Significant effect of accidental pollinations on the progeny of low setting *Prunus* interspecific crosses

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Summary

The incidence of fortuitous pollination on interspecific hybridizations of the plum rootstock Myrobalan with the apricot cultivars 'Moniquí' and 'Moniquí Borde' was assessed in this work. Progeny was originated through hand pollination of emasculated flowers of three Myrobalan clones, without bagging, in 1998 and 1999. Fruit set was low and variable among years (1.8-8.0%), but higher than the level of accidental pollination measured with emasculated and non-pollinated flowers (1.2%). Molecular characterization of the progeny was performed with three SSR markers showing that only 28% of the seedlings, obtained by in vitro germination and culture of immature embryos, were hybrids. This represents a lower percentage than expected, and is explained here by the low viability of hybrid embryos and seedlings. The use of molecular markers is discussed in terms of a convenient method for an early identification of putative hybrids in breeding programs with low setting crosses, where the proportion of non-hybrids is magnified.

Introduction

Interspecific crosses to transfer interesting traits among species are common tools in fruit tree breeding programs (Layne & Sherman, 1986). However, genetic barriers to hybridization are often an obstacle for the obtention of interspecific hybrids (Perez & Moore, 1985) resulting, in most cases, in low ovule fertilization, high embryo abortion, or high fruit abscission rates. Embryo abortion and fruit abscission are frequently the result of postzygotic incompatibility barriers (Stebbins, 1958) that make seeds unviable. However, the development of embryo rescue techniques applied after fertilization has facilitated hybrid obtention (Ramming, 1990), allowing embryo development and *in vitro* germination of unviable seeds, becoming a useful tool in *Prunus* breeding programs (Arbeloa et al., 2000, 2003).

Hybridization is commonly performed by hand pollination of emasculated flowers both to avoid self-pollination and to make flowers unattractive to pollinator insects (Free, 1964), thus increasing the chances of ovule fertilization by the desired pollen. Bagging emasculated flowers to avoid unwanted pollination is a safe practice although it is labour demanding and, consequently, it is often omitted in situations where the risk of accidental pollination is considered as reasonably low (van der Zeet et

al., 1977; Moore & Janick, 1983; Sedgley & Griffin, 1989). Accidental pollinations of emasculated flowers have been reported at low levels (2-5%) in apricot (Viti et al., 1997) or Japanese quince (Kaufmane & Rumpunen, 2002) and as negligible in mango or poplar (Singh et al., 1980; Houston & Joehlin, 1989). However, this situation may be more unfavourable in circumstances where final fruit setting is very low such as interspecific crosses.

Until the development of molecular marker techniques, the assessment of fertilization with unwanted pollen could only be made with morphological traits, but this is only viable when the contamination occurs with pollen from genotypes very different from those used in the controlled pollinations. Moreover, in cases where morphological traits could discriminate among wanted and unwanted crosses, phenotypic traits are usually expressed late in development and they are subject to environmental influences. Consequently, molecular marker techniques are an important tool to speed and increase the accuracy of paternity assessment in controlled crosses. Several molecular marker techniques have been applied for paternity analysis in fruit tree species (Wünsch & Hormaza, 2002). However, microsatellites or simple sequence repeats (SSRs) have become the markers of choice for fingerprinting and paternity analyses in most plant species (Gupta & Varshney, 2000) due to their high polymorphism, codominancy and reproducibility. Over 100 microsatellite markers have been so far reported in peach (Cipriani et al., 1999; Testolin et al., 2000; Sosinski et al., 2000; Aranzana et al., 2002, 2003; Dirlewanger et al., 2002) and many of them are transferable to other related species (Hormaza, 2002; Wünsch & Hormaza, 2002).

In this work, the incidence of fortuitous pollinations on interspecific hybridizations of Myrobalan (*Prunus cerasifera* Ehrh.) x apricot (*Prunus armeniaca* L.) crosses was assessed. SSR markers were used in order to know the extent of fortuitous pollinations on these crosses to define advisable practices that can also be useful for other interspecific crosses, not only in *Prunus*, but also in other fruit tree genera.

