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In vivo fertilization of banana

Fertilização *in vivo* de bananeira

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ABSTRACT

The aim of this research was to study the in vivo *fertilization process of banana cultivars. The diploid hybrid (AA) 091087-01 was the male progenitor. Flower samples were checked for fertilization from the first to the twentieth day after pollination. The size of the diploid ovules increased gradually at the beginning of the seed formation process. On the other hand, in the AAA triploids (Cavendish subgroup), the not fertilized ovules were aborted. In the AAB triploids (Prata subgroup) some ovules were fertilized. The flowers of Grand Naine, Nanicão and 'Pacovan' cultivars presented necrosis in the distal part of the ovary on the first day after pollination. Necrosis can hinder pollen tube growth towards the ovule, which might be related to the low seed yield in 'Pacovan' cultivars and to the absence of seeds in the Cavendish subgroup cultivars.*

Key words: ***Musa*** *spp. L., genetic improvement, hybridization, ovules.*

RESUMO

O objetivo do trabalho foi investigar o processo de fertilização in vivo *em cultivares de bananeira. Utilizou-se como genitor masculino o híbrido diploide (AA) 091087-01. Do primeiro até o vigésimo dia após a polinização, foram retiradas amostras de flores para comprovação da fertilização. Verificou-se que os óvulos de diploides aumentaram de tamanho gradualmente, iniciando o processo de formação de sementes, enquanto, nos triploides AAA (subgrupo Cavendish), ocorreu o aborto dos óvulos não fertilizados. Nos triploides AAB (subgrupo Prata), alguns óvulos são fertilizados. As flores das cultivares 'Grande Naine', 'Nanicão' e 'Pacovan' apresentaram uma necrose na região distal do ovário, detectada desde o primeiro dia após a polinização, a qual pode se constituir em uma barreira para o crescimento do tubo polínico em direção ao óvulo, o que provavelmente pode estar relacionado à baixa produção de sementes em Pacovan e à ausência de sementes em cultivares do subgrupo Cavendish.*

Palavras-chave: ***Musa*** *spp. L., melhoramento genético, hibridação, óvulos.*

INTRODUCTION

Bananas are treelike perennial herbs of great social and economic importance. They are grown in over 120 countries, and can be consumed raw or cooked. The banana fruit is either seeded or parthenocarpic. Wild banana trees are diploid (2n=2x=22), seeded, generally cross-pollinated and have a high number of fertile seeds that allow for the dispersion of the plant. Cultivated bananas, on the other hand, are triploid (2n=3x=33), with a variable degree of parthenocarpy and sterility, and present a low number or complete absence of seeds (SILVA et al., 2002; FORTESCUE & TURNER, 2005a).

Although the majority of bananas produced worldwide are cultivars with AAB and ABB genome constitutions, international trade consists mainly of Cavendish (AAA) cultivars due to the palatability and quality of their fruits, as well as their high yield (FORTESCUE & TURNER, 2005b). However, many of these cultivars present not only female sterility but are also susceptible to leaf diseases and pests that can seriously affect yield (SSEBULIBA et al., 2006).

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Hence, genetic improvement is important to obtain resistant and marketable varieties.

The improvement program developed by Embrapa Cassava and Fruits aims to produce varieties that are more productive, smaller in size, with shorter cycles, resistant to Panama disease (***Fusarium oxysporum*** f. sp. *cubense*), to yellow and black Sigatokas (***Micosphaerella musicola*** and ***Micosphaerella fijiensis***, respectively), to burrowing nematodes (***Rodophilus similis)*** and to banana weevils (***Cosmopolites sordidus)***, through crossings between commercial triploids or tetraploids and improved diploids (SILVA et al., 2001; AMORIM et al., 2013) and more recently between ornamental banana diploids (SANTOS-SEREJO et al., 2012, SOUZA et al., 2012).

