

## Short Communication

## Use of aniline blue stain to observing pollen tubes development in different *Manihot* Mill. species

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The lack of a reliable test to evaluate pollen viability in species of the genus *Manihot* is one of the primary limitations for cassava genetic improvement. Thus, the objective of the present study was to apply aniline blue stain to observing pollen tubes development in different *Manihot* Mill. species, in order to estimate the crossability, pollen germination time, pollen tube size and pollen longevity. Pollen grains from 2 accessions of the cultivated species *M. esculenta* spp. *esculenta* and 4 accessions of 3 wild species (*M. esculenta* ssp. *flabellifolia* (Pohl) Cif, *M. anomala* Pohl, and *M. irwinii* D. J. Roger and Appan) were used. To visualize the pollen tubes, pollinated stigmas was deposited on a glass slide with 3 drops of aniline blue stain and transferred to an optical fluorescence microscope, where they were visualized and photographed using the 10x ocular lens. The pollen crossability rate was estimated by the percentage of germinated pollen, with well-developed pollen tubes in relation to the total number of pollen grains counted per stigma. The experimental design was entirely random with 3 repetitions, each repetition consisting of 100 pollen grains. In addition to the estimation of crossability, the germination time after pollination, pollen tube size, and pollen longevity were also determined to the accession FLA 029V-01. Significant difference was observed between the crossings. Pollen tubes developed after 45 min of contact between the pollen and the stigma. The average pollen tube size 24 h after pollination was 2.9 mm. The studies examining pollen longevity showed that germination did not occur when the pollen grains were evaluated 24 h after anthesis. Thus, the use of aniline blue stain can be considered an effective method for observing pollen tubes development and studying the pollen morphology, longevity, viability and *Manihot* crossability.

**Key words:** Cassava, genetic resources, pre-breeding.

### INTRODUCTION

Cassava, the only cultivated species of the genus *Manihot*, is one of the most important food plants in tropical and subtropical regions of the world, occupying an important position among the primary sources of food

energy in terms of global caloric consumption, classified below flour, rice, corn and sorghum (Adeyemo, 2009). However, despite the importance of cassava and its large contribution as a food source for the poorest populations,

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there is a real demand for improvement, not only in the nutritional quality of the root but also in obtaining plant varieties resistant to biotic and abiotic factors.

Wild species of the genus *Manihot* constitute sources of genes that can be utilized to obtain new varieties (Akinbo et al., 2011). Appropriate pollen conservation strategies are essential to the generation of innovations in breeding programs by the introgression of new genes. Pollen conservation must preserve pollen viability; therefore, the viability must be monitored before, during and after storage. Thus, the maximum period for which pollen grains can be conserved without losing their capacity to germinate must be established (Rajasekharan et al., 2013).

For the evaluation of longevity and the monitoring of the viability of conserved pollen, various tests can be used, which generally consist of four main types: (1) The use of stains; (2) *In vitro* germination; (3) *In vivo* germination; and (4) *In vivo* pollination and the percentage of effective fructification (Galetta, 1983).

In *Manihot*, the most commonly employed method for establishing pollen viability is a colorimetric assay using different types of stains, such as acetic carmine, Lugol's solution, Alexander's solution and acetic orcein (Silva et al., 2001; Vieira et al., 2012). However, no correspondence has been observed between the colorimetric tests and the *in vivo* viability tests, reinforcing theories suggested by some authors regarding the inefficiency of these stains as proof of pollen viability (Rodriguez-Riano and Dafni 2000; Melloni et al., 2013). Aniline blue is a stain specific for callose located on the inner layer of the pollen tube, which fluoresces under ultraviolet light. Until now, no published studies have used this stain to estimate the pollen viability of *Manihot*. The use of aniline blue in fluorescence microscopy allows for the visualization of pollen tubes in the stigma and along the stylet, providing information related to the compatibility of accessions and pollen viability (Fang et al., 2010).