Materials and methods

Interspecific hybridization

Flowers of three different clones (Mb1, Mb2 and Mb3) of Myrobalan (*Prunus cerasifera* Ehrh) were pollinated with pollen of the apricot (*Prunus armeniaca* L.) cultivars ‘Moniquí’ (M) and ‘Moniquí Borde’ (MB) two consecutive years. The trees are maintained at the orchard collection of the Experimental Research Station of Aula Dei, CSIC (Zaragoza, Spain). Flowering took place at the end of February. Myrobalan flowers at balloon stage were emasculated 1 day before anthesis to prevent pollination (Free, 1964) and flowers in earlier or later stages were eliminated from marked branches. Pistils were hand-pollinated the following day with pollen from the apricot cultivars. A total of 3969 flowers in 1998 and 5382 flowers in 1999 were hand pollinated in the three Myrobalan trees. Six different crosses were performed: Mb1xM, Mb2xM, Mb2xMB and Mb3xM in 1998, and Mb1xM, Mb1xMB, Mb2xM, Mb2xMB, Mb3xM and Mb3xMB in 1999 (Table 1, 2). Since emasculated flowers were unattractive to insects (Free, 1964), no bagging was applied to pollinated branches.

Embryo rescue and in vitro culture

Immature Myrobalan fruits from the hand-pollinated flowers were harvested 12 weeks after pollination. Immature embryos were extracted from fruits under aseptic conditions and cultured, for germination, in Chée & Pool (1987) medium without growth regulators (Daorden et al., 2002, 2004). After germination, shoot apices were excised from the seedlings and multiplied in a modified MS (Murashige & Skoog, 1962) medium adding 1.19μM thiamine-HCl, 5μM Benzylaminopurine and 0.5μM Indolbutiric acid (IBA). Germination and survival rates were recorded for each treatment. Shoots were later rooted in vitro in half MS medium with 5μM IBA (Arbeloa et al., 2003). Micropropagated plants were acclimatized (Marín, 2003) and finally transplanted to orchard conditions.

Fruit set assessment

In order to assess the level of accidental pollinations of the three Myrobalan clones, fruit set was determined in 1) emasculated non-pollinated flowers, 2) emasculated and self-pollinated flowers and 3) non-emasculated open-pollinated flowers in each Myrobalan clone (Mb1, Mb2 and Mb3). A set of 416, 558 and 488 emasculated flowers of Mb1, Mb2 and Mb3 respectively was non-pollinated and another set of 478, 566 and 471 flowers, respectively, were self-pollinated, and finally, a set of 509, 463 and 688 flowers, respectively, was controlled for fruit set of open-pollinated branches. Flowers were counted weekly from anthesis until harvest. Fruit set was determined for each treatment and clone.

Progeny characterization

The parental genotype clones, three Myrobalan and two apricots, as well as the putative hybrids, 35 plants grown in the greenhouse and 184 seedlings from in vitro growing shoots, were analysed with SSRs to identify the hybrid status of the progeny. Progeny of every cross combination was studied and the number of analysed seedlings depended on the material available at the moment of the analysis (Table 1, 2). Genomic DNA from all the plant material was isolated according to Hormaza (2002).
For the SSR analysis, the parental genotypes were initially screened with eight SSR loci previously developed in peach by Cipriani et al. (1999) and Sosinski et al. (2000), that have already been shown to be conserved in apricot (Hormaza, 2002) and Myrobalan (Serrano et al., 2002) genotypes: pchgms2, UDP96-003, UDP96-005, UDP96-008, UDP96-018, UDP98-405, UDP98-406 and UDP98-409. Three SSR loci (pchgms2, UDP96-003 and UDP96-008), polymorphic among the parental genotypes, were selected to confirm the hybrid origin of progeny. PCR reactions for SSR amplifications were carried out in 20 μL volumes containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM each dNTP, 0.2 μM each primer, 40 ng genomic DNA and 0.45 units *Taq* polymerase (Invitrogen, Carlsbad, CA), using the following temperature profile: an initial step of 2 minutes at 94 °C, 35 cycles of 45 seconds at 94 °C, 45 seconds at 57 °C and 1 minute at 72 °C, and a final step of 5 minutes at 72 °C. Microsatellite PCR products were separated by electrophoresis using 3% Metaphor (FMC Bioproducts, Rockland, ME) agarose gels in 1X TBE buffer at 5V/cm, stained with ethidium bromide and visualised under UV light. Band scoring was carried out using a standard 10 bp DNA ladder (Invitrogen). Assuming Mendelian inheritance of the SSR loci, each of the progeny genotypes analysed was considered to be hybrid when one of the two SSR alleles amplified with each loci was the same as one of the two

alleles in the maternal genotype and the other was the same as one of the SSR alleles found in the paternal genotype (Figure 1).

Results

In 1998, from a total of 3969 flowers pollinated either with Moniquí or Moniquí Borde pollen, 71 fruits (1.8 % fruit set) were obtained. From these fruits, 67 immature embryos were isolated and placed to germinate *in vitro* (Table 1). An average of 78% of the embryos developed into seedlings and were later maintained *in vitro* through subsequent cultures. During the post-germination growth and multiplication phases, 20% of the genotypes were lost after one year of *in vitro* multiplication. Some of them died soon after germination, once seedlings were transplanted to the culture room, and the rest died along the multiplication period (Table 1)

Table 1

In 1999, from a total 5382 flowers pollinated either with Moniquí or Moniquí Borde pollen, 431 fruits (8.0 % fruit set) were obtained. Immature embryos were isolated and placed *in vitro* onto culture medium to germinate. Germination took place in an average of 85% of the embryos. During post-germination growth and multiplication phases 20% of the genotypes were lost in a similar way than in 1998 (Table 2).

