A liquid 2,4-D pulse increased shoot and root regeneration from leaf explants of adult *Prunus* rootstocks

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Abstract

Regeneration of adult plant material is one of the main limitations for successful *Prunus* rootstock transformation. Results herein show that a liquid pulse (90 min) of 2,4-D (1.7 µM), applied to leaf explants, greatly improved shoot regeneration in Marianna 2624 (*Prunus cerasifera* x *munsoniana*) and Myrobalan 605 AD (*P. cerasifera*); and induced roots in Adafuel (*P. x amigdalopersica*) when placed in regeneration medium. Whole leaves and basal leaf explants of Marianna 2624 regenerated shoots in a higher proportion of explants after the pulse (up to 58.9% in whole leaves) than medium or tip leaf segments, whereas the leaf tip was the explant that showed less regeneration. In the regeneration medium, in the presence of BAP, NAA was more effective than 2,4-D. The application of an auxin pulse is a simple method that could enhance adult plant regeneration in commercial rootstocks.

Key words:

Leaf explant type, induction treatment, genotype effect, adventitious organ differentiation, auxin pulse.

Introduction

Regeneration of stone fruit trees is usually difficult to achieve and, while success has been obtained using juvenile tissues, few reports exist from mature plants (Scorza et al., 1995a). On the other hand, biotechnological gene transfer in fruit trees is a convenient technique, since conventional breeding methods find frequent endogenous barriers to interspecific hybridization that make difficult gene interchange between species (Perez and Moore, 1985). Thus, great effort is being made to get appropriate regeneration protocols for these plants and recent reports have been published for different *Prunus* species, as in almond (Ainsley et al., 2000), apricot (Perez-Tornero et al., 2000), cherry (Gutierrez-Pesce et al., 1998; Hammatt and Grant, 1998; Grant and Hammat, 2000; Tang et al., 2002), and peach (Gentile et al., 2002). Some of these works, as well as other previous reports on apricot and plum (Antonelli and Druart, 1990; Escalettes and Dosba, 1993) involved multiple steps and manipulations that include transfers to different media, successive changes in environmental conditions, and preconditioning of source plant material, suggesting that organogenesis is affected by several factors, some of them not precisely defined. This fact could explain in part the difficulty to reproduce a described protocol when changes are made in any of different factors affecting the plant material and origin, or even the laboratory environment.

A simple, reproducible, and effective protocol for these woody species is needed to apply biotechnology to mature plants successfully. However, differentiation of adventitious organs in adult plant material is difficult to achieve (Rajasekaran and Mullins, 1982). Promising results have been reached by using different strategies including (1) preconditioning of plant material that precedes the regeneration treatment (Sriskandarajah and Goodwin, 1998); (2) several changes in both lighting conditions and hormonal composition of the regeneration medium (Gentile et al., 2002); or (3)

inductive treatments prior to the regeneration phase (Antonelli and Druart, 1990). In a set of experiments searching for a reliable regeneration method for adult material (Pascual, 2003), we observed that an auxin pulse produced a clear effect improving regeneration. In fact, auxin is clearly involved in morphogenesis since it regulates plant cell division, elongation and differentiation (Chen, 2001). Here we test this in several *Prunus* spp., and report the positive effect of a liquid 2,4-dichlorophenoxyacetic acid (2,4-D) pulse on the adventitious regeneration of both, shoots and roots, as well as the effect of α -naphthalene acetic acid (NAA) and 2,4-D in the regeneration medium. This pulse can easily be adapted to other existing protocols, where different kinds of treatments can be applied.

Materials and Methods

Plant material and in vitro culture

Shoot cultures of different *Prunus* rootstocks, established in vitro more than one-year before from mature plants, were used. The rootstocks tested include the apricot A 843 (*P. armeniaca* L.), the almond x peach hybrid Adafuel (*P. x amigdalopersica* (Weston) Rheder), and the plum rootstocks Myrobalan 605 AD (*P. cerasifera* Ehrh.), Marianna 2624 (*P. cerasifera* Ehrh. x *munsoniana* Wight & Hdr.), and Adesoto 101 (*P. insititia* L.).