Pollination is significantly affected by different factors, such as pollen efficiency, which might be related to chromosome translocations that affect fertility and/or growth rate of pollen tubes in the ovaries of triploid cultivars (SHEPHERD et al., 1987a, 1987b). The pollination season also affects seed ploidy: pollinations occurring during the dry summer produce many hybrids with undesired ploidies, such as hexaploids and heptaploids, while pollinations in cooler and more humid seasons have lower seed yield but a higher tetraploid recovery rate (SHEPHERD et al., 1994). Another important and determinant factor that influences pollination is the progenitors' specificity which affects seed quality and quantity.

Outcrossing with Cavendish subgroup cultivars produce no seeds, while the subgroup Prata produces a variable number of seeds depending on the progenitor used (SILVA et al., 2001). In controlled pollinations involving banana and plantain, FORTESCUE & TURNER (2005c) reported that seed production was low, in the order of 1 to 5 seeds per 100 fruits, considering that each ovary could contain at least 300 ovules. The low yield or absence of seeds might be related to the intense agronomic selection and thus might be a consequence of species domestication. This high infertility limits the transfer of desired alleles to the Cavendish subgroup, and impairs improvement by means of the conventional method.

Although different factors that might be responsible for the absence or low production of seeds in banana cultivars have been described in the literature, there is not enough information to understand the physical and/or biochemical barriers that limit or hinder their yield. According to FORTESCUE & TURNER (2005c), flaws in the embryonic sac caused by chromosome imbalance contribute to increase sterility in edible diploid and triploid bananas.

The aim of this research was to study the *in vivo* fertilization process of bananas by comparing ovule development in diploids improved with triploid ovules of Cavendish and Prata subgroup cultivars.

MATERIAL AND METHODS

The reproductive system of the genus ***Musa*** L. was studied by means of hand cross-pollination experiments in the field, with temperatures varying from 25.3ºC to 32.5ºC and relative humidity of 84%, according to the meteorological data provided by the Weather Station of Embrapa Cassava and Fruits. Diploid (AA) and triploid individuals of the Cavendish (AAA) and Prata (AAB) subgroups were used (Table 1). Five AA diploid, five AAA triploid and four AAB triploid plants were used. One plant of each ploidy level was selected as control to evaluate ovule development without pollination. The female inflorescences of the improved diploids (AA) and of the triploids (AAA and AAB) were protected with polyethylene bags one day before anthesis (opening of the flowers) to avoid contamination by insect-carried pollen.

Pollination began one day after the female inflorescences were protected, when they were receptive, i.e., they presented free stigma lobes, being apt for pollination and fertilization. One to two hands were pollinated per day until the last hand emerged.

The pollen was collected from the anthers of the improved AA diploid 091087-01 [(Borneo x Guyod) x (Calcutta 4 x Heva)], which was chosen for its high germination percentage (SOARES et al., 2008). Male flowers were collected during anthesis and were used for manual cross-pollination, when pollen was distributed on the stigma surface. Pollen was collected during anthesis to ensure the normal development of the pollen tube and uniform growth, since pollen grains collected hours or days after anthesis tend to have reduced viability (TANGMITCHAROEN & OWENS, 1997). Depending on the quantity of pollen grains, one male flower was used to pollinate 3 to 4 female flowers.

After pollination, stalks were identified and protected with polyethylene bags until the last hand emerged in order to avoid the contact with ants or other insects that might carry pollen from other plants, or even remove the pollen that had just been distributed on the stigma. Flower samples were collected daily from the first to the twentieth day after pollination to evaluate the ovule size (diameter) using

Table 1 - Ovules diameter (mm) of diploid (AA) and triploid (AAA and AAB) genotypes 1, 10, and 20 days after pollination with the improved diploid hybrid 091087-01.

