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Study of factors affecting direct shoot regeneration of pear (*Pyrus communis* L.)

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Abstract
Conventional methods of pear breeding, largely based on intra- and inter-specific hybridization, are difficult because pear is highly heterozygous, polygenic and has a long juvenile period. Genetic improvements of pear cultivars are possible through induction of mutations and gene transfer by genetic engineering. A general prerequisite for these approaches is to establish an efficient plant regeneration system. In the present study, the effect of two basal media (MS and NN) and different concentrations of TDZ (0, 2.5, 5, 7.5 M) or BAP (0, 4, 8, 16 M) in combination with NAA (1 M) on direct shoot regeneration of two pear (*Pyrus communis* L.) genotypes 'Bartlett' and 'Dargazi' was investigated. The obtained results showed that 'Dargazi' had higher rates of shoot regeneration than 'Bartlett' and in both cultivars the highest percent of shoot regeneration was observed from lower sections of the leaves. Although the highest percent of shoot regeneration in 'Bartlett' (38%) was attained in the NN medium containing 2.5 µM TDZ and 1 µM NAA, the differences in shoot regeneration between this medium and the NN media containing 5 or 7.5 µM TDZ and 1 µM NAA were not significant. The highest percent of shoot regeneration in 'Dargazi' (56%) was obtained in NN medium containing 7.5 µm TDZ and 1 µm NAA. It can be concluded that genotypes, explant types and culture media composition could effect on direct shoot regeneration of pear.
Keywords: direct shoot regeneration, pear, thidiazuron.
Abbreviations: QL- Quoirin and Lepoivre; MS- Murashige and Skoog; NN- Nitsch and Nitsch; TDZ- thidiazuron; BAP-6-benzylaminopurine; NAA- α-naphthalene acetic acid.

Introduction

Pear is one of the most important temperate fruit crops. It belongs to the genus Pyrus, the subfamily of *Maloideae* (*Pomoideae*) in the *Rosaceae* family. Because of the high level of heterozigosity and the long juvenile period, pear breeding by conventional methods is considered to be difficult and time consuming. Therefore, genetic improvement of pear through modern breeding methods like

genetic engineering has been considered as an alternative procedure. *In vitro* direct regeneration is a general prerequisite for this technique. David Lane (1979) reported the regeneration of pear for the first time. Since the first report on *Pear sp. in vitro* culture, various factors have been examined for pear regeneration, including; the type and orientation of explants (Caboni *et al*., 2002; Lane *et al*., 1998), plant growth regulator combinations, basal salt composition and genotype (Caboni *et al*., 1999; Abdollahi *et al*., 2006; Tang *et al*., 2008), gelling agents (Chevreau *et al*., 1997), darkness (Leblay *et al*., 1991; Liu *et al*., 2009), different carbohydrates (Chevreau *et al*., 1989; Leblay *et al*., 1991) and for controlling contamination, the use of some additives like antibiotics (Predieri *et al*., 1989; Caboni *et al*., 1999) or silver nitrate (Liu *et al*., 2009). Furthermore, several studies have shown that the type of cytokinin in the shoot proliferation medium can affect shoot regeneration in *in vitro* explants (Bell *et al*., 2009).

Since, direct shoot regeneration in pear is highly dependent on genotype, the aim of the present investigation was to develop an efficient specific protocol to regenerate adventitious shoots from leaf explants of two commercial pear cultivars ('Bartlett' and 'Dargazi').

Materials and Methods

Plant material and culture conditions

In vitro shoots of two pear cultivars ('Bartlett' and 'Dargazi') were supplied by Agricultural Biotechnology Research Institute of Iran (ABRII). The nodal explants were proliferated on modified QL medium (Quoirin and Lepoivre, 1977) containing 1 µM NAA, 1 µM BA and 2 µM 2ip, 30 g/l sucrose and 7 g/l agar. The *in vitro* leaves were then used in regeneration experiments. The pH of all media was adjusted to 5.8 before adding agar. All the culture media were autoclaved for 15 min at 121°C. The cultures were incubated at 22 ± 2°C and 16 h photoperiod under cool-white fluorescent light with PPFD of 60 mol m^{-2} s^{-1}.

