

Viability of pollen grains of tetraploid banana

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ABSTRACT: Obtaining banana tetraploid cultivars from triploid strains results in total or partial reestablishment of fertility, allowing the occurrence of some fruits with seeds, a feature that is undesirable from a marketing perspective. The objective of this study was to assess the viability of pollen of 12 banana tetraploid hybrids (AAAB) by means of *in vitro* germination and two histochemical tests (acetocarmine and 2,3,5-triphenyltetrazolium chloride). The pollen tube growth was evaluated by germinating grains in three culture media — M1: 0.03% Ca(NO₃)·4H₂O, 0.02% Mg(SO₄)·7H₂O, 0.01% KNO₃, 0.01% H₃BO₃ and 15% sucrose; M2: 0.03% Ca(NO₃)·4H₂O, 0.01% KNO₃, 0.01% H₃BO₃ and 10% sucrose; and M3: 0.015% H₃BO₃, 0.045% Ca₃(PO₄)₂ and 25% sucrose. The acetocarmine staining indicated high viability (above 80%), except

for the genotypes YB42-17 and Caprichosa, which were 76 and 70%, respectively. However, the *in vitro* germination rate was lower than 50% for all the genotypes, except for the hybrids YB42-17 (M1) and YB42-47 (M1). The medium M1 provided the greatest germination percentage and pollen tube growth. Among the genotypes assessed, YB42-47 presented the highest germination rate (61.5%) and tube length (5.0 mm). On the other hand, the Vitória cultivar had the lowest germination percentage (8.2%) in medium M1. Studies of meiosis can shed more light on the differences observed in the evaluated tetraploids, since meiotic irregularities can affect pollen viability.

Key words: *Musa* L., *in vitro* pollen germination, histochemical analysis, plant breeding.

INTRODUCTION

In programs to develop banana tree hybrids, it is necessary to cross different genotypes to test their combinatory capacity. Among the most important factors for the success of these programs are the selection of genotypes and the crosses to be carried out. The efficacy of the crosses, both between varieties and cultivars of the same species and between different species, depends directly on the pollen grains viability.

In the conventional banana genetic improvement strategy used by the Embrapa Mandioca e Fruticultura, most of the desirable traits are concentrated in the diploid germplasm (AA), such as parthenocarpia, good number of hands, long fingers (fruits), well-formed bunches, low plant height and resistance to pests and diseases (Silva et al. 2001).

In general, the literature shows that wild and improved diploids of banana plants produce abundant pollen with high viability, while cultivated varieties show low pollen production as well as occasional viability problems (Fortescue

and Turner 2004). Therefore, a better understanding of the viability of the pollen produced by hybrid tetraploid banana plants is important to identify male genitors with high fertility potential to be used in hybridization programs, in order to increase the range of possible crosses that present some sexual incompatibility, aiming to obtain new cultivars.

Determination of pollen viability can be done using direct methods, such as *in vitro* (Peng et al. 2015) and *in vivo* germination (Soares et al. 2014; Souza et al. 2015), or through indirect methods based on cytological parameters, such as coloration in response to staining (Melloni et al. 2013; Souza et al. 2015). Although the histochemical test is quick, easy and inexpensive, it should not be the only method used to estimate pollen viability, since it does not supply information on the germinative capacity. This information can be obtained by *in vitro* and *in vivo* germination tests.

In vitro germination in culture medium is a technique that simulates the conditions of the style-stigma, inducing germination and growth of the pollen tube (Soares et al. 2008). →

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This information is in accordance with Souza et al. (2015), which found for *Aechmea bicolor* (Bromeliaceae) that high *in vitro* pollen grains germination rates were related to the highest percentages of fruits and number of seeds obtained in *in vivo* conditions. Each species requires a specific protocol to obtain a high germination rate. The success of *in vitro* germination depends on several factors, such as species, nutritional state, season of the year, time of collection, photoperiod, temperature, collection method, incubation period and culture medium, besides adjustments in the composition of the medium for each species (Stanley and Linskens 1974; Sharafi 2010; Moura et al. 2015).

