In Vitro Regeneration of “Carrick” pear from Leaf Plants

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Recebido em: 29 ago. 2016. Revisado 20 fev. 2017. Aceito: 26 fev. 2017.

DOI: http://dx.doi.org/10.21674/2448-0479.31.238-248

Abstract

The aim of the present study was to investigate a two-step procedure as well as the effect of two types of cytokinins in the pretreatment and shoot induction media on in vitro adventitious shoot regeneration of pear leaf explants of ‘Carrick’ pear. The experiment was arranged in a completely randomized design with five replications per treatment. Fully expanded healthy leaves were explanted onto shoot

induction medium (SIM) following a pretreatment in liquid medium (PLM). Both media were supplemented with thidiazuron (TDZ) or 6-benzyladenine (BA) (PLM: 2 mg dm-3; SIM: 3 mg dm-3). The percentage of explants with shoots and the number of shoots per explant were assessed. The two-step proce-dure PLM [BA] + SIM [TDZ] is efficient for in vitro adventitious shoot regeneration from leaves of 'Carrick' pear.

Keywords*:* Pretreatment. Liquid Medium. Cytokinin. Micropropagation. Pyrus Communis L.

Resumo

Regeneração in vitro de pereira 'Carrick' a partir de Explantes Foliares

O objetivo deste trabalho foi de investigar um procedimento em duas etapas, assim como o efeito de dois tipos de citocininas nos meios de indução e pré-tratamento, na regeneração in vitro de brotos adventícios a partir de explantes foliares de pereira 'Carrick'. O delineamento experimental foi inteiramente casualizado com cinco repetições por tratamento. Folhas totalmente expandidas e saudáveis foram inoculadas em meio de indução de brotos (MIB), após pré-tratamento em meio líquido (PML). Ambos meios de cultivo foram suplementados com tidiazuron (TDZ) ou 6-benziladenina (BA) (PML: 2 mg dm-3; MIB: 3 mg dm-3). Foram avaliados a porcentagem de explantes com brotos e o número de brotos por explante. O procedimento em duas etapas PML [BA] + MIB [TDZ] é eficiente para regeneração in vitro de brotos adventícios a partir de explantes foliares de pereira 'Carrick'.

Palavras-chave: Pré-tratamento. Meio Líquido. Citocinina. Micropropagação. *Pyrus communis* L.

Introduction

Pears are the most imported fruits by Brazil. This is due several problems such as excessive tree vigor, floral abortion, lack of rootstocks and adapted cultivars to Brazilian edaphoclimatic conditions (FACHINELLO et al., 2011). However, 'Carrick' pear has shown good crop potential under this conditions and it already has some rootstock op-tions available (PASA et al., 2012; PASA et al., 2011) but further genetic improvement might be necessary in order to achieve competitive yields.

Currently, pear improvement has been accomplished mainly by conventional methods, which are long lasting and difficult due to high levels of heterozygosis and the long juvenile period (ALDWINCKLE; MALNOY, 2009). The development of efficient transformation systems, such as the use of Agrobacterium tumefasciens, could accelerate the improvement process (DJENNANE et al., 2011). However, its success is highly dependent on the availability of efficient and reproducible regeneration protocols (FEI; WANG; DONG, 2009), and most importantly, the lower regeneration ability of transformed tissues compared to non-transformed ones (PAWLICKI-JULLIAN; SEDIRA; WELANDER, 2002). Besides, propagation of plants through tissue culture, including sophisticated techniques of meristem culture and molecular indexing of dis-eases, is of immense use to make available healthy propagules (KAJLA et al., 2013) and conservation of virus-free germplasm (CARRASCO et al., 2013).

The development of an efficient in vitro regeneration protocol depends, among other factors, on mineral composition of regenera-

tion media, growth regulators and explant choice. Adventitious shoot regeneration from leaves have been reported for several pear varieties but it has shown to be genotype-dependent (BELL; SCORZA; LOMBERK, 2012). Leaves are usually the source of explants for transformation through Agrobacterium-mediated protocols and some positive results have been achieved using this method for pears (DJENNANE et al., 2011).

