IDENTIFICAÇÃO DA ORIGEM GENÉTICA DE PLÂNTULAS EM SEMENTES POLIEMBRIÔNICAS DE MANGUEIRA (*Mangifera indica,* L.) CV. ROSINHA POR MEIO DE MARCADORES RAPD[1]

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RESUMO - A mangueira pode ser propagada por sementes ou por enxertia. Para fins comerciais, a propagação por enxertia é a mais apropriada, por manter o padrão genético da cultivar propagada. Na obtenção de mudas enxertadas, é importante o uso de variedades poliembriônicas como porta-enxerto, uma vez que essas produzem uma plântula zigótica e várias nucelares. As plântulas nucelares mantêm as características genéticas da planta-mãe e, por isso, são preferidas na propagação por enxertia por, supostamente, oferecerem maior uniformidade no pomar. Em geral, os viveiristas utilizam a plântula mais vigorosa para enxertar, supondo que o mesmo seja de origem nucelar. Porém, desuniformidades de altura e produção são muito comuns em pomares comerciais de manga na região Nordeste. O objetivo deste trabalho foi identificar a origem genética das plântulas de sementes poliembriônicas da variedade *Rosinha,* utilizando marcadores RAPD (Random Amplified Polymorphic DNA). Além disso, a posição do embrião zigótico e o percentual de plântulas zigóticas e nucelares vigorosas também foram determinados. Foi observada uma elevada taxa de plântulas zigóticas vigorosas a qual, possivelmente, explica a desuniformidade quanto à altura das plantas nos pomares comerciais de mangueira.
Termos de Indexação : identificação de plântulas, embrião, porta-enxerto, uniformidade no pomar.

IDENTIFICATION OF PLANTLET GENETIC ORIGIN IN POLYEMBRYONIC MANGO (*MANGIFERA INDICA*, L.) CV. ROSINHA SEEDS USING RAPD MARKERS

ABSTRACT - Mango can be propagated by seeds or by grafting. For commercial purpose, grafting is the most appropriate method because it maintains the genetic characters from the propagated variety. To obtain grafted mango it is important to use polyembryonic varieties as rootstock since they produce a zygotic and many nucellar plantlets. The nucellar plantlets maintain the genetics of the mother-plant thus, are preferred for grafting since they supposedly give more uniformity to the orchard. In general, nurserymen use the most vigorous plantelet to graft, believing that they are nucellar. But, orchard disuniformities on height and yield are very common among mango trees of commercial orchards in Northeast region. The objective of this paper was to identify the genetical origin of plantlets from polyembryonic seeds of Rosinha variety using Random Amplified Polymorphic DNA (RAPD) markers. Moreover, the position of the zygotic embryo and the percentage of the vigorous zygotic and nucellar plantlets was also determined. It was obtained an elevated taxa of vigorous zygotic plantlets which possibly explains the disuniformity on height of trees at commercial mango orchards.
Index Terms : plantlet identification, embryo, rootstock, orchard uniformity.

INTRODUCTION

Mango is one of the most important fruits of Brazilian agribusiness (Pinto, 2002; Brazilian Fruit Yearbook, 2005). Mango trees can be propagated by seeds or by grafting. Grafting is the best option for commercial mango production since this type of propagation maintains the genetic characters of the propagated variety and, produces consequently, a homogeneous fruit production. Grafting requires rootstock varieties that should be selected and tested appropriately because they can interfere in the yield and physico-chemical characteristics of the fruit (Jauhari et al., 1972; Kohli & Reddy, 1988, Avilán et al., 1996; Ramos et al., 2002). Moreover, rootstocks should be resistant to pathogens and preferably from dwarf and polyembryonic varieties (Ribeiro et al., 1995).

Polyembryonic varieties produce seeds which originate zygotic and nucellar plantlets. Zygotic plantlets are sexual whereas nucellar plantlets come from the maternal tissue which are assexual plantlets. Therefore, nucellar plantlets are preferred for grafting, because they maintain the same genetic background of the rootstock mother-plant which may present disease resistance or dwarfness. Moreover, nucellar plantlets used as rootstock may keep adequate orchard homogeneity. However, it is very common to find out vigour and yield disuniformities on mango plants in orchards from Northeast region of Brazil (Alberto Carlos de Queiróz Pinto, personal communication, 2005).