The majority of articles in the literature covering *in vitro* germination of pollen indicate the use of carbohydrates and stimulant substances (micronutrients and hormones) as the main components of the medium. Many organic and inorganic substances, such as sucrose, boric acid, calcium nitrate, potassium nitrate and magnesium sulfate, have an effect on *in vitro* germination of pollen (Parton et al. 2002; Soares et al. 2008; Sharafi 2010). However, for banana plants, it is not always possible to obtain maximum germination of mature pollen in media with adequate concentrations of sugar or completed with the stimulants mentioned above.

Although several studies have been published on the *in vitro* germination of banana pollen, specially of diploid species, little information is available on the viability and *in vitro* germination of pollen from commercial banana plant cultivars, such as those which are tetraploids.

Therefore, the aim of this study was to evaluate the viability of the pollen from 12 banana tetraploid hybrids by means of *in vitro* germination and two histochemical tests (staining with acetocarmine and with 2,3,5-triphenyltetrazolium chloride — TTC), as well as to examine the capacity to emit the pollen tube, for the selection of male genitors in hybridization programs.

MATERIAL AND METHODS

We used pollen from male flowers collected during anthesis in the morning, from 12 banana tetraploid hybrids (AAAB), from the germplasm bank of Embrapa Mandioca e Fruticultura, as listed in Table 1. We conducted two experiments to compare the viability of pollen: one involving *in vitro* germination in three culture media and the other applying histochemical analyses by the use of two specific stains.

In vitro germination and pollen tube length

Pollen grains, without any disinfestation process, were inoculated into Petri dishes (\emptyset 9 cm) containing 35 mL of each of the following three culture media:

- Medium M1: 0.03% $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.02% $\text{Mg}(\text{SO}_4)_2 \cdot 7\text{H}_2\text{O}$, 0.01% KNO_3 , 0.01% H_3BO_3 and 15% sucrose, solidified with 0.8% agar and pH adjusted to 7.0;
- Medium M2: 0.03% $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.01% KNO_3 , 0.01% H_3BO_3 and 10% sucrose, solidified with 1% agar and with pH adjusted to 6.5;
- Medium M3: 0.015% H_3BO_3 , 0.045% $\text{Ca}_3(\text{PO}_4)_2$ and 25% sucrose, solidified with 0.6% agar and with pH adjusted to 6.5.

With the aid of tweezers, pollen grains were distributed in the Petri dishes as homogeneously as possible. After inoculation, the dishes were kept under controlled temperature conditions of 27 ± 1 °C in the dark for 24 and 48 h; afterwards the germinated grains were counted and the tubes were measured under a stereomicroscope. Pollen was considered germinated when the tube length was greater than or equal to the grain diameter.

For determination of the germination percentage, the experiment was completely randomized in a 12×3 factorial scheme (genotype \times culture medium) with eight repetitions each. In order to determine the tube length, 40 germinated grains of each genotype were selected at random. The length was measured in micrometers under the same stereomicroscope, using a micrometric slide, and data were transformed into millimeters.

Histochemical analysis

Pollen viability was ascertained by staining with two different stains: 2% acetocarmine and 1% TTC; in both cases, stains were diluted in Tris buffer (HCl 0.15 M, pH 7.8) to investigate the most efficient stain for estimation of banana pollen viability.

Pollen was collected from five anthers per genotype and distributed on a glass slide. Then a droplet of the stain was added and a cover slip was mounted to close the set. The observations of the quantities of viable and unviable grains of each genotype were carried out just after preparation of the slides in the case of acetocarmine, while this was done two hours later for the slides stained with TTC, because this substance requires an interval for the enzymatic reactions to occur.

The scanning method was used to obtain a random sample of stained pollen, with examination of 100 grains/slide/genotype →

and three replications each, for a total of 300 grains for each stain. The pollen grains were observed with an optical microscope.

The experimental design was completely randomized in a 12×2 factorial scheme (genotype \times stain), with three replications each.