Effect of a liquid 2,4-D pulse

The effect of a liquid 2,4-D pulse on adventitious shoot or root regeneration was studied using leaf explants from in vitro shoots of different rootstocks. They were subcultured once a month into a modified DKW medium (Driver and Kuniyuki, 1984) with glycine (26.64 μ M), m-inositol (0.59 mM), thiamine-HCl (1.19 μ M), nicotinic acid (4.06 μ M),

pyridoxine-HCl (2.43 μM), benzyl amino purine (BAP, 5 μM), indol 3- butyric acid (IBA, 0.5 μM), and sucrose (30 $\text{g}\cdot\text{l}^{-1}$). pH was adjusted to 5.5 before adding agar (Difco-Bacto) at 7 $\text{g}\cdot\text{l}^{-1}$. Basal, medium and tip leaf explants were obtained by making two transverse cuts perpendicular to the midrib. Leaves were excised under the dissecting microscope to avoid taking stem tissues surrounding the petiole. Leaf explants were placed in Petri dishes (adaxial side up) on regeneration medium immediately (control), or after a short induction treatment (90 min) on liquid MS medium (Murashige and Skoog, 1962) supplemented with 2,4-D (1.7 μM). The regeneration medium consisted on a modified Quoirin & Lepoivre (1977) medium (QL), with half strength macro salts, thiamine-HCl (3 μM), NAA (2.6 μM), BAP (3.5 μM) and sucrose (20 $\text{g}\cdot\text{l}^{-1}$). PPM[®] ('Plant Preservative Mixture'; Plant Cell Technology, Washington, DC, USA) was added at 0.5 $\text{ml}\cdot\text{l}^{-1}$. The pH was adjusted at 5.8, and the medium was gellified with Phytigel (2.8 $\text{g}\cdot\text{l}^{-1}$). All leaves from one in vitro cultured shoot were used per treatment and two replicates were made, thus, a variable number of leaf explants were used: A 843, 12 and 12 leaves (control) and 12 and 14 leaves (2,4-D pulse); Marianna 2624, 20 and 15 leaves (control) and 16 and 14 leaves (2,4-D pulse); Myrobalan 605 AD, 14 and 14 leaves (control) and 12 and 16 leaves (2,4-D pulse); Adafuel, 25 and 30 leaves (control) and 20 and 21 leaves (2,4-D pulse); and Adesoto 101, 14 and 14 leaves (control) and 21 and 20 (2,4-D pulse). Regenerated organs were scored after a 90 days culture period.

Effect of the auxins NAA and 2,4-D in the regeneration medium after the pulse

The regeneration medium tested in the previous experiment was compared with a similar one but containing 2,4-D instead of NAA at the same concentration (2.6 μM). Leaves from different rootstocks grown on MS proliferation medium were used:

Adafuel, Adesoto 101, Marianna 2624, and Myrobalan 605 AD. Forty basal, medium and tip leaf explants, as well as 30 whole leaves per treatment were placed in Petri dishes (adaxial side up) after a short induction treatment (90 min) on liquid MS medium supplemented with 2,4-D (1.7 μ M) as in the previous experiment. The explants were subcultured to the same fresh media at 30 days intervals and regeneration was scored after 90 days. The experiment was repeated three times.

Data analysis

Adventitious regeneration was scored as the percentage of explants with shoot or root formation. A completely randomized design was applied and an ANOVA of the transformed data (arcsine transformation) was performed to study the treatment effects on regeneration. Treatment means were compared when appropriated with Duncan's Multiple Range test. SPSS statistical software (SPSS Inc., Chicago, USA) was used.

Results

Effect of a liquid 2,4-D pulse

A liquid 2,4-D pulse (90 min) of leaf explants placed in a liquid MS medium with 2,4-D (1.7 μ M) greatly increased adventitious shoot and root regeneration from leaf explants of different *Prunus* rootstocks, but regeneration was genotype dependent. Furthermore, while the same tissue –leaf explants– were used in all genotypes, a differentiated response was obtained. Whereas shoots were formed on callus tissues that grew on leaf explants of Marianna 2624 (Figure 1, A) and Myrobalan 605 AD, roots were formed in Adafuel (Figure 1, B), and in a less extent in Adesoto 101 explants. In contrast, in spite of the callus tissues formed, the apricot A 843 did not differentiate any shoot or root.