Polyembryony on mango is considered a genetic feature, although it is not yet known if it is a product of a recessive or dominant single gene (Sturrock, 1968; Aron et al., 1998). Polyembryonic seeds have one zygotic and from one to six nucellar plantlets depending on the variety. Zygotic plantlet in polyembryonic varieties was pointed out as the one which is the closest to the basal side of the seed and it degenerates or, do not develop well (Sachar & Chopra, 1957 In Srivastava et al., 1988). On the other hand, nucellar plantlets are those which develop very well and became the most vigorous in diameter and height. Nurserymen have used vigorous plantlets for grafting taking into account that they are nucellar, which generate morphologically uniform plants.

Mango has been subject of many analysis using different molecular markers types as isoenzymes (Degani et al., 1990; Degani et al., 1992; Degani et al., 1993; Aron et al., 1997), RAPD (Schnell & Kinght Jr., 1993; Schnell et al., 1995; López-Valenzuela et al., 1997; Ravishankar et al, 2000), minisatellite and microsatellites (Zietkiewicz et al., 1994; Adato et al., 1995; Sharon et al., 1995; Eiadthong et al, 1999) and Amplified Fragment Length Polymorphism (AFLP) (Eiadthong et al., 2000). But, the identification or differentiation of zygotic plantlet among nucellar ones in polyembryonic seeds was tried only with isoenzymes (Degani et al., 1993) or selecting a fragment related to polymebriony (López-Valenzuela et al., 1997) using RAPD.

[1] (Trabalho 10-2006). Recebido: 23-01-2006. Aceito para publicação: 22-09-2006.
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This paper represents one of the first attempts to identify zigotic among nucellar plantlets in polyembryonic seeds using RAPD markers considering their fingerprints.

The objective of this work was to identify the type of mango plantlets (zygotic or nucellar) obtained from polyembryonic seeds of *Mangifera indica* , L. cv Rosinha using RAPD markers.

MATERIAL AND METHODS

Local - the study was developed at Embrapa Cerrados which is located in Planaltina (DF), Brazil at 15º35'3"S, 47º42'30" W and 1.100 meters above sea level (m.a.s.l.). The climate presents two well established seasons: a wet and hot one with a temperature range from 25ºC to 30ºC and 1.400 to 1.800 mm of total pluviometric precipitation per year (from September to April); and the other one a dry with a temperature range from 20ºC to 23ºC and 40% of average relative humidity (from May to August).

Genetic Material - seeds from an open pollinated Rosinha fruits grown at the fruit crop experimental area of Embrapa Cerrados was used as plant material. Mango seeds were germinated into polyetilene plastic bags of 40 cm height x 25 cm width x 0.2 mm thickness with lateral perforations, containing soil and sand mixture, in the proportion of 1:1 by volume. The germinated plantlets had been maintained at nursery conditions with irrigation and under 50% of shade. The plantlet position was identified as the number one as being the closest to the basal side and the others numbered in crescent order according to the distance from this side. It was done two experiments. In the first one we selected a sample of nine seeds harvested in 2002 where 3l plantlets germinated from them in total and were identified (zigotic or nucellar and their position). It constituted a preview experiment (Table 1). The second experiment was the one which aimed the plantlet origin identification for only the most vigorous one and their percentual analysis of appearance in the total sample. By so, it was selected fourty plantlets being eigth-month-old from seeds harvested during the years 2002 and 2003 (twenty from 2002 and twenty from 2003), using two parameters: diameter of trunk and height.

Plant DNA extraction and RAPD analysis - Leaves from all analyzed plantlets and its common female parent (Rosinha) were collected and immediately used to extract the genomic DNA as described by Doyle & Doyle (1990) with modifications (Faleiro et al.., 2003). After extraction, DNA concentration and purity was measured by a spectrophotometer (Sambrook et al.., 1989) and samples were diluted to 5 ng μL^{-1}. The DNA integrity was observed by electrophoresis in 0.8% agarose gel in TBE 1X buffer (Tris-Borate 100 mM, pH 8.3; EDTA 2 mM) containing 0.5 $\mu g.ml^{-1}$ of ethidium bromide.

