Embryo rescue from interspecific crosses in apple rootstocks

Adriana Cibele de Mesquita Dantas[1], José Itamar Boneti[2], Rubens Onofre Nodari[1] and Miguel Pedro Guerra[1]

[1]Universidade Federal de Santa Catarina, Dep. de Fitotecnia, Lab. de Fisiologia do Desenvolvimento e Genética Vegetal, Rod. Admar Gonzaga, nº 1.346, Itacorubi, CEP 88034-001 Florianópolis, SC. E-mail: acmdantas@cca.ufsc.br, mpguerra@cca.ufsc.br, nodari@cca.ufsc.br [2]Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina, Estação Experimental de São Joaquim, Rua João Araújo Lima, nº 102, Jardim Caiçara, Caixa Postal 81, CEP 88600-000 São Joaquim, SC. E-mail: boneti@epagri.rct-sc.br

Abstract – The objetive of this work was to rescue immature embryos of apple rootstocks *Malus prunifolia* (Marubakaido) and *Malus pumila* (M9) after 40–60 days of pollination and to put them into MS culture media supplemented with agar (6 g L^{-1}) and casein hydrolysate (500 mg L^{-1}). Embryos originated from interspecific crosses and open pollination showed differences in the in vitro responses, depending on the female parent, the developmental stage of the embryo, and the culture medium composition. Embryos of the *M. pumila* rootstock, rescued within 40 days after pollination and put in culture medium supplemented with indolacetic acid (IAA), gibberellic acid (GA_3), kinetin and maltose, resulted in a normal development of plantlets. However, embryos originating from hand-pollination, cultivated in medium supplemented with 14 µM IAA, 5 µM kinetin and 1.5 µM GA_3 (MS_1), mainly those of *M. prunifolia* x *M. pumila*, showed a high percentage of rusted embryos (96.2%). Embryos from open pollination of *M. prunifolia* and *M. pumila* formed calluses. It was possible to identify the influence of the female parent by the enhanced development of *M. pumila* shoots derived from open or hand-pollination. The crossing of responsive species and the use of the technique of embryo culture provided a rapid and uniform germination and, consequently, the development of fully normal seedlings.

Index terms: *Malus*, Marubakaido, M9, culture medium, carbon source.

Resgate de embriões a partir de cruzamentos interespecíficos em porta-enxerto de macieira

Resumo – O objetivo deste trabalho foi resgatar embriões imaturos de porta-enxertos de macieira *Malus prunifolia* (Marubakaido) e *Malus pumila* (M9) depois de 40 e 60 dias de polinização e colocá-los em meio de cultura MS suplementado com ágar (6 g L^{-1}) e hidrolisado de caseína (500 mg L^{-1}). Embriões originados do cruzamento interespecífico dirigido e de polinização aberta mostraram diferenças significativas in vitro, tendo sido observadas diferenças quanto ao progenitor feminino, quanto à fase de desenvolvimento do embrião e à composição do meio de cultura. Embriões do porta-enxerto *M. pumila*, resgatados aos 40 dias depois da polinização e colocados em meio de cultura suplementado com ácido indolacético (AIA), ácido giberélico (GA_3), cinetina e maltose, resultaram em desenvolvimento normal das plantas. Porém, foi observada alta porcentagem de embriões oxidados (96,2%), originados de polinização dirigida, cultivados em meio com 14 µM de AIA, 5 µM de cinetina e 1,5 µM de GA_3 (MS_1), principalmente para o cruzamento *M. prunifolia* e *M. pumila*. Embriões de polinização aberta de *M. prunifolia* x *M. pumila* formaram calos. Foi possível identificar a influência do progenitor feminino, pelo incremento de brotações de *M. pumila* derivada de polinização aberta e dirigida. O cruzamento de espécies responsivas e o uso da técnica de cultura de embrião promoveram germinação rápida e uniforme e, por conseguinte, o desenvolvimento de mudas normais.

Termos para indexação: *Malus*, Marubakaido, M9, meio de cultura, fontes de carbono.

Introduction

The juvenile period in apples is significantly reduced by grafting on dwarfing rootstocks (Visser, 1970). When the juvenile period is shortened, the time from crossing to seed germination constitutes a relatively larger part of the breeding cycle. It may, therefore, be of interest to investigate whether this period can also be shortened (Hansche, 1983).

The continuous demand for new plant materials requires the upgrading of traditional breeding methods to accelerate the production of new and improved genotypes. Biotechnological tools and traditional breeding techniques may successfully be coupled. These new

strategies allow the early selection of useful traits in apple trees, while avoiding the problems of the long juvenile period and the long generation times (Roen, 1994).