Adventitious shoot regeneration

In vitro leaves were cut perpendicular to the main vein, into three sections; lower (with petiole), middle and upper sections and each was considered as an explant. The explants were placed on the culture media with the adaxial side on the media in 7 mm Petri dishes. Two types of media; MS (Murashige and Skoog, 1962) or NN (Nitsch and Nitsch, 1969) supplemented with different combinations of thidiazuron (TDZ) or 6-benzylaminopurine (BAP) and α-naphthalene acetic acid (NAA) were used. Sixteen treatments including four concentrations of BAP (0, 4, 8 and 16 µM) which were defined as B0, B1, B2 and B3 and four concentrations of TDZ (0, 2.5, 5 and 7.5 µM) which were defined as T0, T1, T2 and T3 were used in combination with NAA (1 µM). The cultures were kept in the dark for 4 weeks, and then they were sub-cultured to the same media composition and were transferred to the light condition. After 8 weeks, the percent of regenerated shoots were recorded. After

60 days, shoots (>5mm) were excised from original leaf explants and were transferred to the proliferation medium (as explained above). Original leaf explants were sub-cultured in the same media. After 4 weeks, the shoots in the proliferation medium were transferred to the elongation medium (the same as proliferation medium without cytokinin) (Figure 1).

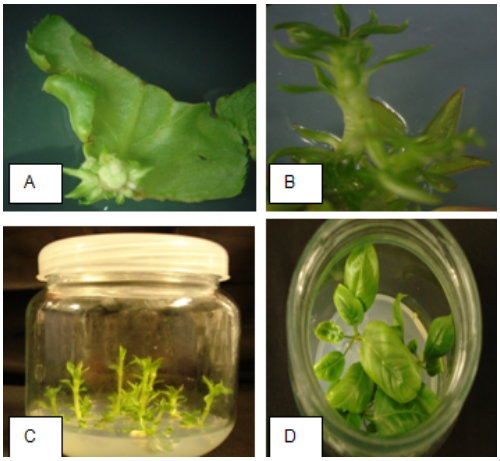

Figure 1. (A) Starting regeneration of shoots; (B) Exceeding the length of shoots; (C) Excising the shoots from maternal explants and transferring to the proliferation medium; (D) Well-developed shoots.

Statistical analysis

The study was designed in a factorial experiment based on a completely randomized design with three replications containing 5 explants per unit. The collected data (explain the kind of data how they has been recorded or calculated) from all experiments were statistically analyzed using MSTAT-C and SAS. Mean values were evaluated at $p<0.01$ level of significance using Duncan's multiple-range test.

Results and discussion

Effect of culture media on adventitious shoot regeneration

Adventitious shoots were developed on the NN media containing various concentrations of TDZ or BAP while the explants with MS culture media in all treatments did not generate adventitious shoots. Effectiveness of NN culture medium for direct regeneration of pear has also been reported by Sun *et al*. (1998) who stated that NN culture medium was more suitable than MS culture medium. The main differences between MS and NN media are in ionic concentration of ammonium and nitrate and their total ionic concentrations. Leblay *et al*. (1991) reported that ammonium/nitrate ratio of 1:3 were essential in direct shoot regeneration of pear. Tang *et al*. (2008) examined six ratios including 1 for ammonium against 2, 3, 4, 5 and 7 fold for nitrate and concluded that the ammonium/nitrate ratio of 1:7 with the rate of 97% shoot regeneration could be the superior one. Since the NN media contains different types of vitamins and also higher amounts of nicotinic acid compared to the MS media it may be considered as another factor in this regard. Other investigations have shown that decreasing the concentration of macro elements (with using of half strength MS media) could have positive effect on regeneration. (Chevreau *et al*., 1989; Leblay *et al*., 1991; Liu *et al*., 2009).