DNA samples from each vigorous plantlets (40 plantlets in total obtained in 2002 and 2003), the female parent and also the plantlets from the first experiment were analyzed by RAPD as described by Welsh & McClelland (1990) and Williams et al. (1990) using 16 10-mer primers for the first experiment (OPD5, OPD15, OPD18, OPE1, OPE6, OPF3, OPF4, OPF6, OPF7, OPF8, OPG2, OPG7, OPG18, OPH5, OPH8, OPH19) (Operon Technol.) and 16 (OPD15, OPD18, OPE1, OPE6, OPF3, OPF4, OPF7, OPF8, OPG2, OPG6, OPG12, OPG13, OPG18, OPG19, OPH4, OPH19) (Operon Technol.) for the second experiment. The amplifications were conducted in a programmable thermocycler (MJ Research, Inc., model PTC100), using a program for 40 cycles of: 15 s at 94 ºC, 30 s at 35 ºC and 90 s at 72 ºC. After the 40 cycles, a final extension step was added during 6 min. at 72 ºC. The amplification reaction contained 15 ng of one DNA, Taq buffer one time concentrated, 1 U Taq polymerase (Invitrogene, Inc.), 3 mM $MgCl_2$, 100 µM dNTP (Amersham) and 0.4 µM primer (Operon Technologies). After amplification the samples were analyzed in 1.2% agarose gels prepared in TBE 1X with ethidium bromide 0.5µg ml^{-1}. The electrophoretic process took place approximately in four hours at 85 volts. At the end of the run the gels were photographed (EDAS 120, Kodak) under UV light (Vilber Lourmat).

The type of plantlet (zygotic or nucellar) was evaluated by comparing the fingerprint of the female parent DNA sample and the different selected plantlets using the same primer. Different pattern of amplification characterized zygotic plantlets. Each 10-mer primer in the second experiment were analyzed in duplicates to confirm data and, to the final result were used only the most reproducible primers (OPE6, OPF3, OPF7, OPG6, OPG12, OPG13, OPG18, OPG19, OPH4). Moreover, the final gels were analyzed by three different people and only the reproducible results were discussed. Plantlets were considered totally polymorphic if different in comparison to the female parent with more than 50% of final primers used or, at least three primers as considered by Novy et al. (1994) .

TABLE 1 - Localization, vigorousness and number of polymorphic primers of zygotic plantlets in polyembryonic Rosinha seeds.

Seeds	Number of plantlets	Position*	Vigour	Number of polymorphic markers
1	3	2	High	4
2	3	3	High	7
3	3	3	High	5
4	3	3	Low	4
5	3	2	Low	3
6	4	4	High	8
7	4	3	Low	6
8	4	3	High	3
9	4	2	High	4

*Position 1 closest to the basal side of the seed

TABLE 2 - Total number of primers which characterized zygotic or nucellar plantlets in 2002 samples using a RAPD diagnostic test.

Year	a	b	C	D
2002	1	1	2	0
	2	1	4	0
	3	3	2	0
	4	2	5	0
	5	1	6	0
	6	3	4	0
	7	3	5	0
	8	1*	6	1
	9	4	5	1
	10	3	3	1
	11	1	4	0
	12	3	6	0
	13	1	6	0
	14	1	5	0
	15	3	4	1
	16	1	6	0
	17	1	5	0
	18	2	4	0
	19	3	4	0
	20	1*	4	0

* monoembryonic seeds, a number of analyzed plantlets, b position of vigorous plantlet in the seed,
c number of polymorphic primers, d number of monomorphic primers

TABLE 3 - Total number of primers which characterized zygotic or nucellar plantlets in 2003 samples using a RAPD diagnostic test

Year	a	b	C	D
2003	1	1	5	2
	2	2	4	0
	3	2	7	1
	4	1	4	0
	5	3	5	0
	6	2	7	0
	7	1	4	0
	8	5	6	0
	9	1	5	0
	10	1	5	0
	11	2	5	0
	12	1*	2	0
	13	1	5	1
	14	1	3	3
	15	1*	4	0
	16	1	5	0
	17	2	5	0
	18	2	6	0
	19	1	7	0
	20	1	7	0