Genotypes	Ovule diameter (mm) / Days after pollination			
	0	1	10	20
AA Diploids				
003004-02 - (Calcutta 4 x Madang)	0.892	0.915	1.518	2.008
042052-05 - (M 53 x Kumburgh)	0.701	0.742	1.433	1.667
058054-03 - [(Calcutta 4 x Pahang) x (Borneo x Madang)]	0.698	0.750	1.585	1.863
091079-03 - [(Borneo x Guyod) x (Tuu Gia x Calcutta 4)]	0.804	0.815	1.439	1.995
091087-02 - [(Borneo x Guyod) x (Calcutta x Heva)]	0.723	0.768	1.393	2.042
Mean	0.764	0.798	1.473	1.915
CV		22.15		
AAA Triploids				
Grande Naine Magario	1.425	1.436	0.912	0.919
Grande Naine Rossete	1.498	1.505	1.360	*
Grande Naine SC-074	1.314	1.327	0.898	0.898
Nanicão Rossete	1.465	1.478	1.116	0.864
Nanicão SC-063	1.321	1.332	1.237	0.949
Mean	1.405	1.415	1.105	0.907
CV		18.30		
AAB Triploids				
Pacovan	0.875	0.894	1.413	1.436
Prata Anã	0.732	0.749	1.108	0.800
Prata Comum	0.897	0.905	1.228	0.907
Prata Graúda	0.745	0.767	1.377	1.463
Mean	0.812	0.828	1.281	1.151
CV		21.13		

* Genotype whose number of hands in the stalk was not sufficient for evaluation during the twenty-day period after pollination.

a stereo microscope. Ovule observation was done with ocular on micrometric plates, and values were then transformed to millimeters.

A 3x5x20 (ploidy x genotype x days after pollination) factorial experiment with a completely randomized design and three repetitions was conducted. Thirty ovules were measured in each repetition. Regression analysis was used, and mathematical models were chosen according to the equations with best adjustments confirmed by the highest values of the coefficient of determination (R^2), and the F test of the regression, both at 5% probability. The means were compared with Tukey's Test. For such, the SAS statistical software system (SAS INSTITUTE INC., 2004) was used.

RESULTS AND DISCUSSION

Analysis of variance indicates that ovule development is not significantly different ($P \leq 0.05$) among the tested genotypes within the genomic group on the subsequent days after pollination (Table 1); and neither was the interaction among factors.

One day after pollination, the average ovule size varied from 0.80mm in AA diploids to 0.83mm in AAB triploids, and 1.42mm in AAA triploids. However, as the number of days after pollination increased, the size of the AA diploid ovule increased, while the size of the AAA triploid ovules decreased. Twenty days after pollination, mean values were 1.92mm and 0.91mm, respectively (Table 1, Figure 1a and 1b). In the Prata subgroup cultivars (AAB), ovules grew until the 10^{th} day followed by a tendency to growth stabilization. On the twentieth day, their average size was 1.15mm (Table 1, Figure 1c and 1d).

The regression analysis indicates that the size of fertilized ovules of AA diploids increased linearly after pollination. Similar results were obtained in the Prata subgroup cultivars which also showed a linear increase of ovule size until the