Thus, the objective of the present study was to apply aniline blue stain to observing pollen tubes development in different *Manihot* Mill. species, in order to estimate the crossability, pollen germination time, pollen tube size and pollen longevity.

## METHODOLOGY

### Study species

For observing pollen tubes development in different *Manihot* Mill studies using the aniline blue stain, accessions from different species of *Manihot*, which were selected according to the availability of material and previous knowledge regarding their crossability, were used. Thus, pollen grains from flowers at anthesis from 2 accessions of the cultivated species *M. esculenta* spp. *esculenta* (BGM 116 and TN 001) and 4 accessions involving 3 wild species: FLA 029V-01 (*M. esculenta* ssp. *flabellifolia* (Pohl) Cif.), ANO 049V-05 (*M. anomala* Pohl), IRW 027-01 and IRW 027-07 (*M. irwinii* D. J. Roger and Appan) were used. All the accessions used

are maintained in the Embrapa Cassava and Fruits collection.

### Crossability tests

To evaluate the crossability, manual crossings between different species and self-pollinations were performed at approximately 9:00 AM, the time at which the flowers open, the pollen grains are most viable, and the stigmas are most receptive (Vieira et al., 2012). Twenty-four hours after pollination, the pistillate flowers were collected and taken to the Laboratory of Biotechnology and Culture of Plant Tissues of Embrapa Cassava and Fruits. The stigmas were fixated by immersion in a 3:1 ethanol-acetic acid (Farmer solution) for 24 h. The samples were then washed with 70% ethanol and subjected to autoclaving for 25 min at a pressure of 1 kg cm<sup>-2</sup> in a solution of sodium sulfite (Na<sub>2</sub>SO<sub>3</sub>) to mollify and clear the tissues. After this procedure, the stigmas were washed with distilled water and submerged in a solution of 0.01% aniline blue for 24 h. To visualize the pollen tubes, each stigma was deposited on a glass slide with 3 drops of aniline blue and covered with a glass cover slip. After preparation, the slides were transferred to an OLYMPUS U-RFL-T optical fluorescence microscope, where they were visualized and photographed using a 10x ocular lens. The crossability rate was estimated by the percentage of germinated pollen, with well-developed pollen tubes relative to the total number of pollen grains counted per stigma.

The experimental design was entirely random with 3 repetitions, each repetition consisting of 100 pollen grains. The averages were compared using the Tukey test at a 5% probability level using the SAS 9.2 program (Cary, NC: SAS Institute INC., 2008).

### Germination time and size of the pollen tube

For germination time, size of pollen tube and pollen longevity, only accession FLA 029V-01 (*Manihot esculenta* spp. *flabellifolia*) was used due to the large production of staminate flowers by this accession. To determine the germination time and size of the pollen grains, at the time of germination, pollinated stigmas at anthesis were fixed in a 3:1 ethanol-acetic acid solution after different time intervals: 0:45, 1:00, 3:00, 6:00, 12:00, and 24:00 h after pollination. The methodology used to visualize the pollen tubes was the same as described previously for the crossability test. Germinated pollen grains were characterized when a pollen tube with a size equal to or greater than the diameter of the pollen was observed.

The sizes of the developed pollen tubes were determined 24 h after the pollination of the stigmas according to the scale in the photographs. Three repetitions including 25 pollen grains each were evaluated.

