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Auxin pulses and a synergistic interaction between polyamines and ethylene inhibitors improve adventitious regeneration from apricot leaves and *Agrobacterium*-mediated transformation of leaf tissues

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Abstract

The effect, on adventitious regeneration from apricot leaf explants and transformation of leaf tissues, of auxins pulses with NAA and 2, 4-D was tested. Addition of the polyamines putrescine and spermidine to the regeneration medium, alone or in combination with the ethylene inhibitors silver thiosulphate and aminoethoxyvinylglycine, were also tested to design a procedure that improved transformation efficiency. Spermidine at 2 mM in combination with 0.5 μM aminoethoxyvinylglycine and four-day pulses with two different concentrations of 2, 4-D increased significantly shoot regeneration. Spermidine at the same concentration but in combination with 60 μM silver thiosulphate and four-day pulses with 9 μM 2, 4-D also increased stable transformation events and GFP-expressing calluses probably by inducing a larger amount of dividing cells where *Agrobacterium* transferred its T-DNA. Since regeneration from apricot leaves occurs mostly from developing calluses, it is important to obtain many GFP-expressing calluses and, given that transformation efficiencies (number of transformed shoots per total number of explants) in woody plants are generally very low, approaches that allow the optimization of T-DNA transfer and total number of transformed cells obtained, will improve probabilities of obtaining transformed shoots.

Abbreviations: AS – acetosyringone; AVG – aminoethoxyvinylglycine; DKW – Driver and Kuniyuki (1984); GFP – green fluorescent protein; QL – Quoirin and Lepoivre (1977); NAA – naphthaleneacetic acid; Put – putrescine; Spd – spermidine; Spm – spermine; STS – silver thiosulphate; TDZ – thidiazuron; 2, 4-D – 2, 4 dichlorophenoxy–acetic acid

Introduction

The lack of efficient adventitious regeneration systems is the major limiting factor preventing the development of gene transfer technologies for perennial crops. Fruit trees are among the most recalcitrant in producing adventitious shoots. The use of ethylene inhibitors to enhance shoot regeneration has been described in some species and an efficient adventitious regeneration procedure from adult apricot leaves has been developed, which include the use of the ethylene inhibitors STS or AVG (Burgos and Alburquerque, 2003).

Polyamines are low molecular weight organic cations implicated in various physiological and developmental processes in bacteria, animals and plants. The diamine Put is the precursor of the triamine Spd and the tetramine Spm. In plants, Put, Spd and Spm are present in amounts

varying from micromolar to more than milimolar (Galston and Sawhney, 1990). Polyamines are involved in stimulation of cell division, regulation of rhizogenesis, embryogenesis, floral development, fruit ripening, etc. (Evans and Malmberg, 1989; Kakkar et al., 2000). The effect of exogenous polyamines on the shoot regeneration in different species (Chi et al., 1994; Pua et al., 1996; Bernet et al., 1998; Bais et al., 2001; Shoeb et al., 2001) and the synergistic effect of ethylene inhibitors and putrescine on shoot regeneration have also been studied (Pua et al., 1996; Bais et al., 2001). Polyamines have been regarded as a new class of plant growth regulators and hormonal second-messengers, at least in cultured tissues (see review by Kakkar et al., 2000).

An adequate balance of cytokinin and auxin in the culture medium is necessary to regulate shoot organogenesis (Litz and Gray, 1992). In apple, the type of auxins, the timing of its application and the length of explant exposure to the specific auxin were critical to reach the highest regeneration rates from leaves (Yancheva et al., 2003).

Although some successful attempts at regeneration from adult material of different *Prunus* species have been reported (Miguel et al., 1996; Hammatt and Grant, 1998; Pérez-Tornero et al., 2000; Gentile et al., 2002; Tang et al., 2002; Burgos and Alburquerque, 2003) only a few publications report the recovery of transformed shoots and most of them used juvenile material. To our knowledge, only transgenic plants from a cultivar of *Prunus subhirtella* (da Câmara Machado et al., 1995) and the cherry rootstocks 'Black Eagle' (Dolgov, 1999) and 'Colt' (Gutiérrez-Pesce et al., 1998) have been obtained. Development of an effective system for gene transfer in apricot (*Prunus armeniaca* L.) depends largely on the availability of tissue culture techniques that permit efficient DNA delivery, selection of transformants and recovery of transgenic plants. Increasing adventitious regeneration and competence in transformation are critical for the application of genetic engineering techniques to the breeding of apricot. This work was designed to test the effect of different factors that could improve the number of stable transformed cells and/or the regeneration percentages in apricot.