Effect of explant type on adventitious shoot regeneration

The results showed that both genotypes, 'Dargazi' and 'Bartlett', have the highest

rates of regeneration (28% and 12% respectively) when lower sections of leaves were used as explants (Figure 2). Tang *et al*. (2008) reported that the maximum regeneration in different cultivars was achieved from basal leaf explants also possessing petioles.

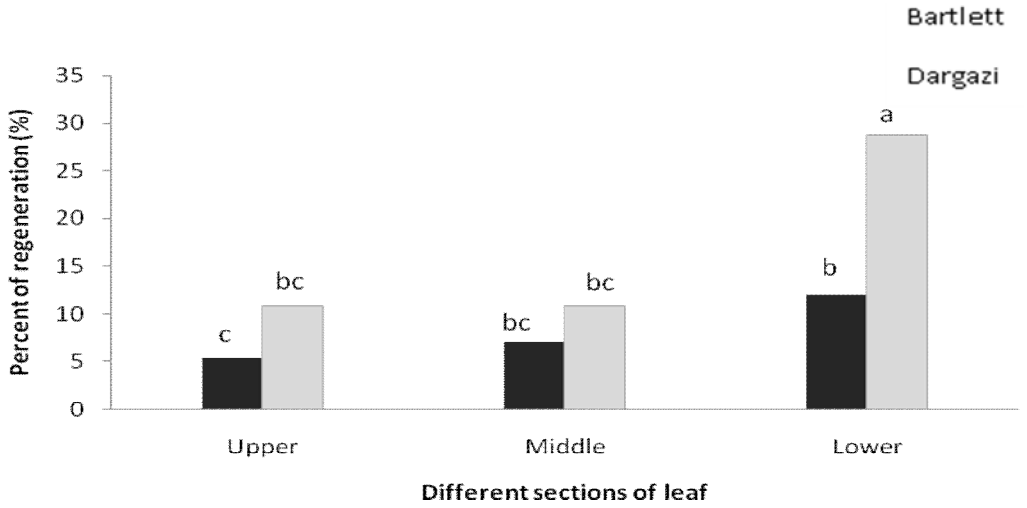

Figure 2. Effect of explant type and genotype on adventitious shoot regeneration of pear. Means in each column with different letters show significant differences according to Duncan's Multiple Range Test ($P \leq 0.05$).

The difference in regeneration ability of explants might be due to differences in the levels of endogenous hormones or an interaction between the endogenous and exogenous hormones (Tang *et al*., 2008). Furthermore, the needs/demands of different leaf sections to hormonal concentration varies for different regeneration procedures. Tang *et al*. (2000) reported that higher concentrations of hormones were needed for organogenesis from distal and middle sections of *Prunus cerasus* cotyledons than from proximal parts.

The interactive effect of hormone and genotype on adventitious shoot regeneration

The presence of a cytokinin in medium is essential for direct shoot regeneration. In all the media containing BAP or TDZ, shoots were regenerated from explants (Figure 4A) whereas in the media lacking cytokinins, only roots were developed (Figure 4B). The highest percentage of shoot regeneration in 'Dargazi' (56%) was obtained on NN medium containing 7.5 µM TDZ and 1 µM NAA (Figure 3). Although the highest percentage of shoot regeneration in 'Bartlett' (38%) was attained in the NN medium containing 2.5 µM TDZ and 1 µM NAA, the differences in shoot regeneration between this medium and NN media containing 5 or 7.5 µM TDZ and 1 µM NAA were not significant (Figure 3). In both cultivars regeneration rates were significantly lower in NN media containing BAP compared to the media containing TDZ (Figure 3). The regenerated shoots from BAP treatments had normal shape

whereas shoots derived from TDZ treatments were short and compact with small leaves (Figure 5).