Pollen grains stained with acetocarmine take on a reddish hue when viable, and the others are considered unviable. In turn, the pollen stained with TTC appears light pink when viable and remains transparent when unviable.

Data analysis

The data on germination percentage and viability were submitted to angular transformation into $\arcsin(\sqrt{x/100})$ before the statistical analysis. For comparison of the means, the data were submitted to analysis of variance, and the Scott-Knott grouping test was also applied to establish groups; in both cases, a 5% probability was considered, using the SAS Institute program (2004).

RESULTS AND DISCUSSION

In vitro germination and pollen tube length

In the *in vitro* germination tests, there were significant differences in the response of the tetraploid hybrids to the culture medium, both for germination percentage and pollen

tube length, as well as for the interaction of these two factors (Table 1, Figure 1).

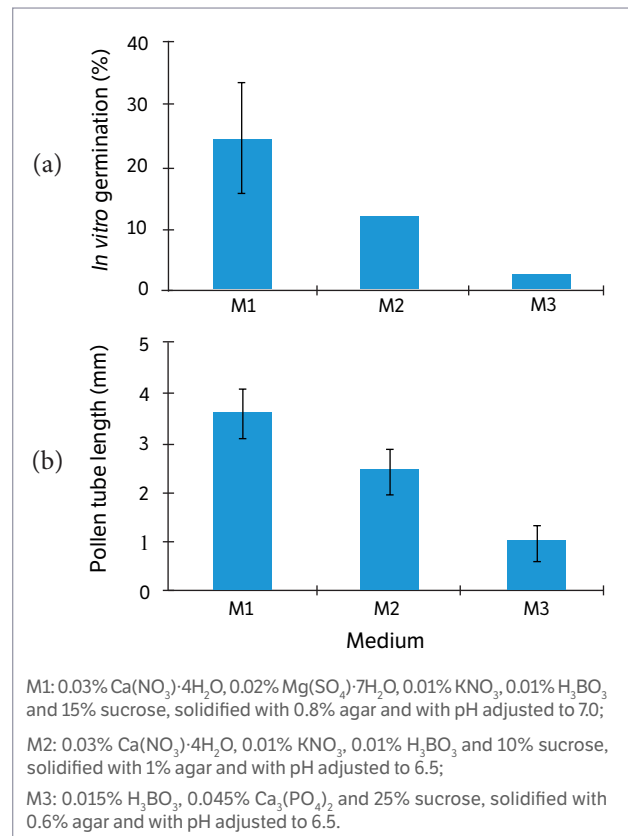


Figure 1. (a) *In vitro* germination percentage and (b) pollen tube length of tetraploid (AAAB) banana (*Musa* spp.) pollen in three culture media.

Table 1. Percentage of *in vitro* germination of tetraploid (AAAB) banana (*Musa* spp.) pollen cultivated in three different media and histochemical test results with two stains.

Genotype	Germination (%)			Pollen tube length (mm)			Histochemical (%)	
	M1	M2	M3	M1	M2	M3	Carmine	TTC
Caprichosa	8.1 dA	3.5 dB	0.4 cC	3.2 cA	1.6 dB	1.2 cC	70.0 bA	35.7 cB
FHIA-21	31.8 cA	31.3 aA	0.1 cB	5.0 aA	4.0 aB	0.1 dC	90.0 aA	45.3 bB
Garantida	9.9 dA	7.4 cA	4.8 aB	3.8 bA	2.5 cB	0.8 bC	90.0 aA	35.7 cB
Japira	11.2 dA	3.4 dB	0.1 cC	2.1 dA	1.4 dB	1.6 aC	84.0 aA	31.0 cB
Preciosa	12.7 dA	5.3 dB	0.0 cC	1.9 dA	1.3 dA	0.0 dB	82.0 bA	45.7 bB
Vitória	8.2 dA	10.6 cA	2.2 bB	2.7 cA	1.8 dB	0.1 dC	88.0 aA	36.3 cB
Tropical	29.4 cA	4.2 dB	2.8 bB	4.2 bA	1.3 dB	0.1 dC	80.0 bA	48.0 bB
YB42-17	54.1 bA	9.2 cB	3.6 bC	3.8 bA	2.5 cB	1.9 aB	76.0 bA	59.0 aB
YB42-47	61.5 aA	25.5 aB	4.9 aC	5.0 aA	3.3 bB	1.6 aC	94.0 aA	62.6 aB
YB42-03	12.3 dA	17.3 bA	2.3 bB	4.2 bA	3.0 cB	1.0 bC	90.0 aA	36.3 cB
YB42-07	29.1 cA	12.4 cB	2.0 bC	4.1 bA	2.7 cB	1.8 aC	85.0 aA	47.7 bB
YB42-08	24.0 cA	9.9 cB	5.1 aC	3.1 cA	3.5 bA	1.5 aB	86.0 aA	41.0 cB

