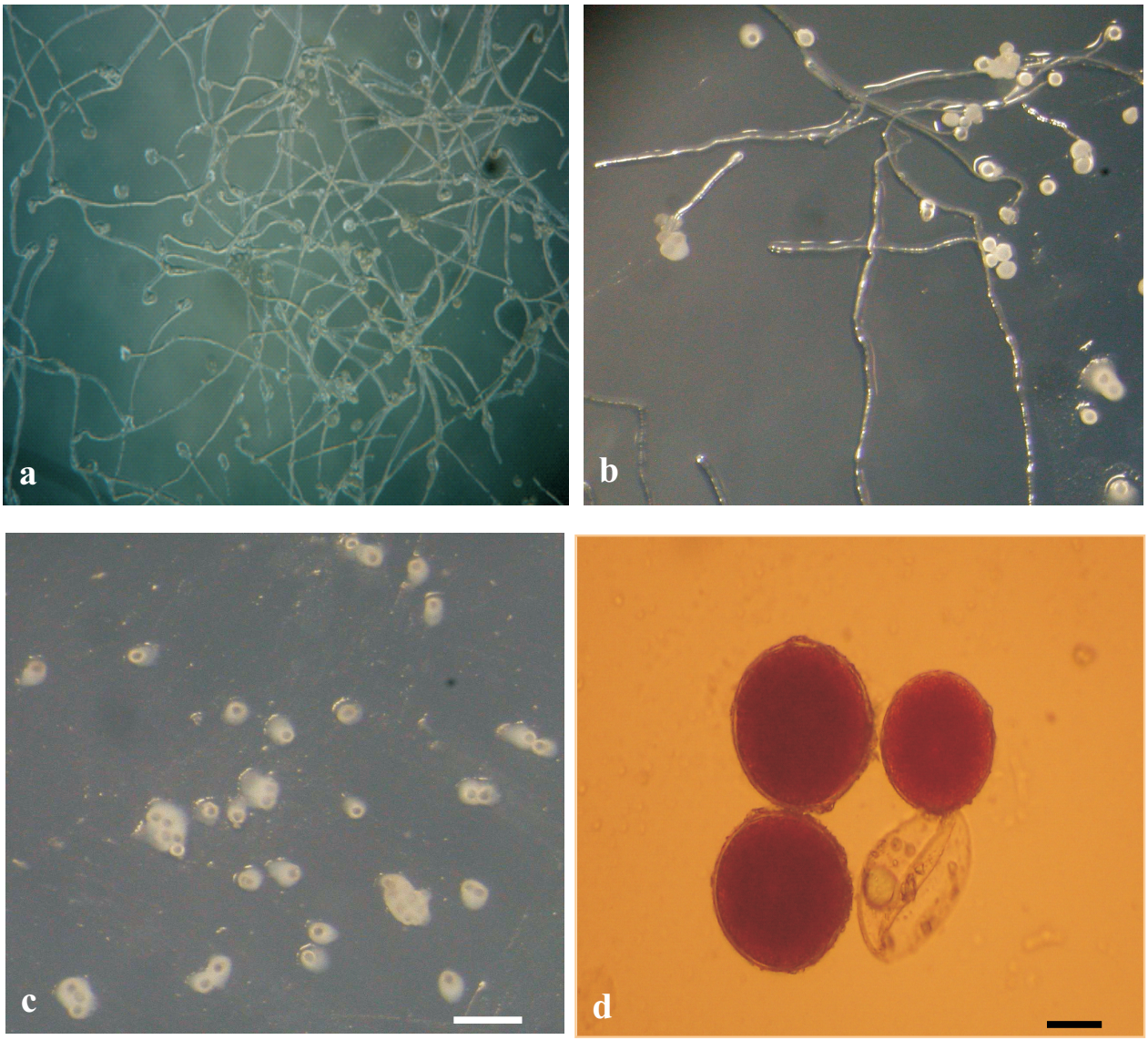

Figure 1. *In vitro* pollen grain germination of diploid banana hybrids, in culture medium with pH 7.0 (a-c). a) 9187-01, high germination percentage and long pollen tube; b) 8987-01, pollen tube growth reduced in comparison with 9187-01; c) 2801-03, breaking of pollen grains. Bar = 1.2 mm; d) Staining with acetic carmine of viable pollen grains. Non- viable pollen grains were not stained (arrow). Bar = 0.9 mm

solution and supply energy in support of the process of pollen tube development (Nunes et al. 2001, Bhattacharya and Mandal 2000).

Aside from the carbohydrate source, bore and calcium play an important role in the pollen germination and pollen tube growth. An absence of calcium or bore normally affects pollen germination in a number of plant species (Baéz et al. 2002). Boric acid is the stimulating agent for pollen germination and pollen tube elongation, since it is involved with the translocation and metabolism of sucrose. Bore, derived from the stigma and style, plays a role in pectin production in the pollen tube (Gibernau et al. 2003). The contribution of boric acid is still unclear, although it is known that this substance is able to form a complex with sugar that improves the translocation of the molecules of this substance. Besides, this ionizable complex reacts faster with the cell membranes (Franzon et al. 2005, Bhattacharya and Mandal 2000).