Table 2

The assessment of the level of accidental pollinations was performed on emasculated flowers. Average fruit set of the three maternal clones in non-pollinated flowers was 1.1%, similar to that of self-pollinated flowers (0.9%), whereas it rose up to 11.5% in open-pollination conditions (Table 3).

Table 3

Progeny characterization

The SSR loci pchgms2, UDP96-003 and UDP96-008, polymorphic for the Myrobalan and apricot clones, were used to confirm the hybrid origin of the progenies (Table 4). From the 219 progeny genotypes evaluated (Table 1, 2), 61 showed hybrid origin and 158 did not (Figure 1). The percentage of hybrids confirmed by microsatellites, among the progeny in both years was 37.1% in 1998 and 26% in 1999.

Table 4
Figure 1

Discussion

In this work we have studied the impact of accidental pollinations in interspecific crosses of three Myrobalan plum clones hand-pollinated with pollen from two apricot cultivars, Moniquí and Moniquí Borde. We have found a high proportion of non-hybrid plants, derived from these fortuitous pollinations. The low fruit settings obtained in these controlled pollinations is due to the incongruity between the applied apricot pollen and Myrobalan (Perez & Moore, 1985). As a consequence, fertilization of Myrobalan ovules by accidental pollen that manage to reach the stigmas will be favoured against the apricot applied pollen.

Lack of protection of flowers through bagging pollinated branches is likely to increase the chance of accidental pollinations. However, bagging of flowers does not guarantee the lack of undesired pollinations, since accidental pollinations have been found even in cases where flowers were bagged (de la Rosa et al., 2004; Neal & Anderson, 2004). Many breeding programs avoid covering the flowers (van der Zeet et al., 1977), since breeders consider that a small level of contamination is an acceptable

price to pay for cost and time saving (Sedgley & Griffin, 1989; Moore & Janick, 1983). In our case, accidental pollination are very low considering the total pollinated flowers (around 1% of the total number of hand-pollinated flowers): 22 non-hybrid seedlings from to 3696 pollinated flowers in 1998 and 158 non-hybrid seedlings from 5382 pollinated flowers in 1999. This suggests that fortuitous pollinations could be a rare phenomenon that goes usually unnoticed in most fruit breeding programs.

However, this small percentage of non-hybrid seeds resulted magnified later due to the advantages of non-hybrid versus hybrid embryos along the process of setting, seed development, seed germination and seedling growth. First, interspecific hybrid seeds are difficult to obtain since reproductive isolation mechanisms may act at the pre- or post-fertilization levels by arresting embryo and/or endosperm development (Bushell et al., 2003). On the other hand, non-hybrid seeds do not suffer incompatibility barriers compared to hybrid seeds, thus most of the accidental pollinated flowers yielded viable seeds. Second, in vitro germination and multiplication are also advantageous for non-hybrid seeds. On the one hand, germination success is highly dependent on seed size (Ramming, 1990; Burgos & Ledbetter, 1993) and, thus, higher germination rates are obtained with larger seeds. Non-hybrid seeds are regularly larger than hybrid seeds since they do not undergo developmental arrest due to post-zygotic incompatibility barriers (Daorden et al., 2002, 2004). A significant percentage of embryos were smaller than 8 mm at harvest, what is considered as abortive seeds (Arbeloa et al., 2000). In optimal in vitro germination conditions, germination rate was 95.6% for embryos larger than 8 mm (non-abortive seeds) and 60% for the embryos smaller than 8 mm (abortive seeds) (Daorden at al., 2002; Arbeloa et al., 2003; Daorden et al. 2004). Moreover, whereas in vitro multiplication of Myrobalan is easy and rapid (Hammerschlag, 1982) apricot is considered a recalcitrant species (Marino et al., 1993). Consequently, due to their apricot genetic inheritance, hybrid seedlings could die along the *in vitro* multiplication phase in a higher proportion than non-hybrid seedlings. We have shown that 20% of the seedlings are lost after one year of cultivation *in vitro*, and most of them are presumed to be hybrid ones. As a result, in this work, low percentages of hybrid plants were identified following the characterization process. Due to the magnification of the rates of non-hybrid seedling obtention along the process, accidental pollinations in this type of crosses should be carefully traced.