Means followed by the same lower-case letter in the column and upper-case letter in the row are part of the same group according to the Scott-Knott test ($p < 0.05$). M1: 0.03% $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.02% $\text{Mg}(\text{SO}_4)_7 \cdot 7\text{H}_2\text{O}$, 0.01% KNO_3 , 0.01% H_3BO_3 and 15% sucrose, solidified with 0.8% agar and with pH adjusted to 7.0; M2: 0.03% $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.01% KNO_3 , 0.01% H_3BO_3 and 10% sucrose, solidified with 1% agar and with pH adjusted to 6.5; M3: 0.015% H_3BO_3 , 0.045% $\text{Ca}_3(\text{PO}_4)_2$ and 25% sucrose, solidified with 0.6% agar and with pH adjusted to 6.5.

Genotype YB42-47 achieved the highest germination percentage (61.5%; Figure 2a), followed by YB42-17, with 54.1%, both in medium M1. In this same medium, the lowest germination rate was 8.1%, observed for the Caprichosa genotype (Figure 2b). With respect to tube length, the variation was from 5.00 mm (YB42-47 and FHIA-21) (Figure 2c) to 1.9 mm (Preciosa) in this same medium (Table 1). According to Scorza and Sherman (1996), good pollen should present 50 to 80% of the pollen germinated with well-developed