Another important prerequisite for in vitro regeneration is the type of cytokinin used to promote shoot organogenesis. The source of cytokinin most used in the reported regeneration protocols for pear is TDZ at different rates (TANG; LUO; LIU, 2008; ALDWINCKLE; MALNOY, 2009), but BA has been shown to be most responsive for some varieties (TANG; LUO; LIU, 2008). Nevertheless, none of these protocols examined the effect of liquid pretreatment of leaf explants with these cytokinins. Pretreatment with liquid medium was already reported for improving in vitro shoot regeneration of the blueberry cultivar Bluecrop (CAO; HAMMERSCHLAG, 2002).

The aim of the present study was to investigate a two-step procedure as well as the effect of two types of cytokinins in the pretreatment and shoot induction media on adventitious shoot regeneration of pear leaf explants of 'Carrick' pear

Material and Methods

Leaves of in vitro-cultured 'Carrick' pear growing in MS medium (MURASHIGE; SKOOG, 1962) were used as source of explants. Fully expanded healthy leaves were excised from 3-week old shoots and the petiole was kept. Each leaf was wounded by making 4 cuts transversely across the midrib with a scalpel. Then they were ex-

planted abaxial side down either onto a semi-solid SIM following PLM, or directly onto semi-solid SIM.

The PLM consisted of WPM (Wood Plant Medium) (LLOYD; MCCOWN, 1981) macro- and micronutrients, supplemented with 30 g dm-3 sucrose, 0.2 mg dm-3 NAA and either 2 mg dm-3 TDZ or 2 mg dm-3 BA. The basal SIM consisted of WPM macro- and micronutrients, supplemented with 30 g dm-3 sucrose, 0.2 mg dm-3 NAA, 2 g dm-3 de gelrite. The pH was adjusted to 5.6 before autoclaving for 20 min at 121ºC. For pretreatment, explants were disposed into Erlenmeyer flasks containing 100 ml of PLM that were wrapped on the top with aluminum foil and then incubated in the dark for 16h with constant shaking (125 rpm) at 25 ± 1°C. After that, they were transferred to SIM, containing either 3 mg dm-3 TDZ or 3 mg dm-3 BA, and returned do the dark for 4 weeks at the same conditions described before. Some treatments were transferred directly to SIM. Each Petri dish contained 25 ml of medium. In the fifth week, explants were transferred to the growing room at 25 ± 1°C, with a 16/8h (light/dark) photoperiod (cool white fluorescent tubes, 42 µmol m-2 s-1). Then, in the seventh week they were transferred to fresh SIM, supplemented with 5 mg dm-3 2-isopentenyladenine (2iP). Explants were sub-cultured to fresh medium with the same composition every 15 days.

After 10 weeks, the percentage of regeneration and the number of shoots per explant was recorded. The experiment was arranged in a completely randomized design with five replications (eight explants each) per treatment. Data on the percentage of regeneration and number of shoots per explants were transformed as arcsin square root and square root (n + 0.5), respectively, to provide a normal distribution. Treatment means were compared using analysis of variance significance was tested at $P \leq 0.5$. Differences among

means for significant effects were tested by Fisher's protected least significance difference test (LSD).

Results and Discussion

After 3 weeks of dark incubation, white nodular callus arose mainly from the wounded edges of the explants. The first adventitious shoot appeared after 4 weeks (Figure 1A). Most shoot development appeared to arise from callus (Figure 1A) rather than by direct organogenesis (Figure 1B).

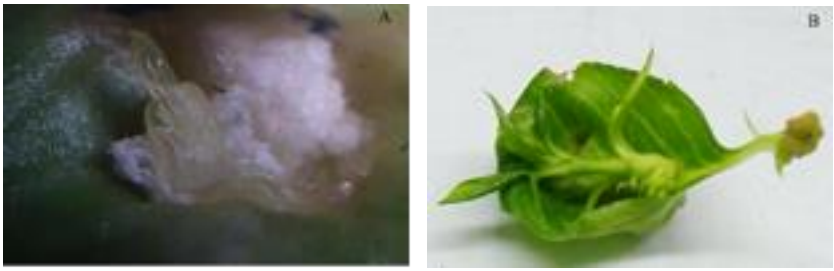

Figure 1 - Adventitious shoot regeneration of 'Carrick' pear leaves after 4 (A) and 10 weeks (B), from callus and directly from leaf surface, respectively, on SIM supplemented with 3 mg dm-3 TDZ, following PLM supplemented with 2 mg dm-3 BA.