Marianna 2624 gave the highest percentage of explants showing shoot regeneration. The effect of the induction treatment, increasing shoot regeneration, was significant ($P < 0.01$) (Table 1, Figure 2 top). An average of 42.9% of basal explants exhibited shoot regeneration after the application of the auxin pulse, 2.8-fold that of control explants, and almost twice that of treated medium or tip leaf explants.

The pulse treatment in Myrobalan 605 AD basal leaf explants showed a similar positive effect on shoot regeneration that reached a 12.5%, more than 3-fold that of control explants. However, this effect was not found in medium- or tip-leaf explants (Figure 2, middle), and these differences were not statistically significant (Table 1).

Adafuel regenerated more roots after the induction treatment, being in basal explants more than 3-fold that of the control, in a similar way as the previous rootstocks, and this effect was statistically significant ($P < 0.05$) (Figure 2 bottom, Table 1). Treated basal leaf explants regenerated more roots than medium or tip leaf explants, but these differences were not statistically significant. However, shoot regeneration was not obtained, in contrast to Marianna 2624 and Myrobalan 605 AD. Adesoto 101 showed, as Adafuel, only root regeneration, but in a small rate (1 basal and 2 medium regenerated leaf explants when the pulse was applied, and only 1 basal leaf explant in the control).

Regeneration was not affected by the leaf age, since no differences between the leaves taken from a shoot were noticed in both, shoot or root regeneration. Most of the differentiated adventitious buds developed into normal shoots that were excised and successfully micropropagated in a MS multiplication medium, and then acclimatized and transplanted to soil, where regenerated plants were indistinguishable from mother plants (Figure 1, C-F).

Effect of the auxins NAA and 2,4-D in the regeneration medium after the pulse

The hormonal composition of the regeneration medium greatly influenced regeneration after the pulse treatment. Marianna 2624 yielded the highest regeneration rate (58.9 %) when whole leaves were placed on regeneration medium with NAA and BAP (Figure 3, top). Both, the hormonal composition of the regeneration medium and the type of leaf explant had a significant effect on shoot regeneration for this rootstock (Table 2). The NAA + BAP medium was more effective than the 2,4-D + BAP medium, giving higher percentages of regeneration in all leaf explants, and these differences were statistically significant ($P < 0.001$). In addition, whole leaves and basal explants significantly regenerated more shoots than medium and tip leaf segments ($P < 0.001$).

Myrobalan 605AD leaf explants regenerated shoots from whole, and basal-leaf explants, in both regeneration media, and from medium-leaf explants in 2,4-D + BAP medium. However no shoot regeneration was obtained from tip-leaf explants (Figure 3, bottom).

Adesoto 101 formed few shoots in all explants but in the tip of the leaves in the NAA + 2,4-D regeneration medium: 3 shoots in both whole and basal explants, and only one in medium explants. However, only one shoot was formed in a whole leaf when the regeneration medium included 2,4-D.

Adafuel leaf explants failed to form adventitious shoots in any regeneration medium, and only few roots were obtained on the 2,4-D + BAP medium: 2 roots in whole leaves and 1 root in basal explants.

Discussion

Data presented here show a striking improvement of adventitious organ regeneration (shoots and roots) from leaf explants of different clonal fruit-tree rootstocks cultured in vitro after an auxin pulse treatment. A high percentage of explants (up to 58.9%) showing shoot regeneration was obtained from whole and basal leaf explants of Marianna 2624, a widely used rootstock, which would facilitate the application of biotechnology to fruit trees. The method is based on an auxin pulse of the explants applied before being placed on a regeneration medium under standard culture conditions. Previous reports with woody fruit trees have described laborious regeneration procedures suggesting that regeneration seems to be affected by several factors that interact between them, as in *P. canescens* (Antonelli and Druart, 1990), apricot and plum (Escalettes and Dosba, 1993), or peach (Gentile et al., 2002). Here, we describe a simple protocol that diminish explant manipulation. The use of liquid induction treatments were reported previously but under different experimental conditions. Thus, a two-days 2,4-D induction treatment was effective in *P. canescens* shoot regeneration (Antonelli and Druart, 1990). On the other hand, the use of liquid pulses (two hours) was reported earlier in gymnosperms utilizing high concentrations of cytokinins (BA or TDZ) to stimulate epidermal cell division and bud formation in *Picea abies* embryos (Arnold et al., 1988) and Douglas fir cotyledons (Goldfarb et al., 1991). The positive effect of a brief passage of the explants in liquid medium has to be studied in depth, since several significant changes in either cytoplasm density, complexity of vacuoles, and porosity of cell membranes of mesophyll cells, have been described in apple and related to an improvement of shoot regeneration and plant transformation (Sriskandarajah and Goodwin, 1998).