Materials and methods

Maintenance of shoot cultures

This study has been carried out with the cultivar Helena, obtained from the apricot breeding programme at the Horticultural Crops Research Laboratory in Fresno (California) and kindly provided by Dr Craig A. Ledbetter. *In vitro* shoots were maintained by sub-culturing at 3-week intervals onto a shoot multiplication medium (Pérez-Tornero and Burgos, 2000).

General strategy for regeneration

The first four apical, expanding leaves from 3-week-old proliferating shoots were placed in sterile water and swirled to randomise. Each leaf was cut transversely three or four times across the midrib without fully separating the segments. Leaves were cultured with the adaxial side in contact with the regeneration medium (Burgos and Alburquerque, 2003). For each treatment, at least five Petri dishes were prepared, each containing seven leaves. Experiments that produced any regeneration were repeated at least twice. After explants were positioned on the medium, the dishes were sealed with Parafilm® and incubated in the dark at 22 ± 1 °C for two weeks before exposure to light, with a 16-h photoperiod and 55 μmol m^{-2} s^{-1} light intensity.

Effect of polyamines on regeneration

The effect of two different polyamines, Put at 0.1, 0.5, 1, 2, 5, 10 and 20 mM and Spd at 0.5, 1, 2 and 5 mM, on regeneration was tested alone or in combination with the ethylene inhibitors STS 60 μM or AVG 0.5 μM. Put and Spd were filter sterilised and added to the medium after autoclaving and cooling to 50 °C.

Effect of auxin pulses on regeneration

The regeneration medium was supplemented with additional amounts of NAA (5.37 or 10.74 μM) or 2, 4-D (4.25 or 9.05 μM) for 4 days and then leaves were transferred to the standard regeneration medium.

Effect of different factors on stable transformation events

All the experiments described above were repeated with an *Agrobacterium*-mediated transformation strategy for leaves described in detail elsewhere (Petri et al., 2004).

When the effect of auxin pulses was tested, additional amounts of NAA (5.37 or 10.74 μM) or 2, 4-D (4.25 or 9.05 μM) were added to the co-culture medium. After that, explants were transferred to the regeneration medium, with the addition of 0.63 mM cefotaxime, 0.13 mM vancomycin and 25.7 μM kanamycin.

To test the effect of those polyamine-ethylene inhibitor combinations that also improved regeneration, the regeneration medium was supplemented with Spd 2 mM and STS or AVG. After two weeks in the dark, explants were transferred to the light, with a 16-h photoperiod and 55 μmol m^{-2} s^{-1} light intensity.

Four weeks after transformation, explants were examined under a Leica MZ75 stereomicroscope equipped with a fluorescence GFP Plus filter module, which contained a 480/40-nm excitation filter, 505-nm LP dichromatic beam-splitting mirror and a 510-nm LP barrier filter. A 50-W high-pressure mercury vapour lamp provided illumination. The red autofluorescence from chlorophyll was not blocked with any interference filter.

Statistical analysis

Total percentages of regeneration and transformation were analysed by a maximum likelihood analysis of variance and, when necessary, specific contrasts of maximum likelihood were designed.

Number of transformation events per explant were analysed by ANOVA and a LSD post-hoc test was used to separate means. Data were transformed by the $(x + 0.5)^{1/2}$ transformation to meet ANOVA requirements.

Results

The application of polyamines to the regeneration medium did not produce any significant improvement of regeneration as compared to a control without polyamines. The application of Put at several concentrations, in combination with STS or AVG, did not make any significant difference with the control and over 2 mM was detrimental to regeneration (Figure 1a). However, Spd significantly improved regeneration, only when applied at 2 mM and in combination with AVG ($p < 0.001$). In combination with STS or when applied at lower concentrations Spd did not have any effect. Application of higher concentrations, in combination with both STS and AVG, had a detrimental effect, as compared to control with only the ethylene inhibitors (Figure 1b).