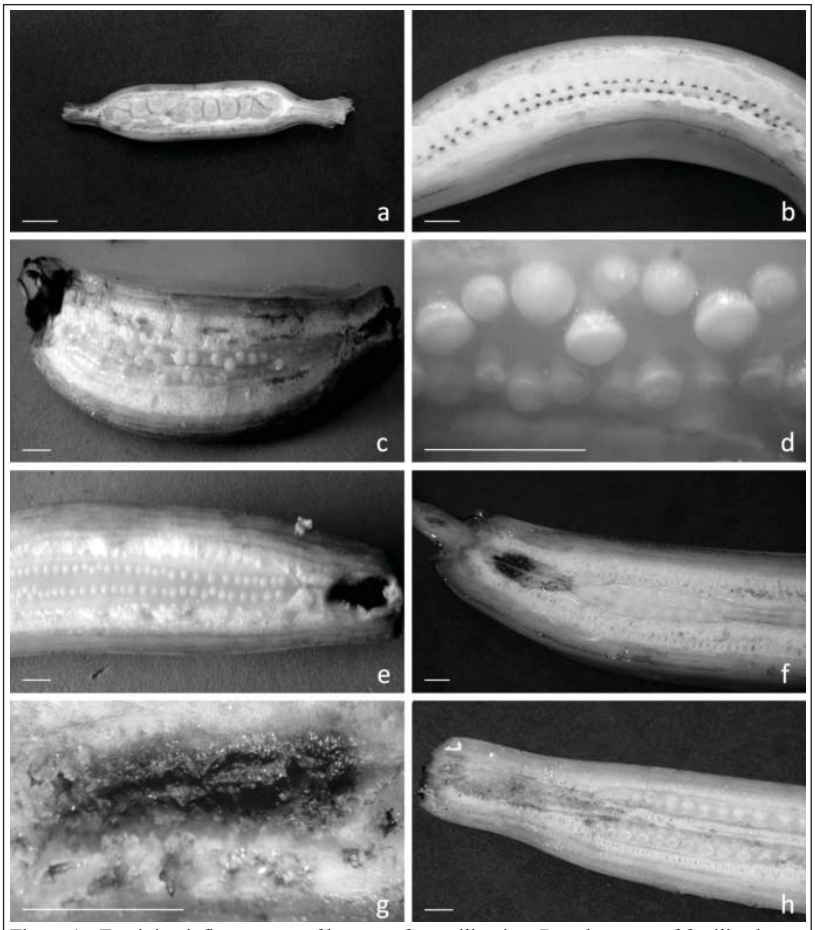

Figure 1 - Feminine inflorescence of banana after pollination. Development of fertilized eggs twenty days after pollination in the diploid 003004-02 (a); in the cultivar 'Nanicão Rossete' (b) and in the cultivar 'Pacovan' (c-d). Necrosis in the distal part of the ovary of the 'Pacovan' triploid (e) and 'Grande Naine Rossete' (f-g). Diploid 091087-02, without necrosis in the distal part of the ovary (h). Bar 1.00cm.

tenth day after pollination, remaining fairly constant thereafter. The opposite occurred with the AAA triploid cultivars: the ovule size decreased with time (Figure 2) as a consequence of the abortion of the not fertilized ovules (Figure 1b).

During pollination evaluation, it was noted that in diploids the pollen grains deposited on the stigma emitted and developed pollen tubes faster than in triploids. Four hours after pollination, pollen grains were not visible on the stigma anymore, which indicates that germination was complete. In triploids, pollen germinated on the stigma, but apparently it didn't penetrate into the style, which suggests the lack of some sort of stimulus to direct the pollen tube, or the presence of a physical barrier. One day after pollination, the diploid stigmas fell off the fruit, while the triploid stigmas fell off on the third day.

Long cross section views of female flowers showed signs of necrosis on the distal part of the ovary in AAA and AAB triploids (Figure 1e-g) one day after pollination, either in the pollinated plants and in the controls. No necrosis was observed in diploid cultivars (Figure 1h). The necrosis can be consequence of an inhibitory process of the pollen tube growth toward the ovule, which might be related to the low seed yield in Prata subgroup cultivars and to the absence of seeds in Cavendish subgroup cultivars, as suggested by SHEPHERD et al. (1987a). The deposition of exudates (necrosis) in the distal region of the ovary could act as mechanical obstruction, or as permeability barrier to prevent the development of the pollen tube along the style and ovary and consequently the embryonic bag penetration and fertilization. Therefore, experimental studies are needed to assess whether