In this work we have shown that whole leaves have performed better than leaf segments in Marianna 2624, and no previous reports on that statement have been found. The type of leaf explant used affected regeneration rate when a pulse was used, being the basal part, including the petiole, the most reactive leaf part for both shoot and root regeneration. Whole leaves and basal leaf explants showed a significantly better regeneration than medium or tip leaf explants. In addition, basal treated explants regenerated more shoots than medium or tip leaf explants in Marianna 2624 and Myrobalan 605 AD, as well as roots in Adafuel, but these differences faded when comparing control explants, suggesting that basal tissues in the petiole area were stimulated by the auxin pulse more than other leaf tissues. Care has been taken to avoid the inclusion of meristematic cells, from developing axillary buds next to the petiole, when the leaf explants were obtained, therefore, regeneration took place exclusively from leaf tissues. However, no statistically significant differences between leaf segments were found in the first experiment, indicating that more experimental work would be needed to clarify this effect. Other reports have described higher regeneration from basal explants, being the proximity of the petiole or the petiole itself the most morphogenic part (Antonelli and Druart, 1990; Druart, 1990a; Escalettes and Dosba, 1993; Yepes and Aldwinckle, 1994). In contrast, Sarwar and Skirvin (1997) described in apple that mid leaf segments regenerated better than basal explants, showing tip explants the lowest regeneration rate, and they suggested that it could be related to the presence of two cut surfaces in mid segments that could increase the absorption of media components. However, this effect was not found in our conditions, where shoots arose from callus tissues that were in contact with the culture medium. A gradient on shoot regeneration capacity was described in apple by Welandar (1988) who found the lowest regeneration in the tip, increasing towards the base. In our work, a gradient on

the regeneration competence cannot be concluded, since no significant differences in the regeneration rate were found between the treated explants. The second experiment of this work, which confirms the positive effect of the auxin pulse, showed that whole leaves and basal leaf explants of Marianna 2624 regenerated significantly more shoots than medium or tip leaf segments. But whole leaves have performed better than basal leaf explants, suggesting a possible detrimental effect of wounding or a beneficial effect of the presence of a higher amount of leaf tissues. Both, whole and basal leaf explants exhibited high variability (Figure 3, top) that impeded to find a significant difference between their transformed mean values with Duncan's Multiple Range test. The election of whole leaves as explants simplify the protocol minimizing the manipulations of plant material.

Previous reports found a detrimental effect of leaf age on regeneration. Thus, it is a common practice to use only the youngest leaves: as in almond (Miguel et al., 1996), apricot (Druart, 1990a; Escalettes and Dosba, 1993), cherry (Druart, 1990b; Hammatt, 1993), peach (Gentile et al., 2002; Declerck and Korban, 1996), and in plum (Bassi and Cossio, 1991; Escalettes and Dosba, 1993). However, in the first experiment of this work all leaves from each donor shoot were used and no effect of leaf age on regeneration were noticed, which suggests that the competent plant material is not restricted to the youngest leaves when the 2,4-D pulse is applied, increasing the explant availability.

The role of auxin in regeneration has been stressed here, both in the pulse treatment and in the regeneration medium, NAA being an effective auxin when combined with BAP after the 2,4-D pulse. However, the presence of 2,4-D in the regeneration medium decreased the regeneration rates obtained, suggesting that 2,4-D is effective only as a pulse treatment to stimulate the start of the morphogenetic response.