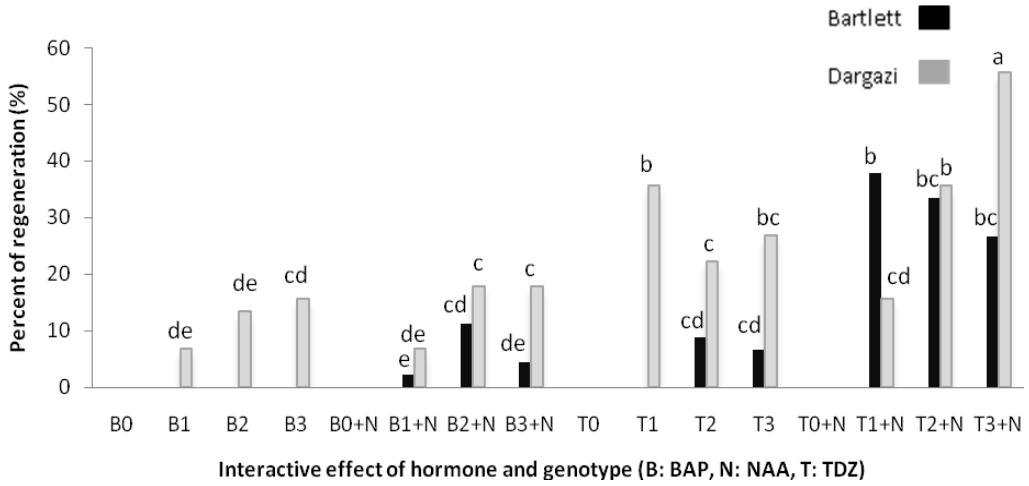

Figure 3. Interactive effect of hormone and genotype on adventitious shoot regeneration of pear. Means in each column with different letters show significant differences according to Duncan's Multiple Range Test ($P \leq 0.05$).

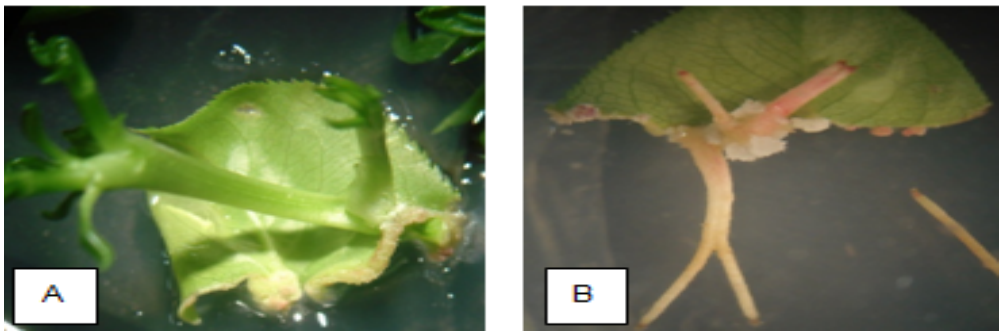

Figure 4. (A) Direct shoot regeneration from explant in medium containing cytokinin; (B) Direct root regeneration from explant in medium lacking cytokinin.

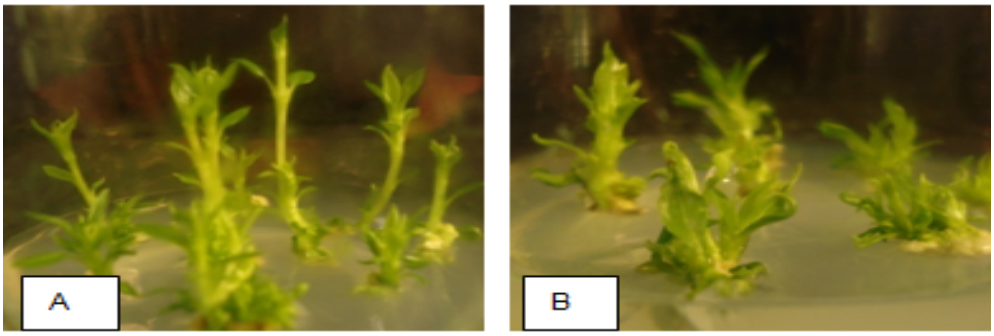

Figure 5. (A) Normal shoots growing from adventitious shoots originated in the medium containing BAP; (B) Compact and short shoots growing from adventitious shoots originated in the medium containing TDZ.

