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EFFECTIVE *IN VITRO* REGENERATION SYSTEMS FROM LEAF EXPLANTS FOR COMMERCIALLY IMPORTANT PEAR CULTIVARS (*PYRUS COMMUNIS* L.)

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Abstract

Breeding Programs are focused on creating new improved cultivars and rootstocks of pears. One of the disadvantages of creating new pear cultivars is the long duration of the breeding process. One of the modern methods that can significantly speed up the breeding process is genetic transformation. One of the possible and most frequently used methodological approaches for the introduction of genetic constructions into the genome is agrobacterial transformation.

For pear (*Pyrus communis* L.), the transformation was achieved using various types of explants, but its effectiveness depended very much on the cultivar. High regeneration frequency values were not always reproducible. This is due to the fact that the organogenesis of pear leaves is influenced by a large number of factors. To solve this problem, we have collected and summarized detailed information on the effect of explant orientation, optimal compositions of culture media, concentration of growth regulators and antibiotics for the main commercial pear cultivars grown worldwide. For the first time, optimal protocol parameters have been collected to create effective *In vitro* regeneration systems for the main commercially important pear cultivars. The summarized results of this review will be useful in the development of future genetic transformation trials for commercially significant cultivars and for the application of basic NGTs for the genetic improvement of pears.

Key words: *Pyrus Communis*, *In vitro*, Organogenesis, Transformation, *Agrobacterium tumefaciens.*

Introduction

Pear is one of the traditional and widely distributed fruit crops in the regions with the temperate climate. Pear belongs to the *Pyrus* genus, *Spiraeoideae* subfamily, *Pyreae* tribe of the *Rosaceae* family (Potter *et al.,* 2007). *Pyrus* is mainly divided into European and Asian pears (Silva *et al.*, 2014). Varieties of four *Pyrus* species are grown commercially for fruit production: Chinese pear (*P. bretschneideri* Rehd. и *P. ussuriensis* Maxim.), Japanese pear (*P. pyrifolia* Nakai) and European pear (*P. communis* L.) (Bell *et al.,* 1996).

Due to taste qualities, common pear cultivars are in great demand among consumers. Pear fruits are valuable dietary products and are an excellent source of dietary fiber, amino acids, vitamins and minerals, such as sodium, potassium, calcium, magnesium and iron. They are of interest to processing industry as raw materials for obtaining products of high nutritional quality (Verkhoturov & Baykova, 2009; Yim & Nam, 2016).

Pear (*Pyrus* spp.) is the fifth most common fruit crop in the world. The annual global production in 2020 was approximately 23.1 million tons, of which 2.8 million tons were produced in Europe and mainly consisted of European pears (*P. communis* L.). Unlike crispy Asian pears, European pears usually have a soft and smooth flesh (Hancock, 2008; He *et al.*, 2022).

China is the leader in pear fruit production, followed by Italy and the USA. In China, Asian pear cultivars are mainly produced, while other countries produce European cultivars. In Europe, the main cultivar is Conference, which accounted for 42% of total production over an average of five years (2015-2019), followed by Abbé Fétel with 13% and Williams with 12%. In the USA, Williams (Bartlett) (51%) and Beurré Anjou (34%) alone account for about 85% of total production (Musacchi *et al.,* 2021). Among the producers of the southern hemisphere, which are also the leading exporters of pears, it is worth mentioning Argentina, where pears Williams (40%), Packham's Triumph (30%) and Beurré Anjou (15%) account for 85% of production. Among the commercial cultivars, it is also worth highlighting 'Spadona', one of the most important cultivars of European pear (*Pyrus communis* L.) grown in Israel. This pear cultivar is very popular in the markets of countries with hot climates (Yancheva *et al.*, 2006.).

Although the selection activity in the last several centuries has produced several hundred cultivars, only a few pear cultivars are currently grown (Dondini & Sansavini, 2012). As we can see, the modern pear assortment is limited to traditional and commercially significant cultivars, and the rate of emergence and spread of new hybrid forms on the market is quite low.

In the 20th century, the purpose of pear breeding programs was to improve the complex of morphological and agronomic characteristics. Currently, it is becoming most urgent to increase the resistance of plants not only to biotic, but also abiotic environmental factors (Bellini & Nin, 2002; Dondini & Sansavini, 2012).

