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Two methods of haploidization in pear, *Pyrus communis* L.: greenhouse seedling selection and in situ parthenogenesis induced by irradiated pollen

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Abstract. Seedlings of 12 crosses involving pear varieties or hybrids were observed for the presence of haploid plants. On the basis of phenotypic characteristics, 17 plants corresponded to the haploid condition and, of these, 12 were determined by chromosome counting to be haploid ($2n = x = 17$). In addition, and in order to induce in situ parthenogenesis, several pear varieties were pollinated with a selected clone carrying a homozygous dominant marker gene for the colour of red. This pollen had previously been irradiated with γ-rays of cobalt 60 at 0, 200, 250 and 500 Grays. The immature embryos were cultured in vitro, whereby 1 haploid and two mixoploid plants were obtained. Numerous diploid plants with the maternal phenotype were also obtained, and their genetic origin was subsequently studied by means of isozyme analysis.

Key words: Pear – Haploids – Irradiated pollen – In situ parthenogenesis

Introduction

The improvement of perennial fruit trees through traditional breeding methods is a long-term effort because of their long generation time and high level of heterozygosity. The production of haploid plants should offer new possibilities for genetic studies and reduce the time required for breeding. Homozygous clones may also be of interest for the production of uniform rootstocks by seed propagation from the F1 stage.

The most advanced haploid research studies in perennial species have involved *Malus* ssp (Zhang et al. 1990; Zhang and Lespinasse 1992). As regards the obtaining of haploid plants via in vitro androgenesis, embryoids have been observed to result from anther cultures (Kubicki et al. 1975; Hidano 1982; Zhang et al. 1987), and androgenic plants have been reported by Fei and Xue (1981) and Xue and Niu (1984). The other method used successfully on apple to obtain haploids is in situ parthenogenesis, in which the use of irradiated pollen is coupled with the in vitro culture of immature seeds (Zhang and Lespinasse 1991). In pear, *Pyrus communis* L., Jordan (1975) induced multicellular structures after the in vitro culture of anthers. The production of spontaneous haploids on pear has been reported only once (Braniste et al. 1984), and this material died a few months later and was not doubled or used for any genetic study (N. Braniste, personal communication). This paper reports the production of haploid plants of pear.

Materials and methods

Screening of haploid plants

Twelve crosses were carried out for this study (Table 1). At ripening, the seeds were harvested and stratified for 3 months at 2 °C and then placed in the greenhouse for germination. The parents of the crosses used were: 'Abbé Fétel', 'Bautomne', 'Beurré' d'Anjou', 'Conférence', 'Delbias', 'Delwilmor', H03.68 ('Précoce de Trevoux' x 'Beurré précoce Morettini'), 'Harrow-Sweet', HW608 ('Williams' × US309), 'Mac', 'Notaire Lepin', P2105 ('Passe Crassane' × 'Grand Champion'), TNR12.40 ('Max Red Bartlett' × 'Royal Red Hardy'), TN19.25 ('Beurré Hardy' × 'Passe Crassane') and 'Williams'. TNR12.40 is a homozygous clone for the marker gene C coding for anthocyanin production (Decourtye 1967); its phenotype is completely red (leaves, wood and fruits).

Progenies were observed and haploid plants were determined on the basis of phenotypic characteristics. Haploid plants

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Table 1. Pear haploid plants obtained from several crosses

Cross	Observed seedlings	Plants with 'haploid phenotype'	Plants counted as x = 17/dead plants before counting	Haploid plants per 1000 observed seedlings
Harrow-Sweet × H03.68	1,019	5	5/0	4.91
Harrow-Sweet × TN19.25	1,248	4	1/3	0.80
Harrow-Sweet × Conference	1,203	2	1/1	0.83
Harrow-Sweet × P2105	1,028	1	1/0	0.97
Harrow-Sweet × Delwilmor	717	1	1/0	1.39
Harrow-Sweet × Beurré d'Anjou	1,187	1	0/1	0
Harrow-Sweet × Mac	1,171	1	1/0	0.85
Harrow-Sweet × Bautomne	840	0	–	–
Harrow-Sweet × Notaire Lepin	433	0	–	–
Harrow-Sweet × Abbé Fétel	604	0	–	–
Delbias × HW608	433	1	1/0	2.31
Williams × TNR12.40	195	1	1/0	5.13
Total	10,078	17	12/5	1.19

Table 2. Percentage of germination of pear pollen (TNR12.40) in vitro

Dose of irradiation	% of germination
0	74.13
125	57.38
200	58.58
250	55.24
500	54.03

are smaller than diploids because of their short internodes; they have also a slender stem and narrow, thin leaves of a pale green colour.