Calcium added to the culture medium for pollen germination influences the physiological traits: pollen

tube and pollen grain are less sensitive to variations of the basic medium, have a lower permeability, linear growth and rigid appearance of the pollen tube (Bhojwani and Bhatnagar 1974). In the absence of calcium the permeability of the pollen tube membrane is greater, causing the release of internal metabolites to the external medium (Stanley and Linskens 1974). Besides, calcium is involved with pectin synthesis and the control of the osmotic conditions (Gibernau et al. 2003).

Reports that a medium containing only pure water and sucrose provided good pollen germination are rather rare. Studies by Souza-Lang and Pinto Júnior (1997), with different sugars in the pollen germination of araucaria (*Araucaria angustifolia*) showed that the highest germination percentages were observed in media without any sugar. Probably, the concentrations used may have had no effect, owing to the osmotic imbalance between the pollen and the culture medium. On the other hand, Sahar and Spiegel-Roy (1984), avocado pollen did not germinate *in vitro* in culture medium containing only sucrose and agar. According to the authors, for germination, the medium should contain some nutrients (e.g., calcium, magnesium sulphate, potassium nitrate and boric acid). Similarly, pollen grains of *Sorghum bicolor* (L.) did not germinate when cultured with sucrose only and in the few that did germinate, the pollen tube development was reduced (Tuinstra and Wedel 2000). In general, the medium used for pollen germination varied according to the plant species (Dane et al. 2004).

The frequent opening of the pollen grains and tube is the greatest difficulty in studies with pollen culture (Baloch et al. 2001). There are several reports on the breaking of pollen grains *in vitro*, cultured in medium containing only water or low sucrose concentrations and on high germination percentage in sucrose concentration varying from 20-30%. For the pollen germination of *Agrostis stolonifera* L. in medium with sucrose at 0.025, 0.05 and 0.1%, Fei and Nelson (2003) observed no breaking of the pollen grain in any of the treatments. In our study, breaking was observed in pollen grains of the genotypes 2803-01, 9179-03, 4279-13 and TH03-01 (Figure 1c). Pollen grains break up, among other factors, due to high moisture and variations in the medium, caused by an increase of the osmotic pressure and a low resistance of the cell wall, according to Pio et al. (2002).

The analysis of variance showed a significant difference between the two groups of genotypes ($P<0.05$) by the test of Scott-Knott (level of 5% probability). The pollen grains of the different genotypes were collected from inflorescences at the same physiological stage. Staining with carmine proved efficient to differentiate viable from non-viable pollen grains (Figure 1d). In 15 of the 20 diploid under study, the viability was over 90% (Table 1). In this group, the percentage of viable pollen was highest in the genotypes M-53 and 9187-01 (98.3 and 97.7%, respectively), although statistically there was no difference. In the group of the five others, the viability varied from 86.3 to 90%. For Adhikari and Campbell (1998) pollen viability is strongly influenced by temperature, moisture, genotypic differences, plant vigor and physiological stage, and flower age. Here, the different responses of pollen viability can be ascribed to the genotype, since temperature and moisture conditions were controlled and the male inflorescences were sampled at the same physiological stage.

Data on the viability and development of pollen grains are fundamental for studies of reproductive biology and genetic breeding of banana, ensuring safer crosses, performed to generate new hybrids and/ or increase the fertility.

The pollen grain viability of the different tested genotypes did not correspond to the *in vitro* germination percentage, indicating that cultivation conditions were not adequate for all tested genotypes, which calls for adjustments of the culture medium.

An optimization of the medium of *in vitro* germination by new tests involving different sugar sources and pH levels could be applied to investigate if these factors exert a stimulating effect on the germination process, with a view to identify the best male parents for the use in conventional breeding as well as in the process of *in vitro* fertilization of banana.

The relation between *in vitro* germination and in vivo fertilization capacity is little documented since there are plants that do not germinate *in vitro,* but are able to fertilize *in vivo.* Therefore, studies on *in vitro* and *in vivo* germination are necessary to work out adequate breeding strategies.

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Germinação *in vitro* e viabilidade de grãos de pólen em bananeiras diplóides

RESUMO - *O objetivo deste trabalho foi avaliar a influência do pH na germinação* in vitro *e a viabilidade de grãos de pólen de bananeiras diplóides (AA), geradas pelo programa de melhoramento da* Embrapa Mandioca e Fruticultura Tropical. *Os grãos de pólen foram inoculados em meio de cultura containing 15% de sucrose, 0,01% H_3BO_3, 0,01% de KNO_3, 0,03% $Ca(NO_3)_2.4H_2O$, 0,02% $MgSO_4.7H_2O$, solidificado com 0,8% de ágar, e pH ajustado para 5,8 ou 7,0. A viabilidade do pólen foi avaliada pela coloração com carmin acético a 1%. As mais altas percentagens de germinação foram obtidas nos genótipos 9187-01 (90,0%) e M-53 (89,7%), em meio com pH 7,0. Entretanto, o comprimento do tubo polínico do genótipo 9187-01 foi aproximadamente duas vezes menor (1,79 mm), quando comparado ao M-53 (3,84 mm). A viabilidade nos genótipos avaliados foi superior a 85%, mesmo para os diplóides que apresentaram baixa percentagem de germinação* in vitro.

Palavras-chave: *Musa acuminata***,** diplóides, melhoramento genético, tubo polínico, meio de cultura.

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