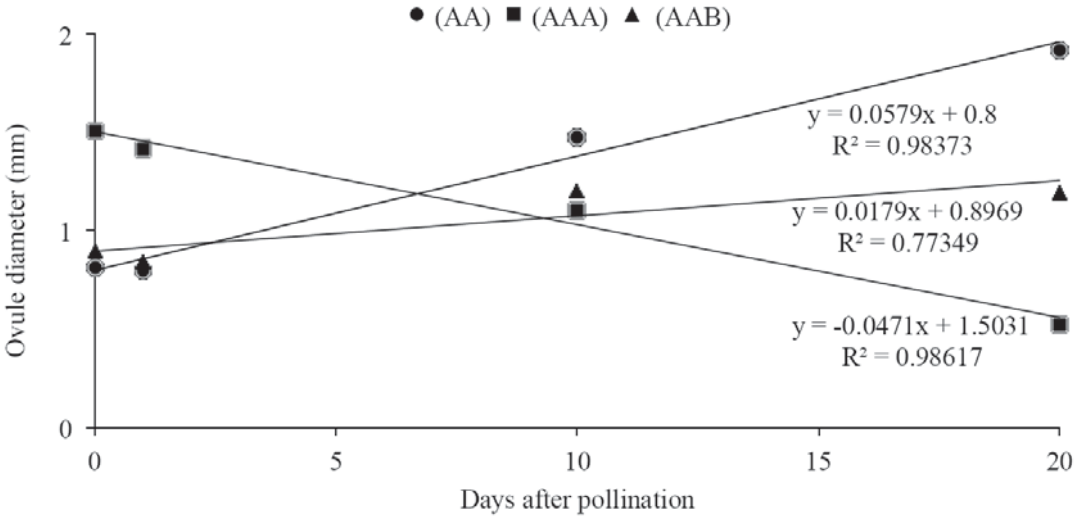

Figure 2 - Diameter alteration in the ovaries of diploid (AA) and triploid (AAA, AAB) bananas until the twentieth day after hand cross-pollination.

necrosis or others factors are associated to the sterility in this subgroup.

Literature offers various causes for banana sterility. According to SIMMONDS (1959), ***Musa*** sterility would be due to meiotic errors that would lead to errors in the development of the embryonic sac, as well as morphological and physiological alterations that would hinder the penetration of the pollen tube into the style and ovary. SHEPHERD et al. (1987a) reported that the absence or low production of seeds would be a consequence of: a) irregular growth of the pollen tubes in some varieties; b) pollen tubes that are too short and cannot reach the ovule; c) the occurrence of seeds solely in the first hands and in the distal portion of the fruits in some cultivars; and d) the premature necrosis of the nectary region in the female flower which hinders the passage of the pollen tube.

While studying the ovule anatomy, FORTESCUE & TURNER (2005c) observed that ovule development of diploids and triploids was similar until anthesis. After anthesis, the triploid ovules started to wither in the same way not fertilized ovules do, acquiring a brownish color as observed in the center of ripe fruits. The authors also reported that the ovules of the AAA Gros Michel cultivars withered as a consequence of embryonic sac degeneration. During anthesis, the majority of embryonic sacs of edible cultivars do not have nuclear membrane, the nuclear content is disorganized and the embryonic sac deteriorates after anthesis.

In the present study, controlled pollination of diploids allowed for the adequate amount of pollen in terms of quality and quantity during the period in which the stigma was highly receptive, thus ensuring fertilization and seed formation. Controlled pollination can be a routine procedure, and when high seed yield is required, the number of pollinations can be increased.

Prezygotic barriers resulting from pollen-pistil incompatibility, where pollen grain germination and pollen tube growth of one species is inhibited by the stigma of another species (BURSON & YOUNG, 1983) are quite common. Both pollen and pistil produce essential compounds to ensure successful pollen germination (QIN et al., 2011; BOAVIDA et al., 2011). In many interspecific crosses, the incompatibility reaction of the pollen-pistil occurs because the pollen tube growth of one species is hindered in the stigma of the other. This reaction may be partially inhibited by proteins that are frequently released by the compatible pollen walls, as a result of genic disharmony interactions due to genetic divergence among species (HODNETT et al., 2005; KUMAR et al., 2009).

