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Propagation

Illustrated guide to the classification of banana seeds and embryos

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Abstract - Considering there is no illustrated scale of the principle anomalies observed for banana seeds, the objective of this guide is to cover in detail, for the first time, the categories to classify the most common types of anomalies observed in banana embryos and seeds, as well as *in vitro* embryo rescue and culture, taking into account the experience acquired by the banana breeding program at Embrapa in Brazil. Four classes to classify the seeds were proposed and with regards to classifying the embryos, eight classes were established.
Index terms: *In vitro* culture; Germination; Genetic improvement; *Musa* spp.; embryo rescue.

Guia ilustrado para a classificação de sementes e embriões de banana

Resumo – Considerando que não há uma escala ilustrada das principais anomalias observadas para as sementes de bananeira, o objetivo deste guia é mostrar em detalhes, pela primeira vez, as categorias para classificar os tipos mais comuns de anomalias observadas em embriões e sementes de banana, bem como no resgate in vitro de embriões, levando em consideração a experiência adquirida pelo programa de melhoramento de banana da Embrapa, no Brasil. Quatro classes para classificar as sementes foram propostas e, no que diz respeito à classificação dos embriões, foram estabelecidas oito classes.
Termos de indexação: Cultivo *in vitro*; Germinação; Melhoramento genético; Musa spp.; resgate de embriões.

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Seed germination in the genus *Musa* is variable and limited due to physical, physiological and genetic barriers (Chin, 1996; Ahmed et al., 2006). The reduced number or absence of seeds in some *Musa* species occurs because of pre- and post-zygotic barriers that interfere with the fertilization process under natural conditions (Shepherd et al., 1994). According to Fortescue and Turner (2011), failures that result in seeds not forming in *Musa* spp. can occur in the gametophytic phase, sporophytic phase, or even during the interaction between male and female gametes.

Banana seeds can vary in size, shape and color, depending the species and cultivar, and are irregular in shape, generally flat, grayish to brownish, and have a coat surrounded by a wrinkled membrane that makes it rigid (Chin, 1996). These seeds consist of an embryo that can vary size but is normally small with endosperm surrounded by two integuments (the outer with multiple layers and the inner thin), which protect the seed during maturation, dispersal and dormancy. The outer integument limits the germination of the embryo due to its composition (Graven et al., 1996).

The high germination variation and low number or even absence of seeds in crosses to produce new hybrids are the major limitations that genetic improvement programs face, which is based on crossing triploid and tetraploid genotypes and improved or wild diploids, followed by selecting promising hybrids from the progenies (Amorim et al., 2013). Among these programs, the following are notable: Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA, Brazil), the Fundación Hondureña de Investigación Agrícola (FHIA, Honduras), the National Research Centre For Banana (NRCB, India), the International Institute of Tropical Agriculture (IITA, Nigeria), and the Centre de Coopération Iinternationale en Recherche Agronomique pour le Développement (CIRAD, France) (Amorim et al., 2013).

The difficulty of obtaining viable seeds through crosses has increased interest in studies about *in vitro* germination of zygotic embryos, since most of the seeds have some form of abnormalities because of the absence or deformation of the embryo and/or endosperm (Asif et al., 2001). Due to seed mortality caused by these anomalies, even before *in vivo* germination, culturing embryos has become a strategy to rescue embryos before they die, making it possible to genetically improve bananas (Asif et al., 2001). *In vitro* cultivation of banana embryos also depends on the maturation stage of the embryo and the culture medium used (Uma et al., 2011). However, it is necessary to optimize the methods used in tissue culture to rescue embryos of *Musa* spp. seeds.

Considering there is no scale of the main anomalies observed in banana seeds, this work aims to propose an illustrated guide to classify these anomalies aiming to assist in the selection of seeds and embryos that are most suitable for embryo rescue. The guide is subdivided into six steps. It starts with processing seeds, followed by embryo rescue, classifying seeds and embryos, embryo cultivation and seedling growth, and ends with seedling acclimatization.

Step 1: Seed processing and disinfection-The banana seeds are removed from fruits in an advanced stage of maturation (brown patches on the peel), which means they are completely developed (Figure 1, step 1A). The seeds are then washed in water to remove any pulp that is adhered to the surface (Figure 1, step 1B). The seeds are placed in a container with water and those that float are discarded because they may not present an embryo or endosperm (Uma et al., 2011). After processing, the sunken seed disinfection was performed under sterile conditions in a laminar hood, where they are treated with 70% alcohol for 5 minutes, immersed in sodium hypochlorite (2.5% active chlorine) for 30 minutes, and then triple washed with sterile distilled water. Finally, the seeds are suitable for embryo rescue (Figure 1, step 1C and 1D).