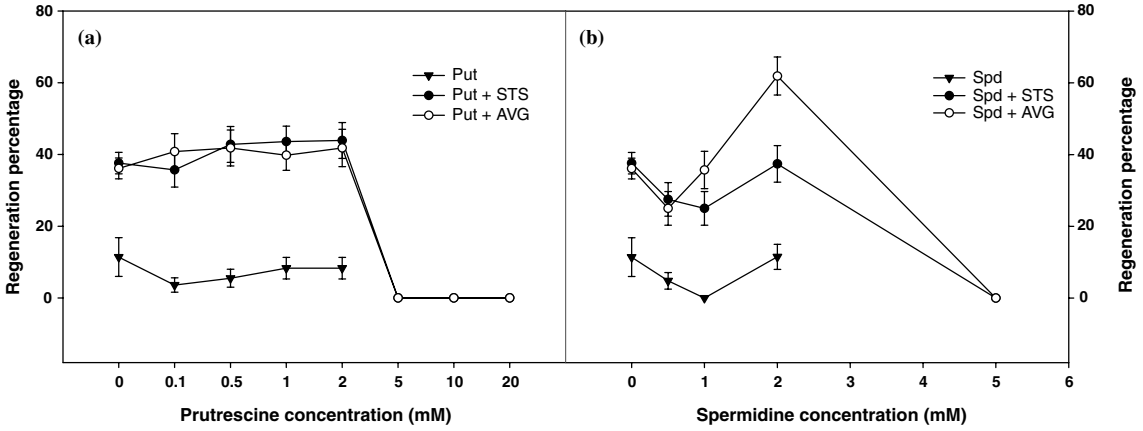

Figure 1. Effect of different concentrations of the polyamine putrescine (*a*) or spermidine (*b*), alone or in combination with the ethylene inhibitors STS (60 μM) or AVG (0.5 μM), on the adventitious regeneration from apricot leaves. A total of 1722 and 1085 leaf explants were included in the study to study the effect of putrescine and spermidine, respectively. Bars indicate standard errors.

The addition of polyamines to the regeneration medium in concentrations ranging from 1 to 2 mM produced very healthy, dark green explants and many calluses on the leaf surfaces.

Four-day pulses with additional auxins produced healthy explants and many calluses on the leaf surfaces and a significant increase ($p < 0.001$) in the regeneration rates (Table 1). When comparing separately the different treatments with the control, the significant regeneration improvement was due only to the addition of 2, 4-D ($p < 0.001$).

The effect on transformation percentages and number of transformation events per explant of all treatments that increased regeneration (significantly or not) was tested (Table 2). Transformation rates were over 75% in all cases and were not significantly affected by treatments. Total number of transformation events and number of GFP-expressing areas or calluses were significantly affected by treatments ($p < 0.01$ for both independent variables). According to a Dunnett's test (that compares each treatment with the control), only auxin pulses with 2, 4-D at 9.05 μM and the combination of Spd 2 mM and STS 60 μM produced a significant increase in the number of transformation events and GFP-expressing calluses.

Discussion

The addition of the ethylene inhibitor silver nitrate to culture media can improve regeneration of different *Prunus* species (Escalettes and Dosba, 1993), and recently we have described a positive effect of STS and AVG in shoot organogenesis in apricot (Burgos and Alburquerque, 2003). It has been demonstrated that ethylene produced by cultured explants inhibits shoot organogenesis (Chi et al., 1990; Arigita et al., 2003).

The addition of polyamines increased plant regeneration in different species (Shoeb et al., 2001; Bernet et al., 1998; Chi et al., 1994). Cellular polyamine levels and their Put:Spd ratio have even been suggested as important determinants (biomarkers) of plant regeneration ability in indica rice (Shoeb et al., 2001). However, in this work the use of Put or Spd alone did not improve regeneration from apricot leaves which is also in agreement with results in other species where Put did not have any effect on shoot regeneration when added alone to the medium (Pua et al., 1996).