Future research might investigate other *in vivo* pollination techniques that could overcome the incompatibility of crosses to improve controlled pollination efficiency, as well as overcome the prezygotic barriers observed in the Cavendish subgroup in order to obtain hybrids that are resistant to the main pathogens, productive, and of high quality.

CONCLUSIONS

The AA diploid banana ovules develop normally after pollination and produce seeds. In the

AAA triploid, however, the not fertilized ovules abort their development and size reduction is observed. In the AAB triploids some ovules are fertilized but their size are smaller than the diploid ovules. Necrosis of the distal part of the ovary might be related to the sterility observed in some Cavendish subgroup varieties and to the low seed yield of the Prata subgroup cultivars.

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REFERENCES

AMORIM, E.P. et al. Banana breeding at Embrapa Cassava and Fruits. **Acta Horticulturae**, v.986, p.171-176, 2013. Available from: <http://www.actahort.org/members/showpdf?booknrarnr=986_18&session=4768&justloggedin=2>. Accessed: May 15, 2013.

BOAVIDA, L.C. et al. Whole genome analysis of gene expression reveals coordinated activation of signaling and metabolic pathways during pollen-pistil interactions in ***Arabidopsis***. **Plant Physiology**, v.155, p.2066–2080, 2011. Available from: <http://www.plantphysiol.org/content/155/4/2066.long>. Acessed: Jul. 10, 2013. doi: 10.1104/pp.110.169813.

BURSON, B.L.; YOUNG, B.A. Pollen-pistil interactions and interspecific-incompatibility among ***Panicum antidotale***, ***P. coloratum***, and ***P. deustum***. **Euphytica**, v.32, p.397-405, 1983. Available from: <http://rd.springer.com/article/10.1007/BF00021448>. Accessed: Nov. 15, 2011. doi:10.1007/BF00021448.

FORTESCUE, J.A.; TURNER, D.W. Growth and development of ovules of banana, plantain and enset (***Musaceae***). **Scientia Horticulturae**, v.104, p.463-478, 2005a. Available from: <http://www.sciencedirect.com/science/article/pii/S0304423805000105>. Accessed: Jan. 11, 2012. doi:10.1016/j.scienta.2005.01.007.

FORTESCUE, J.A.; TURNER, D.W. The occurrence of a micropylar exudate in ***Musa*** and ***Ensete*** (***Musaceae***). **Scientia Horticulturae**, v.104, p.445-461, 2005b. Available from: <http://www.sciencedirect.com/science/article/pii/S0304423805000099>. Accessed: Jan. 11, 2012. doi: 10.1016/j.scienta.2005.01.006.

FORTESCUE, J.A.; TURNER, D.W. The anatomy of ovule ontogeny of banana, plantain and enset (***Musaceae***). **Scientia Horticulturae**, v.104, p.479-492, 2005c. Available from: <http://www.sciencedirect.com/science/article/pii/S0304423805000117>. Accessed: Jan. 11, 2012. doi: 10.1016/j.scienta.2005.01.008.

HODNETT, G.L. et al. Pollen-pistil interactions result in reproductive isolation between ***Sorghum bicolor*** and divergent ***Sorghum*** species. **Crop Science**, v.45, p.1403-1409, 2005. Available from: <https://www.soils.org/publications/cs/pdfs/45/4/1403>. Accessed: May 13, 2012. doi:10.2135/cropsci2004.0429.

KUMAR, R.S. et al. Investigation on cross-compatibility barriers in the biofuel crop ***Jatropha curcas*** L. with wild ***Jathopha*** species. **Crop Science**, v.49, p.1667-1674, 2009. Available from: <https://www.crops.org/publications/cs/articles/49/5/1667?highlight>. Accessed: May 13, 2012. doi: 10.2135/cropsci2008.10.0601.