Genotype had a strong influence in adventitious organ differentiation. Thus, in the experimental conditions tested here, shoots were obtained in the plum rootstocks Marianna 2624 and Myrobalan 605 AD, whereas the almond x peach hybrid Adafuel formed only roots. The plum rootstock Adesoto 101 regenerated shoots or roots depending on the experiment, but only in very few explants. No clear explanation is available to account for this fact since the same plant material and the same protocol was applied to these genotypes.

We have found here that the growth regulators combination in the regeneration medium, combined to the auxin pulse, was competent for Marianna 2624 shoot regeneration, with regeneration rates that suggests the possible use of this protocol for biotechnological applications. Nevertheless, in spite of the positive effect of the auxin pulse on regeneration, the protocol is not applicable yet to other rootstocks to get satisfactory regeneration rates, so more work is necessary in these rootstocks to fit their requirements. The auxin pulse can easily be adapted to other existing protocols to study its possible beneficial effect to expand the use of biotechnology to adult stone-fruit trees in a similar way as it was done with juvenile tissues, where the expression of genes for agricultural traits has been performed (Scorza et al., 1995b).

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Table 1. ANOVA table of the effects of leaf explant type and a 2,4-D pulse treatment on Marianna 2624 and Myrobalan 605 AD shoot regeneration, as well as in Adafuel root regeneration, with F-values and significance

<i>Source</i>	<i>d.f.</i>	<i>S.S.</i>	<i>F</i>	<i>P</i>
<i>Marianna 2624</i>				
Explant type	2	173.34	3.89	0.083
Pulse treatment	1	437.53	19.63	0.004
Interaction	2	66.62	1.49	0.30
Error	6	133.76		
<i>Myrobalan 605 AD</i>				
Explant type	2	52.81	0.36	0.71
Pulse treatment	1	27.47	0.37	0.56
Interaction	2	52.81	0.36	0.71
Error	6	443.28		
<i>Adafuel</i>				
Explant type	2	140.76	0.78	0.50
Pulse treatment	1	978.08	10.82	0.017
Interaction	2	2.81	0.02	0.99
Error	6	542.92		

Table 2. ANOVA table of the effects of leaf explant type and the growth regulatros
 combiantion in the regeneration medium on Marianna 2624 and Myrobalan 605 AD
 shoot regeneration, with F-values and significance.

<i>Source</i>	<i>d.f.</i>	<i>S.S.</i>	<i>F</i>	<i>P</i>
<i>Marianna 2624</i>				
Explant type	3	1293.20	9.39	0.001
Regeneration medium	1	5554.67	120.93	0.000
Interaction	3	143.91	1.04	0.400
Error	16	734.92		
<i>Myrobalan 605 AD</i>				
Explant type	3	304.03	2.090	0.14
Regeneration medium	1	0.70	0.014	0.91
Interaction	3	54.45	0.374	0.78
Error	16	775.67		

Figure captions

Figure 1. A) Adventitious shoot regeneration from a Marianna 2624 medium leaf explant after a 2,4-D pulse (1.7 μ M , 90 min). B) Adventitious root regeneration from Adafuel tip leaf explants after the 2,4-D pulse. C) Adventitious shoot regeneration from a Marianna 2624 basal leaf explant. D) Marianna 2624 shoots developed from adventitious buds and ready for micropropagation. E) Multiplication phase of Marianna 2624 regenerated shoots in vitro. F) Potted in vitro regenerated Marianna 2624 plants after the acclimatization in the greenhouse.

Figure 2. Shoot regeneration percentages from leaf explants of Marianna 2624 (top), and Myrobalan 605 AD (middle), and root regeneration percentages from Adafuel (bottom), with or without a 2,4-D pulse treatment (bars: s.e.).

Figure 3. Shoot regeneration percentages from leaf explants of Marianna 2624 and Myrobalan 605 AD on regeneration media with a different auxin (NAA or 2,4-D) after a 2,4-D pulse treatment (bars: s.e.). Different letters showed significant differences between leaf explant types after a Duncan's Multiple Range test ($P < 0.05$) of transformed data.