* monoembryonic seeds, a number of analyzed plantlets, b position of vigorous plantlet in the seed,
c number of polymorphic primers, d number of monomorphic primers

RESULTS AND DISCUSSION

Sachar & Chopra (1957) cited by Srivastava et al. (1988) described the zygotic plantlet as being the weakest in polyembryonic mango seeds because it probably degenerates due to competition with nucellar plantlets. In this paper it was observed an opposite result. The pattern of amplified fragments obtained by RAPD technique, in comparison to the female parent, demonstrates that the zygotic plantlet might be the vigorous one (Table 1). Moreover this results were confirmed in further analysis (Tables 2, 3). The vigour of the zygotic plantlet can be explained by a heterotic effect of crosses between the female plant and another unidentified mango tree nearby. Regarding the position of the zygotic plantlet, it is expected to be the first position, the one closest to the basal side of the seed which is the side of the peduncle. This position probably is the one in which favours the fecundation phenomenon in the embryo sac. However, it was observed that the zygotic plantlet can be found also in the second, third or fourth position (Table 1, 2, 3).

Concerning the identification and percentual of vigourous plantlets it was observed during 2002 and 2003 that almost all selected vigorous plantlets were considered polymorphic (zygotic) when compared to the female parent (Table 2, 3). A total of 50% and 70% plantlets in 2002 and 2003, respectively, were polymorphic considering more than 50% of primers used for the final result. It was also found that 40% and 20% plantlets were considered polymorphic with less than five primers. But, some of these plantlets could be also considered polymorphic according to Novy et al. (1994). These authors, using RAPD and studying the genetic relation among cranberry plants, reported that possibly only three primers are needed to differentiate them. Therefore, in the case of Rosinha plantlets during 2002 and 2003 it was expected at least 90% of vigorous plantlets being from zygotic type. These results might explain the tree disuniformity found at commercial mango orchards. Besides, the result reported by Degani et al. (1993) which refers to the zygotic plantlet as the one having in media more weight can favour the result described here, which means that the zygotic plantlet is the more vigorous one. The remaining 10% of plantlets were considered doubtful results as they showed differences only with two primers or equal number of primers polymorphic or monomorphic. These plantlets in which were found a few number of polymorphism could have the highest similarity with the female parent. They could produce more uniform rootstocks than the most polymorphic ones and then could be used to generate homogeneous grafted plants. However, more evaluations in the field are needed to confirm this supposition. Moreover, the result observed with Rosinha may be reproduced in other varieties, since in one-year study it was found a similar result for Comum do Cerrado, however it need more evaluations to confirm these data.

The RAPD described in this paper seemed to be useful as the pattern of fragment amplification at least in the final selected primers of the second experiment were reproducible. Moreover, the strategy of three different people gel analysis seemed to confirm the obtained reproducible data. Amplified fragment reprodutibility was also evaluated by Schnell et al. (1995) and López-Valenzuela et al. (1997). Both authors analyzed only reproducible bands in duplicate samples. The reaction conditions reported here are similar to Schnell et al. (1995) and also, the percentual of final reproducible primers corresponds to the number described by Valenzuela et al. (1997) (over 50% of total primers used).

Considering this two-year study, it can be expected that most of the vigorous rootstocks come from zygotic vigorous plantlets which could partly explain disuniformities among mango trees at commercial orchards. However this study should be repeated in different climate conditions and, with different varieties for more than two years in order to confirm the percentage of vigorous zygotic plantlets.

CONCLUSIONS

1) Zygotic plantlets may not be necessarily in the first position or considered the weakest in the mango polyembryonic seed;

2) 90% of vigorous plantlets analyzed in 2002 and 2003 were considered zygotic, and the remaining 10% were considered ambigous;

3) The RAPD markers can be used as a diagnostic tool to identify plantlets in polyembryonic seeds but, at least duplicates and reproducible primers should be utilized to enhace the working efficiency;

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