The rescue of hybrid embryos resulted from intra and interspecific crosses is commonly applied in apple breeding programs aimed at increasing the efficiency of the seed germination and the number of individuals obtained through sexual hybridization (Rubio Cabetas et al., 1997).

To provide an efficient alternative for dormancy suppression, when isolated embryos can be rescued at different developmental stages (Ramming, 1990; Burgos Ortiz, 1997), the low-temperature treatment can be partly replaced by the use of gibberellin (GA_3), alone or in combination with cytokinin or auxin, added to the culture medium as a supplement (Zhang & Lespinasse, 1991; Dantas et al., 2002).

Interspecific hybrids are useful for the transfer of desirable genes from wild to cultivated species. However, in many cases, successful wide crosses between species are difficult to accomplish. Post-zygotic barriers, such as endosperm abortion, are commonly observed, but this has been overcome through the use of embryo rescue (Lu & Bridgen, 1996; Palmer et al., 2002).

The objective of this work was to evaluate the application of embryo rescue technique and the effect of different growth regulators and carbon sources on the origin of embryo and time of rescue of M9 apple rootstocks (*Malus pumila* Mill.) and Marubakaido (*Malus prunifolia* Borkh.).

Material and Methods

Forty and sixty days after the open and hand pollinations of *M. pumila* (M9) and *M. prunifolia* (Marubakaido), the young fruits were collected at the Experimental Station of Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina (Epagri), in São Joaquim, Santa Catarina State, Southern Brazil. For wide crossing, the flowers were emasculated and immediately hand-pollinated. Flowers were then closed with a paper bag. Flowers that were not hand-pollinated were subjected to open pollination by bees.

Seeds were removed from the fruits and immersed in ethanol 70% for 30 seconds, followed by treatment in sodium hypochloride 1.25%, for 15 minutes, after which they were washed with sterile distilled water three times and cut open aseptically. Embryos were then dissected out with the use of forceps. Intact embryos were sterilized by the same procedures and were put in test tubes (150x25 mm) containing 15 mL of MS (Murashige & Skoog, 1962) basal medium supplemented with the compounds described below. The cultures were incubated in the darkness at 25±2ºC, for the first ten days, and then transferred to a culture room under a 16-hours light period using a cool-white fluorescent lamp at 40 µmol m^{-2} s^{-1} light intensity.

Embryos of *M. pumila* with 40–60 days derived from open pollination were put into MS salts and vitamins, supplemented with myo-inositol (100 mg L^{-1}), agar (6 g L^{-1}) and casein hydrolisate (CH) (500 mg L^{-1}). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 minutes. Different types and levels of plant growth regulators and carbon sources used were: MS_1: indolacetic acid (IAA) (14 µM), kinetin (Kin) (5 µM), gibberellic acid (GA_3) (1.5 µM) plus sucrose (30 g L^{-1}); MS_2: GA_3 (1.5 µM) plus sucrose (30 g L^{-1}); MS_3: IAA (14 µM), Kin (5 µM), GA_3 (1.5 µM) plus maltose (30 g L^{-1}); and MS_4: GA_3 (1.5 µM) plus maltose (30 g L^{-1}).

Embryos were rescued after 60 days of pollination from open pollinated *M. pumila* and *M. prunifolia*, open pollinated *M. pumila* x *M. prunifolia* and hand-pollinated *M. prunifolia* x *M. pumila*. Embryos were put in MS basal medium, supplemented with agar (6 g L^{-1}), maltose (30 g L^{-1}), CH (500 mg L^{-1}) and two combinations of plant growth regulators: 14 µM IAA, 5 µM Kin and 1.5 µM GA_3 (MS_1) and 1.5 µM GA_3 (MS_2).

All experiments were arranged in a completely randomized design, with five embryos per experimental unit and five repetitions per treatment. Following traits were evaluated after 60 days in culture: length of shoots and radicle, number of buds, calluses induction, oxidation rates and percentage of embryos germinated. The data were submitted to analysis of variance and SNK multiple range test at 5% of probability. No statistical analysis was carried out for data of the number and percentage of plantlets formed.

Results and Discussion

The rescue of *M. pumila* embryos after 40 and 60 days of open pollination resulted in values of 48 and 80% of seedling recovering, respectively. The greatest length of shoots (3.7 cm) was observed in response to the culture medium MS_1 (kinetin, GA_3 and IAA), for embryos rescued 40 days after the pollination (Table 1). The culture

media MS_1 and MS_3 (kinetin, GA_3 and IAA) promoted an enhanced growth of the shoots, when compared to the results obtained in response to the MS_2 and MS_4 (GA_3) media. Maltose was more efficient than sucrose as carbon source (Table 1).