Induction of haploid plants through the use of irradiated pollen

The pollination experiments with irradiated pollen were based on previous work conducted on apple (Zhang and Lespinasse 1991). Four genotypes were chosen as female parents: 'Doyenné du Comice', 'Harrow-Sweet', 'Président-Héron' and 'Williams'. The male parent used throughout the experiments was TNR12.40.

Pollen was subjected to gamma irradiation, in small petri dishes (35 mm) from cobalt 60 at doses of 0, 125, 200, 250 and 500 Gy (Grays). After manual emasculation at stage F1 (Fleckinger 1964), pollination was performed on orchard trees isolated by a cage for each genotype. Three or four flowers per cluster were pollinated.

Fruits were collected 3 months after pollination. Immature embryos were removed from the seeds and disinfected according to Zhang et al. (1987). These immature seeds were cultured on medium MN+, which contains the macro salts of Lepoivre (Quoirin et al. 1977) plus a salts supplement (in $g\,l^{-1}$) of NH_4NO_3 (8), $MgSO_4 \cdot 7H_2O$ (7.2), KH_2PO_4(5.4) and $Ca(NO_3)_2 \cdot 4H_2O$ (12), and the micro salts of Lepoivre plus (in $mg\,l^{-1}$) thiamine (0.8), pyridoxine (1), myoinositol (10), nicotinic acid (1), glycine (4), benzylaminopurine (0.5) and indole butyric acid (0.1). All cultures were kept in a cold dark chamber at 3 °C, for 3 months to break the embryo dormancy, they were then transferred to a culture room at 24 °C with a 16 h light photoperiod (40–60 $\mu Mol\ m^{-2}\ s^{-1}$) for embryo germination and plant regeneration. All of the red plants were discarded. The green plants were kept, and their chromosomes were counted on root tips according to Lespinasse and Salesses (1973).

The pollen was germinated on a liquid medium containing 20% sucrose and 20 $mg\,l^{-1}$ boric acid at pH 5.7 (Calzoni et al. 1979) in petri dishes (55 mm) at 30 °C and 100% hygrometry. The germination rates were recorded 4 h later. Three replicates of 100 pollen grains each were observed.

The percentage of pollen-tube germination in vitro is summarised in Table 2. The effect of irradiation on the pollen was slight; the percentage of germination of non-irradiated pollen was 75%, whereas it was about 55% for the irradiated pollen whatever the dose of irradiation.

Isozyme analysis was performed on leaf samples according to the technique developed on apple (Chevreau and Laurens 1987).

Results

More than 10,000 seedlings were observed (Table 1). Of these 17 plants showed the haploid phenotype: 12 of them were determined by chromosome counting to be haploid (2n = x = 17), the other 5 died before chromosome counting could be performed. Out of the 12 surviving haploid plants 10 were obtained from crosses using 'Harrow-Sweet' as the female parent.

The rate of spontaneous haploid plants was relatively high; on average, it reached 1.19 per 1,000 observed plants. When at least 1 haploid plant was detected, the rate of spontaneous haploid plants reached 1.71 per 1000 observed plants. In the crosses 'Harrow-Sweet' X H03.68 and 'Williams' X TNR12.40, the number of haploid plants per 1,000 seedlings observed approached 5.

Results of in situ-induced parthenogenesis obtained with 'Doyenné du Comice' are shown in Table 3. The higher was the irradiation dose, the lower the number of embryos per fruit. With the untreated pollen, the number of embryos per fruit was genotype dependent. This num-

Table 3. Results of induced parthenogenesis on 'Doyenné du Comice' after pollen irradiation and in vitro culture of immature embryos

Genotype	Doses (Gy)	Fruits collected	Embryos cultivated	Number of embryos/fruit	Green diploid 2x=34	Haploid plants x=17	Haploid plants per 1,000 emb. cultivated
Doyenné du Comice	0	134	792	5.91	1	0	–
	125	208	1,045	5.02	11	0	–
	250	160	594	3.71	12	2 (x=17/34)	3.37
	500	421	78	0.18	5	1	12.82

ber was only 0.67 for 'Harrow-Sweet' (data not shown) but reached 5.91 for 'Doyenné du Comice'.