QIN, Y. et al. Sulfinylated azadecalins act as functional mimics of a pollen germination stimulant in Arabidopsis pistils. **Plant Journal**, v.68, p.800-815, 2011. Available from: <http://onlinelibrary.wiley.com/doi/10.1111/j.1365-313X.2011.04729.x/pdf>. Accessed: Jul. 4, 2013. doi: 10.1111/j.1365-313X.2011.04729.x.

SANTOS-SEREJO, J.A. et al. Selection and use recommendation in hybrids of ornamental banana. **Crop Science**, v.52, p.560-567, 2012. Available from: <https://www.crops.org/publications/cs/abstracts/52/2/560?access=0&view=pdf>. Accessed: Jul. 13, 2012. doi: 10.2135/cropsci2011.06.0325.

SAS Institute Inc. **SAS/STAT user's guide**. Version 9.1.3. Cary, NC, USA, 2004. 404p.

SHEPHERD, K. et al. Aspects of banana breeding at the Centro Nacional de Pesquisa de Mandioca e Fruticultura, Brazil. In: GALINDO, J.J.; JARAMILLO, R. (Eds). **Proceedings of ACORBAT 7**. San José, Costa Rica 1985. Turrialba: CATIE, 1987a. p.78-86.

SHEPHERD, K. et al. Banana breeding in Brazil. In: PERSLEY, G.; LANGHE, E. (Eds.). **Banana and plantain breeding strategies ACIAR proceedings**. Canberra: ACIAR, 1987b. N.21, p.78-83.

SHEPHERD, K. et al. Breeding prata and maçã for Brazil. In: JONES, D.R. (Ed.). **The improvement and testing of *Musa***: a global partnership. Montpellier: INIBAP, 1994. p.157-168.

SILVA, S.O. et al. Bananeira. In: BRUCKNER, C.H. (Org.). **Melhoramento de fruteiras tropicais**. Viçosa: UFV, 2002. p.101-158.

SILVA, S.O. et al. Banana breeding program at Embrapa. **Crop Breeding and Applied Biotechnology**, v.1, p.399-436, 2001. Available from: <http://www.sbmp.org.br/cbab/siscbab/uploads/bd6b9012-2d97-ac26.pdfZx>. Accessed: May 7, 2011.

SIMMONDS, N.W. **Bananas**. London: Longman. 1959. 466p.

SOARES, T.L. et al. In vitro germination and viability of pollen grains of banana diploids. **Crop Breeding and Applied Biotechnology**, v.8, p.111-118, 2008. Available from: <http://www.sbmp.org.br/cbab/siscbab/uploads/bd6b9df0-14d5-c291.pdf>. Accessed: Jul. 11, 2011.

SOUZA, E.H. et al. Genetic variability of banana with ornamental potential. **Euphytica**, v.184, p.355-367, 2012. Available from: <http://www.springerlink.com/content/lg940612g3571878/fulltext.pdf>. Accessed: June 10, 2012. doi. 10.1007/s10681-011-0553-4.

SSEBULIBA, R. et al. Reproductive efficiency and breeding potential of East African highland (***Musa*** AAA-EA) bananas. **Field Crops Research**, v.95, p.250-255, 2006. Available from: <http://www.sciencedirect.com/science/article/pii/S0378429005000705>. Accessed: Dec. 22, 2011. doi: 10.1016/j.fcr.2005.03.004.

TANGMITCHAROEN, S.; OWENS, J.N. Pollen viability and pollen-tube growth following controlled pollination and their relation to low fruit production in teak (***Tectona grandis*** Linn. f.). **Annals of Botany**, v.80, p.401-410, 1997. Available from: <http://aob.oxfordjournals.org/content/80/4/401>. Accessed: Dec. 18, 2011. doi: 10.1006/anbo.1996.0440.