Several studies have demonstrated the positive effects of TDZ on pear regeneration. Chevreau *et al.* (1989) reported that TDZ was more effective than BAP. Leblay *et al.* (1999) investigated different concentrations of TDZ (up to 48 µM), and concluded that TDZ with concentrations more than 12 µM were preventive for pear regeneration. Liu *et al.* (2009) demonstrated that using cytokinin in combination with auxin would promote pear regeneration. They showed that the combination of TDZ with IBA was more effective than the combination of TDZ with NAA. Caboni *et al.* (1999) reported that NAA had positive effect whereas IBA was ineffective in pear regeneration. The effect of genotype on the capacity of pear shoot regeneration and organogenesis has also been reported by many authors (Chevreau *et al.*, 1989; Lane *et al.*, 1998; Caboni *et al.*, 2002), Therefore, it is necessary to develop an efficient specific shoot regeneration protocol for each pear cultivar.

In conclusion, present study demonstrated that direct adventitious shoot regeneration in pear was highly dependent on genotype, explants types and culture media. The maximum rate of regeneration was observed in lower sections of the leaves of 'Dargazi' cultivar in NN medium containing 7.5 µm TDZ and 1 µm NAA.

Acknowledgements

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مطالعه فاکتورهای موثر بر باززایی مستقیم شاخه در گلابی (*Pyrus communis* L.)

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چکیده

اصلاح گلابی با استفاده از روش‌های سنتی به طور عمده بر اساس هیبریداسیون درون و بین گونه‌ای می‌باشد، که به علت سطح بالای هتروزیگوسی در گلابی، پلی‌ژنیک بودن صفات و دوره جوانی طولانی بکارگیری این روش‌ها مشکل است. بهبود ژنتیکی ارقام گلابی از طریق روش‌های القای موتاسیون و انتقال ژن با استفاده از مهندسی ژنتیک امکان‌پذیر است. پیش‌نیاز اساسی برای این روش‌ها پایه‌ریزی یک سیستم باززایی گیاهی کارآمد است. در مطالعه حاضر اثر دو محیط کشت پایه (MS و NN) و غلظت‌های مختلف TDZ (۰، ۲/۵، ۵، ۷/۵) میکرومولار و BAP (۰، ۴، ۸، ۱۶) میکرومولار همراه با NAA (۱ میکرومولار) بر باززایی مستقیم شاخه‌ی دو ژنوتیپ گلابی "بارتلت" و"درگزی" بررسی شد. نتایج به دست آمده نشان داد که در رقم درگزی میزان باززایی شاخه نسبت به رقم بارتلت بالاتر بود و در هر دو رقم بالاترین درصد باززایی شاخه از بخش‌های پایینی ریز نمونه برگ مشاهده شد. گرچه بالاترین درصد باززایی شاخه در رقم بارتلت (۳۸٪) در محیط کشت پایه NN حاوی ۲/۵ میکرومولار TDZ و۱ میکرومولار NAA به دست آمد، تفاوت باززایی شاخه بین این محیط کشت و محیط کشت پایه NN حاوی ۵ یا ۷/۵ میکرومولار TDZ و ۱ میکرومولار NAA معنی‌دار نبود. بالاترین درصد باززایی شاخه در رقم درگزی (۵۶٪) در محیط کشت NN حاوی ۷/۵ میکرومولار TDZ و ۱ میکرومولار NAA به دست آمد. مطالعه حاضر نشان داد که ژنوتیپ، نوع ریز نمونه و ترکیب محیط کشت می‌توانند بر باززایی مستقیم شاخه در گلابی مؤثر باشند.

کلمات کلیدی: باززایی مستقیم شاخه، گلابی، تیدیازورون.