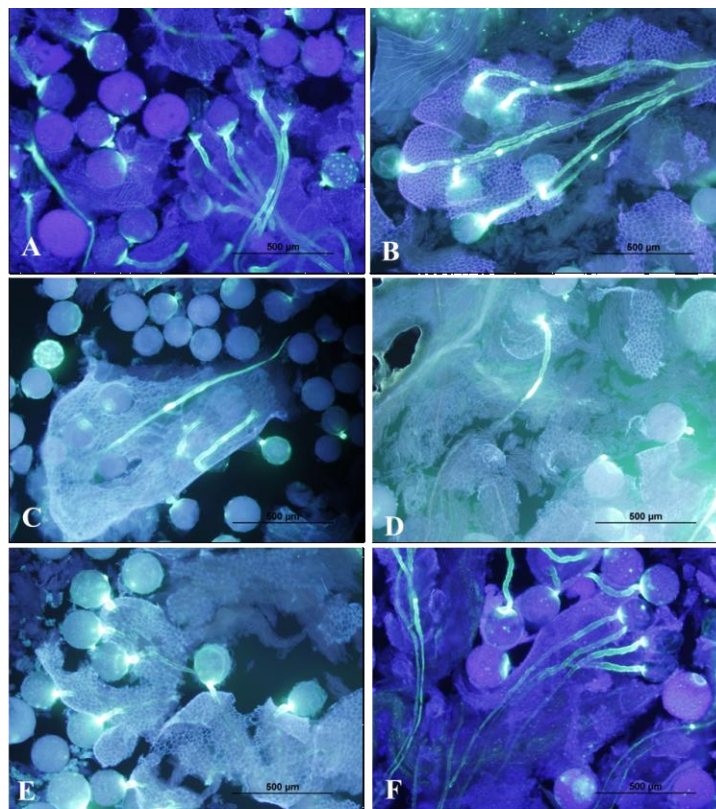
### Pollen longevity

To evaluate the pollen longevity, staminate flowers were used at 4 distinct stages: (1) Pre-anthesis; (2) Anthesis; (3) 24 h after anthesis, and (4) 48 h after anthesis. Two distinct stains (acetocarmine and aniline blue) were used in this experiment to measure the pollen viability in each of these stages.

## RESULTS AND DISCUSSION

The use of aniline blue to stain callose located in the inner layer of the pollen tube permitted clear visualization of the germinated pollen (Figure 1).

The use of aniline blue showed significant differences between crossings (Table 1). The highest rates of pollen



**Figure 1.** Development of the pollen tube in the crossings: A, FLA 02-01 x TN 001; B, BGM 116 x TN 001; C, IRW 027-01 x IRW 027-07; D, FLA 029V-01 x ANO 049V-051; E, BGM 116, self-pollinated; and F, FLA 029V-01, self-pollinated.

**Table 1.** Average percentage pollen germinated (%) and coefficient of variation (CV) in crossings (female parent x male parent) between accessions of the genus *Manihot* using the aniline blue stain.

Crossing	Pollen germinated (%)
FLA 029V-01 x TN 001	77 <sup>A</sup>
BGM 116 x TN 001	84 <sup>A</sup>
IRW 027-01 x IRW 027-07	32 <sup>C</sup>
FLA 029V-01 x ANO 049V-05	27 <sup>C</sup>
BGM 116 x BGM 116 (self-pollinated)	56 <sup>B</sup>
FLA 029V-01 x FLA 029V-01 (self-pollinated)	81 <sup>A</sup>
CV	3.7 <sup>B</sup>

\*Averages followed by the same letter do not differ by Tukey's test at probability of 5%.

tubes developed were observed in BGM 116 x TN 001 (84%) and FLA 029V-01 x TN 001 (76%) crossings and in self-pollinated FLA 029V-01, with 81% of the pollen tubes being germinated. This high percentage of developed pollen tubes found when the same accession was used as the female and male parents indicates that pollen-stigma self-incompatibility does not occur in the evaluated subspecies (*M. ssp. esculenta* and *M. spp.*

*flabellifolia*). Nassar and O'Hair (1985) also did not observe self-incompatibility between cassava clones, and staminate and pistillate flowers frequently opened together.

The lowest percentage of developed pollen tubes was observed in the FLA 029V-01 x ANO 049V-05 crossings, with 27% of the grains being germinated (Table 1). A comparison between data involving the same parent

**Table 2.** Pollen viability (%) of the FLA 029V-01 accession of *M. esculenta* spp. *flabellifolia* at different physiological stages using the acetocarmine and aniline blue stains.