Our results may suggest that exogenous Spd is more effective to decrease ethylene production than Put when combined with AVG. The possible explanation could be that SAM is the direct precursor of Spd, but not of Put that is formed from amino acids like ornithine or arginine (Evans and Malmberg, 1989). The decarboxylated SAM, which is produced from SAM by SAM decarboxylase (SAMDC), is used as an aminopropyl donor for the formation of Spm from Spd and the latter from Put (Kumar et al., 1997).

Differences in regeneration induced for the combination of Spd and both ethylene inhibitors, may be related to the different action mechanism of AVG and silver ions. AVG inhibits ethylene biosynthesis (Yang and Hoffman, 1984), while STS is believe to inhibit ethylene action (Beyer, 1976). The lack of synergistic effect, found here between Spd and STS, may also be explained if our optimize STS concentration produced a maximum inhibition of ethylene action and supplying Spd to compete for SAM did not have an additional effect.

Pretreatments of the plant material with plant growth regulators have increased regeneration

Table 1. Regeneration percentages from apricot leaves after four-day pulses with additional auxins in the regeneration medium. A total of 245 leaf explants were included in the study

Auxin	Concentration (μM)	Regeneration (%)	Number buds/regenerating explant
None (control)	–	25.7 ± 7.4	1.6
NAA	5.37	44.3 ± 5.9	1.8
	10.74	42.9 ± 8.4	1.6
2, 4-D	4.52	67.1 ± 5.6	2.1
	9.05	68.6 ± 5.5	2.0

Table 2. Effect of auxin pulses during co-culture or the combination of spermidine and ethylene inhibitors on transformation of apricot leaves

Treatment	Additional auxin concentration (μM)	Ethylene inhibitor and concentration (μM)	Transformed explants (% ± SE)	Total gfp + /explant (mean + SE)	Gfp + zones[a]/explant (mean + SE)
None (control)	–	STS (60)	85.0 ± 4.0	10.3 ± 1.1	6.7 ± 0.8
NAA	5.37	STS (60)	75.6 ± 6.7	10.3 ± 1.3	7.6 ± 1.1
	10.74	STS (60)	80.0 ± 8.9	14.3 ± 3.9	10.1 ± 3.1
2, 4-D	4.52	STS (60)	89.2 ± 5.1	12.8 ± 2.3	8.0 ± 1.3
	9.05	STS (60)	94.6 ± 3.7	15.1 ± 2.0	9.5 ± 1.3
Spermidine (2 mM)	–	STS (60)	100	15.1 ± 2.1	13.0 ± 2.0
		AVG (0.5)	80 ± 6.8	7.9 ± 1.6	5.7 ± 1.2

[a] gfp + zones are large areas or calluses expressing gfp.

(Burnett et al., 1994; Yancheva et al., 2003) and transformation (McHughen et al., 1989; Villar et al., 1999) results in different species. In this study, auxin-pulses pretreatment increased significantly shoot regeneration. The type of auxin and timing of application have been found to critically affect regeneration from apple leaves (Yancheva et al., 2003).

Woody plants have a tendency to be recalcitrant regarding tissue culture and transformation (Schuerman and Dandekar, 1993). Efforts to improve the efficiency of *Agrobacterium*-mediated transformation have focused on increasing the virulence of the *Agrobacterium* strain used (Ghorbel et al., 2001) or modifying the physiological conditions of the target plant material so that they are optimal for the bacterial infection. *Agrobacterium* may preferentially transform metabolically active and/or actively dividing cells (Dandekar, 1995). Some compounds, which promote cell division, have induced virulence genes in *A. tumefaciens* and also exogenously applied plant growth regulators, mainly auxins, enhanced hairy root and tumor induction (see review by Dandekar, 1995). The role of polyamines in cell division, growth and differentiation has been widely reported (Chi et al., 1994) and in apricot leaves, addition of polyamines to the regeneration medium produce large amounts of deep-green calluses suggesting higher cellular division than in controls without polyamines.

To our knowledge, this is the first report on the effect of polyamines on regeneration and transformation in a fruit tree species. Since regeneration from apricot leaves occurs mostly from developing calluses, it is important to obtain many gfp-expressing calluses and given that transformation efficiencies (number of transformed shoots per total number of explants) in woody plants are generally very low, approaches that allow the optimization of T-DNA transfer and total number of transformed cells obtained, will improve probabilities of obtaining transformed shoots.

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