Standard genetic improvement of pear cultivars includes interspecific crosses to transmit necessary traits (Bell & Hough, 1986; Sun *et al.*, 2011). The pear is heterozygous and has a long-lasting juvenile period, for this reason traditional breeding programs for pathogens resistance equire a lot of time and resources. Spontaneous mutations occurring in nature, which can lead to genetic changes in fruit crops, for example, a change in fruit color (Teskey & Shoemaker, 1978; Walsh & Volz, 1990), rarely cause resistance to pathogens, since they are accidental and exclude the possibility of targeted receipt of genetic changes.

In connection with the above, new genomic technologies with various biotechnological tools make it possible to improve important commercial cultivars (Ricci *et al*., 2023) in a shorter time by modifying certain traits

and will help to get more highly productive plants with improved fruit quality (Sabbadini *et al*., 2021). In this case, new genomic technologies are a potent tool to speed up the breeding process and obtain improved cultivars.

Nevertheless, the successful implementation of new biotechnological methods demands the optimization of protocols suitable to modify the genome of the cultivar of interest. In particular, to implement genome modification such as agrobacterial transformation, the optimization of successful and repeatable *In vitro* regeneration protocols is necessary (Ricci *et al.*, 2020a, b; Ricci *et al*., 2023).

Although the sufficient levels of regeneration have been achieved in some pear genotypes, organogenesis remains a difficult task at the moment and apparently is largely determined by the genotype. Thus, the creation of a stable and effective *In vitro* regeneration system is a basic requirement for the success of the genetic transformation of the pear (San *et al.*, 2015)

The goal of this review is to analyze and systematize the parameters of effective *In vitro* regeneration protocols for commercially significant pear cultivars in order to increase the frequency of transformation mediated by *Agrobacterium* for further solving important problems of these cultivars. The main factors influencing the organogenesis of pears and their optimal parameters are discussed. The review includes detailed information on the optimal compositions of culture media, the choice of the explant, the concentration of growth regulators and antibiotic sensitivity.

Research on the regeneration and transformation: emerging problems: Pear is the natural host of *Agrobacterium tumefaciens*, which by definition makes it possible to carry out an agrobacterial transformation. However, there are relatively few studies aimed at the development and use of pear transformation methods with *A. tumefaciens* (Zhu & Welander, 2004).

Genetic modification of pears is a more difficult task, especially compared to the same apple, due to factors such as low regeneration ability, low transformation efficiency, which also depend on the genotype. In most of the applied methods, pear transformation is carried out using strains of A. tumefaciens EHA101 or EHA105. The first published research on this topic was the report of Mourgues (Mourgues *et al.*, 1996). Pear cultivars Doyenne du Comice, Conference and Passe-Crassane were used as research objects, of which Conference had the greatest regenerative ability (Mourgues *et al.*, 1996; Yancheva *et al.,* 2006).

To date, a large number of regeneration protocols are described for various pear cultivars (da Silva *et al.,* 2018). however, high regeneration frequency values have not always been repeatable, as evidenced by significant variability among repeats of the same study and among trials repeated at different time intervals (Leblay *et al.*, 1991; Bell *et al*., 2012).

Factors can strongly influence pear organogenesis: Many studies have described how various factors can strongly influence pear organogenesis, including the selection and orientation of the initial explant (Chevreau & Leblay, 1992; Yousefiara *et al.,* 2014), plant growth regulators (particularly cytokinins) and their concentrations, the main salt composition of the medium for the induction of shoots (Caboni *et al.*, 1999; Abdollahi *et al.*, 2006; Tang *et al.*, 2008; Yousefiara *et al.*, 2014), gelling agents (Chevreau *et al.*, 1997), carbohydrate source and genotype (Leblay *et al.*, 1991; Chevreau *et al.*, 1997; Zhu, 2000; Abdollahi *et al.*, 2006; Tang *et al*., 2008; Yousefiara *et al.*, 2014).

Depending on these factors, the optimal parameters of *In vitro* regeneration protocols for commercially significant pear cultivars are presented below (da Silva *et al.,* 2018).

The type and orientation of explants: Main categories of plant material which use as explants for regenerate transformed lines are seed-derived and somatic tissues.

The organogenesis of pear shoots has been noted with the use of various initial explants: roots (Viseur, 1990), the axes of the embryos (Browning *et al.*, 1987), cotyledons (Browning *et al.*, 1987), protoplasts (Ochatt *et al.*, 1993), the tops of the shoots (Caboni, 2002), embryos obtained from anthers (Kadota *et al.*, 2002) and leaves (Predieri *et al.*, 1989; Abu-Qaoud *et al.*, 1991; Chevreau & Leblay, 1992; Zhu, 2000; Poudyal *et al.*, 2008; Tang *et al*., 2008; Bell *et al.*, 2012; Yousefiara *et al.,* 2014).