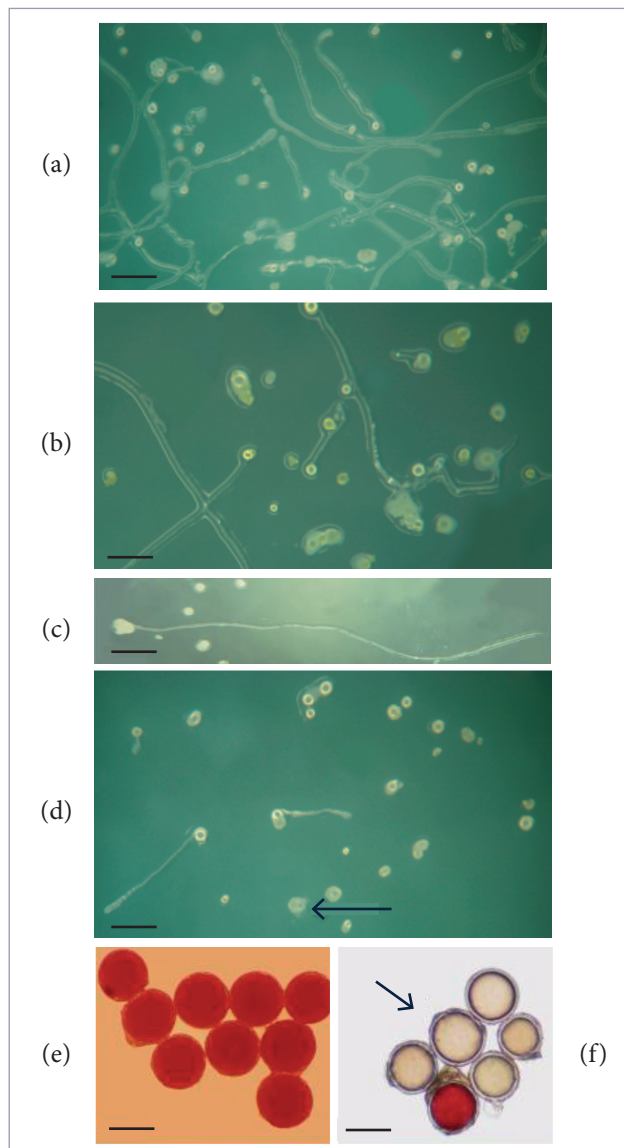


Figure 2. Micrographs of pollen of tetraploid banana (*Musa* spp.). (a) High germination pollen percentage of the YB42-47 genotype; (b) Low germination pollen percentage of Caprichosa; (c) Detail showing the tube length of YB42-47; (d) Pollen of the Vitória genotype with exine rupture in culture medium M3; (e) Pollen of the YB42-17 genotype stained with acetocarmine; (f) Pollen of the Caprichosa genotype stained with TTC: viable (red) and unviable (not stained – arrow). Bars: 0.9 mm.

tubes. By this criterion, genotype YB42-47, which showed good germination and tube development, can be used in improvement programs or be preserved in germplasm banks.

Overall, medium M1 provided the highest germination percentage (24.37%) and the longest average tube length (3.60 mm), probably due to its more complete chemical composition (Figure 1). In contrast, the lowest overall germination rate (2.35%) and average tube length (0.99 mm) occurred in medium M3, the poorest in nutrients which are essential for germination and tube development (Figure 1). Besides, exine rupture was observed in many pollen grains cultured in this medium (Figures 1, 2d - arrow). We believe this result was caused by the lower agar concentration in the medium compared to the other two media tested (M1 and M2).

Some authors have reported germination of pollen cultivated *in vitro* in just water or water with low sucrose concentrations. According to Loguercio (2002), pollen tubes rupture mainly due to osmotic pressure and low cell wall resistance, allowing a rapid influx of water, which, in turn, causes loss of soluble substances and ions in the cytoplasm, a situation known as imbibition damage. This inadequate rehydration causes disorganization and dehydration of the membrane and impairs the pollen's metabolism, reducing viability (Souza et al. 2015).

To prevent rupture of pollen, it is necessary to maintain osmotic balance between the culture medium and the contents of the pollen. This balance is determined by the ratios between the sucrose concentration and the concentrations of other substances in the medium, such as boric acid, calcium nitrate and agar. Therefore, an excess or deficiency of any of these components can lead to rupture of the pollen (Galletta 1983).

The purpose of including sucrose in all the culture media tested was to assure osmotic balance and to supply energy to promote development of the pollen tube (Stanley and Linskens 1974). From the results obtained, a sucrose level of 15% in the medium is sufficient to provide the necessary energy for good tube development.

According to some authors, the culture medium should include, besides carbohydrates, stimulating elements like boric acid, calcium nitrate, potassium nitrate and magnesium sulfate (Sharafi 2010).

According to Pfahler (1968), boron maximizes germination, with variable responses depending on the species. It works by interacting with sugar to form an ionizable sugar-boron

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complex, which reacts faster with the cell membranes. Sharafi (2010) found an increase in the germination percentage and tube length of the pollen of various fruits species grown in temperate climates with the addition of boron to the medium.

The calcium added to the culture medium affects physiological traits. The grain and tube become less sensitive to variations in the basic medium and have lower permeability. Additionally, in particular, the tube grows more uniformly with time (linear growth) and becomes more rigid (Bhojwani and Bhatnagar 1974). An important factor for successful *in vitro* germination is the consistency of the culture medium. Liquid media have the disadvantage of tending to cause detachment of the tube, impairing the evaluation, besides causing underestimation of gamete viability because pollen without tubes is counted as ungerminated. On the other hand, high agar concentrations can pose a physical osmotic barrier, preventing germination of the tube (Alvim et al. 2012).