Method	Pre-anthesis	Anthesis	24 h after anthesis	48 h after anthesis
Acetocarmine	82 <sup>Aa</sup>	90 <sup>Aa</sup>	84 <sup>Aa</sup>	75 <sup>Ba</sup>
Aniline blue	50 <sup>Bb</sup>	81 <sup>Aa</sup>	0 <sup>Cb</sup>	0 <sup>Cb</sup>

\*Averages followed by the same letter do not differ by Tukey's test at a probability of 5%. Capital letter for comparing averages between lines.

(FLA 029V-01) allows for inferences to be made regarding the potential compatibility of the parent with accession TN 001 and the reduced compatibility of the parent with the accession ANO 049V-05.

Regarding the germination time and pollen tube size, the use of aniline blue enabled the visualization and evaluation of the developed tubes. Studies aimed at assessing the development of pollen tubes are essential because the pollen tube is responsible for the transport of two sperm cells to the embryo sac where fertilization occurs. Thus, molecular, biochemical and physiological analyzes of germination and pollen tube growth are important for fundamental studies in plant reproduction (Boavida and McCormick, 2007) In this work, the results from the fixation of pollen grains at different time intervals after pollination revealed the presence of developed pollen tubes after 45 min of contact with the stigma, demonstrating that the germination of pollen grains in *Manihot* occurs rapidly. The size of the pollen tubes that developed 24 h after pollination was approximately 2.9 mm, on average, varying from 1.3 to 4.2 mm.

Studies of pollen longevity have demonstrated that the viability estimated using acetocarmine is higher for all of the physiological stages compared with aniline blue in the fluorescence test, varying from 90% of the pollen in anthesis to 75% in the grains examined at a period greater than 24 h after anthesis. With the aniline blue stain, the percentage of viable pollen was 81% for the pollen at anthesis and 50% for the pollen at pre-anthesis; germination did not occur when the pollen grains were evaluated 24 h after anthesis (Table 2). These results confirmed that aniline blue is more efficient than acetic carmine for monitoring *Manihot* pollen.

The results observed in this study did not differ from those reported by Vieira et al. (2011), who evaluated the pollen longevity of *M. esculenta* accessions using the *in vivo* pollination method and the percentage of seeds formed. They observed that the largest percentage of viable pollen was recorded in crossings in which the pollen grains were used in anthesis; furthermore, as the storage time increased, the viability and, consequently, the crossings performed were compromised. A 20% decrease in viability was observed after 1 h of storing fresh pollen at room temperature, and after 24 h, a total loss of pollen viability was recorded. In the present study, a similar result was observed using the aniline blue stain, which is a faster and less labor-intensive methodology

because there is no need to wait for maturation of the seeds, which takes approximately 90 days after pollination, to obtain the results.

As demonstrated in this study, the application of aniline blue to different accessions of the *Manihot* genus provides useful information that may be applied in a practical manner to various studies that support the genetic improvement of the culture of cassava. The importance of developing precise techniques for analyzing pollen has generated a series of recent studies, such as those performed with sunflower (Atlagiê et al., 2012), where a comparison between methods has demonstrated positive results when using fluorescence. However, no record of this type of analysis exists for cassava, prior to the present investigation. Studies related to pollen conservation from the genus are underway, with the objective of forming a pollen bank to subsidize the improvement of cassava, making the generation of new hybrids possible. This study was not feasible until now due to the absence of a reliable method for evaluating pollen viability, which is essential for this type of analysis.

## Conclusion

The use of aniline blue is a rapid, easy and efficient test for observing pollen tube development in different *Manihot* Mill. species. The application of this methodology will facilitate pollen-monitoring studies of conserved pollen grains of cassava, which will positively affect the crop breeding.

## Conflict of interest

The authors declare that they have no conflict of interest.

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