Only 1 haploid plant was obtained with the genotype 'Doyenné du Comice' at the dose of 500 Gy. With this same genotype, nevertheless, two mixoploid plants (2n=17/34) were also obtained, at a dose of 250 Gy. These two mixoploid plants may have originally been haploids that doubled spontaneously. Unfortunately, neither the haploid plant nor the mixoploid plants survived more than 3 months in vitro.

More than 50 green diploid plants were also obtained from in situ-induced parthenogenesis with the four genotypes. To establish whether or not these plants were of maternal origin, isoenzymatic analyses were performed on a sample of them. In our study, which involved 13 enzymatic systems, 61 band positions were registered. Among the 19 studied samples, polymorphism (presence/absence) was observed for 37 bands. Our interpretations are limited to the presence or absence of bands: the presence of any additional band compared to the control indicates that the origin of the plant cannot be maternal. For 3 plants from 'Doyenné du Comice' and 1 from 'Williams', all of the patterns were in agreement with the hypothesis of a maternal origin.

Discussion

'Harrow-Sweet' seems to be a favourable genotype for producing haploid plants. Indeed, 15 plants were obtained from the crosses involving this genotype as female parent, and 10 of these were determined by chromosome counts to be haploid (2n=x=17).

These pear haploid plants have proven to be very weak, and their survival rate is very low in the greenhouse. Only 1 haploid plant from the cross 'Delbias' X HW608 has survived until now, through conventional grafting on diploid pear seedlings. Nevertheless, the production of haploid plants from the genotype 'Harrow-Sweet' is promising. A method to transfer these haploid pear plants in vitro is under investigation.

These haploid plants arise through gynogenesis. Numerous cellular processes can be involved. For example, the sperm cell enters into the cytoplasm of the egg cell, but the fusion between the two nuclei does not occur and the plant originates only from the egg cell. This phenomenon was observed by Solntzeva (1978) on *Solanum*. Another mechanism was reported by Mogensen (1982) on barley: the sperm cell cannot enter into the egg cell and stays in the intracellular space; this phenomenon is under the control of the gene "hap" (haploid induction gene). The frequency of gynogenetic haploid plants is variable; Chase (1969) estimates it to be 0.1% in maize, but Lashermes and Beckert (1988) reported an increase in its frequency to 5% by using specific genotypes as the male parent. Zverzhanskaya et al. (1980) have reported the production of haploid plants of maize by the autonomous development of the egg cell and the central cell before fertilisation. Histological studies should be performed to determine the precise origin of the haploid pear plants.

Studies on the behaviour of apple irradiated pollen in vivo have demonstrated the capacity of pollen irradiated up to 1,000 Gy to germinate on the stigma, grow within the style and reach the embryosac (Carreau 1988). Other studies, also on apple, (Lecuyer et al. 1991) have shown that the pollen tube of irradiated pollen grew in the same way as that of control pollen, only more slowly. Sniezko and Visser (1987) observed the same phenomenon on pear; after irradiation at 500 Gy the pollen tube was slower to grow, but within 2 weeks as many embryos were initiated as in the case of untreated pollen.

In our study, with 'Doyenné du Comice', 1 haploid and two mixoploid plants were obtained, but none with the other genotypes (including 'Harrow-Sweet'). The number of embryos collected on 'Harrow-Sweet' was very low and is unlikely to be representative. The success of in situ parthenogenesis may be genotype dependent and, as this is the first time that haploid plants of pear have been obtained by this method, it will be very interesting to develop this methodology on several other genotypes. In any case, it is noteworthy that the promising results obtained with haploid plants from 'Harrow-Sweet' by normal crossing process are not correlated with those obtained through induced parthenogenesis with the same cultivar.

The occurrence of diploid plants which do not have a maternal origin could be explained by some outcross pollen that succeeded in entering under the cage despite the precautions taken. The presence of mixoploid plants amongst the green diploid plants supposed to be of maternal origin indicates that some may have doubled spontaneously.

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