Embryos rescued after 40 days and put in culture medium supplemented with maltose (MS_3 and MS_4) resulted in the highest number of buds, differing statistically from the results observed in the culture medium supplemented with sucrose (MS_1 and MS_2) (Table 1). MS_1 culture medium produced the longest roots, however, it provoked the highest rate of callus induction in the root (Table 2).

Successful production of seedlings through embryo culture largely depends on both the developmental stage of the embryo and the composition of the culture medium (Sharma et al., 1996). In the present work, immature embryos rescued after 40 days produced plantlets within four weeks of culture.

The best results for radicle emergence and seedling development resulted from the MS culture medium supplemented with Kin, GA_3 and IAA (MS_3). For *M. pumila*, the addition of either cytokinins and auxins or gibberellins enhanced the in vitro development of rescued embryos (Dantas et al., 2002). The promotion of precocious embryo germination promoted by growth regulators has also been reported in other species (Anderson et al., 1990; Bajaj, 1990; Das et al., 1999).

Maltose has proved to be superior to other carbon sources in the culture of isolated microspores (Scott & Lyne, 1995) and for the induction of somatic embryos (Ladyman & Girard, 1992). It is postulated that maltose maintains the osmolarity in the culture medium over the whole culture period due to its slow hydrolysis to glucose, while sucrose is rapidly hydrolyzed (Indrianto et al., 1999).

The rate of the germinated interspecific hybrids was higher in open pollinated *M. pumila* (60.4%) than in open pollinated *M. prunifolia* (47.9%). The crossing between *M. pumila* and *M. prunifolia* resulted in 26.7% of germinated embryos. For the reciprocal cross, the rate of germination was 12.8%. From open pollination condition, *M. pumila* resulted in 52% of plantlets (Table 3).

Rescued embryos from the open pollination of *M. prunifolia* and *M. pumila* put in MS_1 culture medium showed rates of callus induction of 23.8 and 12.5%, respectively (Table 4). In general, embryos originating from open pollination were more efficient in the calluses induction than those resulting from hand-pollination.

The different in vitro responses of genotypes may be related to the nature of the parental species and the degree of compatibility between parents. Genotypic differences in the capacity of cultured immature embryos were found in wheat (*Triticum aestivum*) (Maddock

Table 1. Length of shoots and number of buds from embryos of apple rootstock *Malus pumila* rescued 40 and 60 days after open pollination and cultivated in vitro[(1)].

Medium	Days after pollination			
	Length of shoots (cm)		Number of shoots	
	40 days	60 days	40 days	60 days
MS_1	3.7aA	0.0dB	8.6bA	0.0cB
MS_2	2.2bA	1.0cB	8.1bA	3.0bB
MS_3	2.7bA	3.4aA	23.0aA	10.4aB
MS_4	2.6bA	2.2bA	21.0aA	6.0bB

[(1)]Averages followed by the same lower case letter, comparing the medium, and capital letters, comparing 40 and 60 days, do not distinguish each treatment from the test SNK at 5% of probability.

Table 2. Length of roots in embryos of the rootstock *Malus pumila* from open pollinated plants and cultivated in vitro[(1)].

Medium	Length of roots (cm)	Callus formed (%)
MS_1	3.0a	26.1a
MS_2	2.4ab	10.0b
MS_3	2.3ab	10.0b
MS_4	2.1b	8.9b

[(1)]Averages followed by the same letter are not different by SNK test at 5% of probability.

Table 3. Relative efficiency of embryo cultures from *Malus* isolated 60 days after pollination.

Origin of the embryo	Percentage of embryos germinated[(1)]	Number of plantlets formed	Percentage of plantlets formed
M. pumila open pollination	60.4a	26	52
M. prunifolia open pollin.	47.9ab	18	36
M. pumila x *M. prunifolia*	26.7bc	12	24
M. prunifolia x *M. pumila*	12.8c	5	10

[(1)]Averages followed by the same letter are not different by SNK test at 5% of probability.

Table 4. Percentage of callus formed and oxidation in embryos rescued from apple rootstocks in open and hand pollinations[(1)].