For each treatment, we used pollen from five flowers in each Petri dish to reduce the heterogeneity of the data. In a previous study, Soares et al. (2008) found that the use of a sample of two to three flowers resulted in a very large variation of the *in vitro* germination data. According to them, the quantity of pollen varied greatly between genotypes, and they used only two flowers for genotypes that produced abundant pollen and three flowers otherwise.

A few authors have reported that the germination rate tends to increase with the pollen sample size, explaining the finding by the synergetic effect of the grains, i.e. mutual stimulation, so that the pollen grains germinate better when grouped in larger quantity (Stanley and Linskens 1974).

Specifically regarding banana plants, various articles have reported that the viability and total quantity of pollen vary among cultivars and that diploids produce more viable grains than tetraploids, which, in turn, produce more viable grains than triploids. According to Fortescue and Turner (2004), diploid species produce three times more viable grains than tetraploids, with rates of 88 *versus* 29%. According to them, tetraploids produce roughly three times more viable grains compared to AAA and ABB triploids and four times more in relation to AAB cultivars (respective percentages of 29, 9, 10 and 6%).

Our results regarding banana pollen viability confirm that diploid genotypes normally produce a higher percentage of fertile pollen than tetraploids. A factor explaining this finding is that the meiotic behavior of tetraploids tends to be more complex in relation to diploids. The tetraploid condition contributes to irregular segregation of the chromosomes

during meiosis, causing formation of unbalanced microspores and unviable gametes. However, genotype YB42-47, because it attained the greatest viability and tube length, can be considered a good choice for use as a male genitor in hybridization programs.

Histochemical analysis

In the histochemical analyses, different groups were formed by the Scott-Knott test at 5% probability in relation to the stains tested and the percentage of viable grains, as well as for the interaction of these two factors (Table 1).

Although there was a perceptible difference in the pollen viability between genotypes of the same group (AAAB), the greatest variations were found when comparing the two stains. With respect to the chromatin integrity with acetocarmine, ten of the tetraploids studied presented viability greater than 80%, with the only exceptions being the YB42-17 and Caprichosa genotypes, with respective rates of 76% and 70% (Figure 2e). In turn, the test with TTC indicated the presence of dehydrogenase enzymes with variation from 31 to 62.6% by means of the red coloration of pollen (Table 1, Figure 2f). The pollen grains that were not colored were considered inviable (Figure 2f - arrow).

According to Parfitt and Ganeshan (1989), staining with acetocarmine does not produce reliable results, because they subjected pollen to high temperatures to render them unviable, but the test with this stain still indicated a high level of viability.

The test with TTC produced results nearer the viability estimated by the *in vitro* germination test (Table 1). Various authors have argued that staining with TTC allows a more reliable estimate of pollen viability, nearer that provided by *in vitro* germination tests (Huang et al. 2004; Abdelgadir et al. 2012). Besides this, TTC is widely used because the procedure is relatively fast and simple, although some authors have expressed reservation about the fact that the method can produce ambiguous results, since aborted pollen can take on similar coloration to that of viable pollen (Heslop-Harrison and Heslop-Harrison 1970).

Staining tests, although they are simple and inexpensive, can produce results that overestimate the viability values (Galletta 1983). On the other hand, although *in vitro* germination involves the control of the experimental conditions, it does not fully reproduce the conditions of *in vivo* pollen tube growth, since interactions can occur between the substances in the culture

medium and different plant materials. This fact probably explains the higher viability values found by the staining method compared to those from *in vitro* germination (Table 1).

Nevertheless, it is possible to agree with Soares et al. (2008) by the fact that the *in vitro* germination method produces results nearer those that occur *in vivo*.

CONCLUSION

The culture medium containing 0.03% $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.02% $\text{Mg}(\text{SO}_4) \cdot 7\text{H}_2\text{O}$, 0.01% KNO_3 , 0.01% H_3BO_3 and 15% sucrose is the most recommended for analyzing the pollen germination and tube growth of tetraploid banana genotypes.

Genotypes YB42-47 and YB42-17 can be used as male parents in hybridization programs.

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