The two-step procedure PLM [BA] + SIM [TDZ] showed the highest percentage of explants with shoots (35 ± 2.5), i.e., greater regeneration, followed by 'no pretreatment' + SIM [TDZ] (17.5 ± 5.0) and PLM [TDZ] + SIM [BA] (15 ± 4.7) (Table 1).

Table 1 - Regenerating explants (%) and shoot number (explant-1) of pear leaf explants on the two-step procedure PLM [TDZ or BA] + SIM [TDZ or BA], as well as on SIM [TDZ or BA] alone.

Growth regulator		**Explants with shoots (%)**	**Number of shoots per explant**
Pretreatment in liquid medium (PLM)	Shoot Induction medium (SIM)		
No pretreatment	TDZ	17.5 ± 5.0 b	0.8 ± 0.2 ab
	BA	0.0 ± 0.0 c	0.0 ± 0.0 c
TDZ	TDZ	7.5 ± 5.0 bc	0.4 ± 0.2 bc
	BA	15 ± 4.7 b	0.8 ± 0.2 ab
BA	TDZ	35 ± 2.5 a	1.0 ± 0.0 a
	BA	2.5 ± 2.5 c	0.2 ± 0.2 c
***P* > F**		<0.0001	<0.01

*Data were presented as means of 5 replicates + SE (n=8). Means separation within columns by Fisher's protected least significance difference test (LSD) at P ≤ 0.05.

These results were consistent to the results previously reported by Cao and Hammerschlag (2002) which found that a two-step growth regulator pretreatment in liquid medium (PT1 - 5 µM TDZ; PT2 - 20 µM zeatin) significantly enhanced the efficiency of shoot organogenesis from leaf explants of blueberry cv. Bluecrop. On the other hand, in previous studies with the same pear variety, Erig and Schuch (2003) found zero percent of regeneration using leaf explants on semi-solid MS medium supplemented with similar rates of TDZ. Other studies have also reported increased efficiency of in vitro regeneration from leaf explants of Alstroemeria (LIN; DE JEU; JACOBSEN, 1997) and lowbush blueberry (DEBNATH, 2009) using a two-step procedure.

The results also indicate a possible synergistic effect between the cytokinins TDZ and BA when combined in the two-step procedure. This hypothesis is based on the increased percentage of regeneration of leaf explants on PLM [BA] + SIM [TDZ] and PLM [TDZ]

+ SIM [BA], when compared with their regeneration percentages on SIM with TDZ or BA without PLM (Table 1). Adventitious shoot regeneration from leaves of some pear varieties has been reported when TDZ (SUN et al., 2011, BELL et al., 2012) and BA (TANG; LUO; LIU, 2008) were used as the source of cytokinin on SIM, but the possible synergistic effect of these two growth regulators in a two-step procedure, as found in the present study, has not been reported.

Considering the number of shoots per explant, the treatment PLM [BA] + SIM [TDZ] showed greater value (1.0 ± 0.0) than PLM [BA] + SIM [BA] (0.2 ± 0.2), PLM [TDZ] + SIM [TDZ] (0.4 ± 0.2) and SIM [BA] without PLM (0.0 ± 0.0) (Table 1). Tang, Luo and Liu (2008) observed the greater number of shoots per explant when with TDZ (3 mg L-1) was added to NN (NITSCH; NITSCH, 1969) medium, but they have not studied a two-step procedure. A great number of shoots per explants is desired because it increases the chance of achieving a whole plant, since more propagation material would be available for further multiplication.

Conclusion

The results show that the two-step procedure PLM [2 mg dm-3 BA] + SIM [3 mg dm-3 TDZ] increases the in vitro adventitious shoot regeneration from leaf explants of 'Carrick' pear, thus being a potential tool to be used in its future improvement through genetic transformation.

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