Step 2: Embryo rescue -The seed embryos are excised and classified, with using a stereoscopic microscope in a chamber under laminar flow (Figure 1, step 2A). A longitudinal fissure is made in each seed for excision of the embryo (Figure 1, step 2B).

Step 3: Seed classification - The following scale is proposed to classify the seeds after making a longitudinal cut: embryo and endosperm present (PP); embryo absent and endosperm present (AP); embryo present and endosperm absent (PA); and embryo and endosperm absent (AA) (Figure 1, step 3).

Step 4: Embryo classification - After extraction, the embryos are classified using the following proposed scale: 1. Embryo normal; 2. Embryo with deformed base; 3. Embryo base absent; 4. Embryo with deformed apex; 5. Embryo apex absent; 6. Embryo with deformed base and apex; 7. Embryo with oxidation; and 8. Embryo normal but small (Figure 1, step 4).

Step 5: *In vitro* embryo cultivation and seedling growth - Seeds classified as PP and embryos classified as 1 are placed in Petri dishes containing MS medium (Murashige & Skoog 1962) supplemented with 6-benzylaminopurine (BAP) 2.0 mg L^{-1} and gibberellic acid (AG_3) 0.035 mg L^{-1} (Figure 1, step 5A and Table 1). The dishes are initially incubated in a growth chamber in the dark at a temperature of 27 ± 2ºC. After germination, the embryos are transferred to test tubes (25 x 150 mm) containing 15 ml of MS culture medium without regulators (Table 1). The cultures are maintained in a 16/8-h (light/dark) photoperiod under white fluorescent lamps with a light

intensity 1,600 lux, at 26 ± 2ºC (Figure 1, step 5A and 5C), until the seedlings can complete the *in vitro* development.

Step 6: Seedling acclimatization-Rooted seedlings are potted in the sterilized soil mixture containing vermiculite, sand and organic matter (1:1:1), and placed in a greenhouse (Figure 1, steps 6A and 6B) until they reach the appropriate size (ca. 25 to 30 cm tall with 5 to 6 leaves). Subsequently, they are transplanted to plastic bags (35x35cm, 2 liters) with the same substrate, where they remain for about 30 to 45 days (Figure 1, step 6C), and then taken to the field for agronomic evaluation and selection of promising hybrids.

Table 1. Composition of the basal culture media MS (Murashige & Skoog 1962) and plant regulators for the in vitro cultivation of banana zygotic embryos and plant growth. For each medium, pH is adjusted to 5.7-5.8 with 0.1 N KOH prior to autoclaving for 20 min at 120 °C.

Components	Elements	Concentration mg.L^{-1}
MS Macronutrients	Ammonium nitrate (NH_4NO_3)	1650
	Potassium nitrate (KNO_3)	1900
	Calcium chloride ($CaCl_2.2H_2O$)	440
	Magnesium sulfate ($MgSO4.\ 7H_2O$)	370
	Potassium phosphate monobasic (KH_2PO_4)	170
MS Micronutrients	Manganese sulfate ($MnSO_4.H_2O$)	22.3
	Zinc sulfate ($ZnSO_4.7H_2O$)	8.6
	Boric acid (H_3BO_3)	6.2
	Potassium iodide (KI)	0.83
	Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.25
	Copper sulfate ($CuSO_4.5H_2O^*$)	0.025
	Cobalt chloride ($CoCl_2.6H_2O^*$)	0.025
Fe-EDTA	Iron sulphate ($FeSO_4.7H_2O$)	27.8
	$Na_2EDTA.2H_2O$	37.3
Vitamins	Thiamine-HCl	0.1
	Pyridoxine-HCl	0.5
	Nicotinic acid	0.5
	Myo-inositol	100
Amino acid	Glycine	2
Carbohydrate	Sucrose	30 000
Gelling agent	Phytagel	2.6
Embryo germinating medium	6-benzylaminopurine (BAP) ($C_{12}H_{11}N_5$)	2.0
	Gibberellic acid (AG_3) ($C_{19}H_{22}O_6$)	0.035
Plant growth medium	6-benzylaminopurine (BAP) ($C_{12}H_{11}N_5$) Gibberellic acid (AG_3) ($C_{19}H_{22}O_6$)	0 0

Figure 1. Steps for classification of banana seeds and embryos. 1) Collecting and washing of seeds; 2) Rescue of embryo; 3) Classification of the seeds according to the presence or absence of embryo and endosperm; 4) Classification of embryos according to your morphology; 5) In vitro embryos cultivation and seedling growth; and 6) Acclimatization of seedlings.

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