The pollen origin of accidental pollinations may be diverse and, among different sources, self-pollination is an acceptable possibility. However Myrobalan has been described as a total or partial self-incompatible species (Layne & Sherman, 1986; Esmenjaud et al., 1996; Lecouls et al., 1999); this is in agreement with the low fruit set rate obtained in self-pollinated flowers. In emasculated but non-pollinated flowers accidental pollinations also occurred since an average of 1.1% of the flowers set fruit, a value close to that obtained in self-pollinated flowers (0.9%) indicating that a similar accidental process could happen. Consequently, it is likely that pollen for accidental pollinations in both cases arrives by wind or gravity from adjacent trees as it has been shown in other species (Visser & Vergaegh, 1980).

We have found that most of the progeny from interspecific crosses appeared to be non-hybrid seedlings. Consequently, accidental pollinations should be carefully avoided. One option is to bag the flowers before and after the application of the desired pollen; however, this technique is costly and time consuming. Another alternative is to carry out early hybrid identification in breeding programs with low setting crosses. Thus, microsatellites applied to paternity identification have revealed here as a very useful tool as in other species (de la Rosa et al., 2004). A quick test on tissues of the cotyledon after embryo isolation (Hormaza, 1999) would allow screening for hybrids in

a very early stage of the selection process, since embryos can germinate *in vitro* even without cotyledons (Arbeloa et al., 2000). This would allow the elimination of non-hybrid seeds at early stages avoiding tedious techniques as bagging or morphological hybrid identification.

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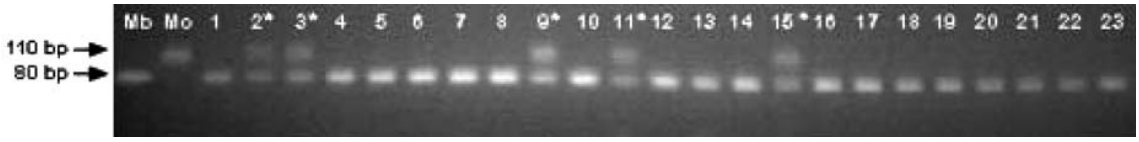

*Figure 1. - PCR amplification of the SSR locus UDP96-003 in Myrobalan and Moniquí parental genotypes, and in 23 progeny genotypes. Amplification of one allele of 80 bp in Myrobalan (line Mb), one allele of 110 bp in Moniquí (line Mo), two alleles of 80 and 110 bp in hybrid progenies (lines 1 to 23 marked with *) and a single allele of 80 bp in non hybrid progenies (lines 1 to 23 not marked with *).*

Table 1. - Number of pollinated flowers, fruit set, in vitro germination rate, survival of seedlings, and SSR analysed seedlings in 1998

	Number of pollinated flowers	Number of fruits	Fruit set (%)	Germinated seeds	Germination rate (%)	Growing seedlings (1 year later)	SSR analysed seedlings
Mb1 x M	1072	13	1.2	10	83	8	8
Mb2 x M	567	26	4.6	18	72	15	14
Mb2 x MB	1130	19	1.7	13	76	13	9
Mb3 X M	1200	13	1.1	8	62	7	4
Total	3969	71	1.8	49	78	43	35

Table 2. - Number of pollinated flowers, fruit set, in vitro germination rate, survival of seedlings, and SSR analysed seedlings in 1999

	Number of pollinated flowers	Number of fruits	Fruit set (%)	Germinated seeds	Germination rate (%)	Growing seedlings (1 year later)	SSR analysed seedlings
Mb1 x M	912	40	4.4	36	90	30	25
Mb1 x MB	870	33	3.8	30	88	24	19
Mb2 x M	630	21	3.3	9	64	3	2
Mb2 x MB	859	8	0.9	5	71	5	2
Mb3 X M	1202	159	13.2	142	88	96	59
Mb3 x MB	909	170	18.7	144	86	132	77
Total	5382	431	8.0	366	85	290	184

Table 3. - Fruit set (%) in the three Myrobalan clones (Myrobalan 1, 2 and 3) following open-, non- or self-pollination of flowers.

	Myrobalan 1	Myrobalan 2	Myrobalan 3	Mean
Open-pollination	4.4	22.2	10.4	11.5

Non-pollination	0.5	0.7	2.4	1.1
Self-pollination	0.2	1.4	1.1	0.9

Table 4. - SSR alleles detected in the paternal genotypes.

SSR locus	**Myrobalan**	**Moniquí**	**Moniquí Borde**
pchgms2	130/140/160 bp	150/160 bp	150/160 bp
UDP96-003	80 bp	110 bp	110 bp
UDP96-008	120 bp	130 bp	130 bp