Origin of the embryo	Percentage of callus formed		Percentage of oxidation	
	MS_1	MS_2	MS_1	MS_2
M. pumila open pollination	12.5bA	0.0aB	0.0cA	0.0bA
M. prunifolia open pollination	23.8aA	0.0aB	59.6aA	0.0bB
M. pumila x *M. prunifolia*	10.6cA	0.0aB	12.3bA	0.0bB
M. prunifolia x *M. pumila*	0.0dA	0.8aA	62.7aB	96.2aA

[(1)]Averages followed by the same small letter, comparing the pollinations among culture media, and capital letters, comparing the medium inside each pollination type, are not different by SNK test at 5% of probability.

et al., 1983). Kapila & Sethi (1993) reported that in vitro responses such as the percentage of embryo germination, callus development and plantlet regeneration were affected by parental wheat genotypes in the embryo rescue of bread wheat x hexaploid triticale hybrid.

In *Alstroemeria* the percentages of embryo germination, and callus and shoot developments were significantly affected by the parental genotype in the interspecific crosses (Lu & Bridgen, 1996). In the present study, genotypic differences in the in vitro response of immature hybrid embryo were observed.

Embryos rescued from the *M. prunifolia* and *M. pumila* cross resulted in a 96.2% oxidation rate in culture medium MS_2, differing statistically from results observed in the culture medium MS_1 for the same cross and also from open pollinated *M. prunifolia* (Table 4).

The stem axis length of plantlets obtained from *M. pumila* x *M. prunifolia* embryos cultivated in MS_1, or originating from open pollinated *M. pumila* cultured in MS_2, showed the values 2.5 cm and 3.0 cm, respectively (Table 5). For both rootstocks originated from open pollination, the observed values were higher in plantlets cultured in MS_2 compared to the values observed in MS_1 culture medium.

Embryos resulted from *M. pumila* x *M. prunifolia* presented the greatest number of buds (11.5) when cultured in MS_1. Rescued embryos resulting from open pollination demonstrated that they were able to differentiate according to the medium used, since those from *M. pumila* in MS_2 showed the highest number of buds (4.4) (Table 5).

The results obtained in the present work showed the female progenitor and the genotype influences on the morphogenetic responses. For example, in *M. pumila*, the presence of normal seeds was observed, while for *M. prunifolia* a number of aborted embryos were verified. The supplementation of the culture medium with a source of auxin (IAA), cytokinin (kinetin) and gibberellin (GA_3) was effective in increasing the germination rates of rescued embryos. However, in some cases, the presence of enlarged hypocotyls, epicotyls and calluses was observed.

The little production of plantlets resulting from the rescue of immature hybrid embryos is to be expected, and a number of previous studies have reported this occurrence in apples (Dantas et al., 2002) as well as in other species (Anderson et al., 1990; Palmer et al., 2002). The failure of many of the immature hybrid *Malus* rescued embryos to germinate is common in noncompatible interspecific crosses, possibly due to the often reduced viability to rescue embryos, which happens because of the activation of post-zygotic abortion mechanisms (Bajaj, 1990).

Apple is self-incompatible and need allelic polymorphism for the fertilization and subsequent embryo and fruit developments. Several hypotheses have been proposed to explain the failure of the normal embryo development in interspecific crosses, such as endosperm breakdown or collapse (Camadro & Masuelli, 1995).

The results reported here are valuable for breeders, since crossing of responsive species and using of the technique of embryo rescue culture can transfer desirable genes to different species. Further extension of the cross range within the *Malus* genus can be successfully achieved by embryo rescue techniques, but this rests mainly on the development of techniques to overcome pre- and post-fertilization barriers involved in the interspecific hybridization process (Palmer et al., 2002).

Table 5. Length of shoots and number of buds derived from embryos rescued from rootstocks in open and hand pollinations[(1)].

Origin of the embryo	Length of shoots (cm)		Number of buds	
	MS_1	MS_2	MS_1	MS_2
M. pumila open pollination	0.7bB	3.0aA	1.8cB	4.4aA
M. prunifolia open pollination	1.5bA	2.2bA	5.0bA	5.0aA
M. pumila x *M. prunifolia*	2.5aA	0.0cB	11.5aA	0.0bB
M. prunifolia x *M. pumila*	1.0bA	0.0cB	4.0bA	0.0bB

[(1)]Averages followed by the same small letter, comparing the pollinations among each medium, and capital letters, comparing the medium inside each pollination system, are not different by SNK test at 5% of probability.

Conclusions

1. Embryos rescued 40 days after the pollination, in culture medium MS_3 perform better development in the number of buds and root lengths.

2. Embryos originated from open pollination are more potent in the induction of calluses than those resulted from hand-pollination.

3. The apple rootstock *Malus pumila* shows a better number of normal seeds, while *M. prunifolia* presents a number of aborted embryos.

4. Embryos resulted from interspecific crosses *M. pumila* x *M. prunifolia* present the higher number of buds.

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Received on February 17, 2005 and accepted on February 24, 2006