Usage adult somatic tissues for regeneration from is strongly recommended for cloning-propagated crops in order to maintain the genetic uniformity of cloned plants, especially for highly heterozygous species (Ricci *et al.*, 2020a).

Leaves are the most significant source of explants used for pear regeneration protocols (Chevreau & Leblay, 1992; Yousefiara *et al.*, 2014), as well as for genetic transformation mediated by Agrobacterium spp (Yancheva *et al.,* 2006; Sun *et al.*, 2011; Nakajima *et al.*, 2013; Tomes *et al.,* 2023).

Selection of a nutrient medium for reproduction: In order to obtain regenerable explants (for example, leaves and petioles), it is necessary to create an effective micropropagation system before conducting regeneration studies.

To carry out this stage successfully, it is necessary to fulfill a number of conditions, the main of which is the selection of a nutrient medium. The composition of the medium is crucial because it directly affects the growth and viability of cells.

The most commonly used nutrient medium for clonal micro-propagation of pears is MS (pH 5.8) (Murashige & Skoog, 1962)

Researchers also reported that explants of six pear cultivars (Kaiser, Harrow Sweet, Abate Fetel, Williams, Dar Gazi and Conference) (Abdollahi *et al.*, 2006) demonstrated a higher ability to regenerate when they were collected from shoots propagated on a modified QL medium (Quoirin *et al.*, 1977). This medium contributed to the growth of leaves and to obtain 90% regeneration in explants of Williams and Dar Gazi.

In other reports (Ricci *et al.*, 2023), the use of QL medium also proved to be more effective for Conference and Abate Fétel cultivars, the percentage of regeneration of which on this medium was 87.3% and 68%, respectively.

Basal salt composition: The regeneration process in *In vitro* culture strongly depends on the mineral composition of the nutrient medium.

The media that are most often found in literary sources for the regeneration of pear cultivars (Abdollahi *et al*., 2006) are the following: MS (Murashige & Skoog, 1962) (Chevreau *et al.*, 1989, Gao *et al.*, 2002; Abdollahi *et al.*, 2006; Ricci *et al.*, 2023) and Nitsch (Nitsch & Nitsch, 1969) (Matsuda *et al.*, 2005; Gao *et al*., 2007; Yousefiara *et al.,* 2014).

For the regeneration of the cultivars 'Bartlett', 'Packham's Triumph', 'Williams', the researchers recommended using the MS medium (Abdollahi *et al.*, 2006, Poudyal *et al.*, 2008, Tang *et al.*, 2008,), while a high percentage of regeneration of 'Conference' (87.3%) and 'Abate Fétel' (68%) was achieved at MS half strength.

For 'La France' and 'Old Home', among the various base salts tested, the Nitsch medium (Nitsch & Nitsch, 1969) was recognized as the most effective for regeneration in most experiments (Gao *et al*., 2007; Sun *et al.*, 2011).

It has been proved that ammonium and total nitrogen play an important role: the balance between NH4 + and NO3 - also influenced regeneration; optimal regeneration occurred on media with a ratio of NH4+/NO3 - 1:3 (Leblay *et al.*, 1991; Wada *et al.*, 2015).

Type, concentrations and combinations of growth regulators: The process of callus formation and regeneration of tissues, organs and the whole plant from differentiated specialized cells can be reproduced in plant cell culture on artificial nutrient media containing certain phytohormones or growth regulators (PGRs). The selection of the appropriate type and concentrations of growth regulators added to the medium for basic regeneration is one of the important stages in determining the appropriate *In vitro* stimulus capable to influence favorable on organogenes (Ricci *et al.*, 2020a, b).

Auxins induce the process of dedifferentiation of specialized cells. However, in order for dedifferentiated cells to begin dividing, cytokinins are needed.

Among growth regulators, tidiazuron (TDZ) in combination with naphthylacetic acid (NAA) proved to be the most effective for stimulating adventitious shoots (Chevreau *et al.*, 1989; Poudyal *et al.*, 2008). TDZ is a synthetic growth regulator (Sun *et al.*, 2011), it has a high ability to stimulate the formation of adventitious shoots and somatic embryos in a wide range of plants (Sajid *et al.*, 2009; Sharma *et al.*, 2013). However, the effectiveness of TDZ in inducing the appearance of accessory shoots in pears depends on the genotype. Researchers have established, that TDZ was to be more effective than 6-BAP (BA) in inducing organogenesis in many commercial pear cultivars (Leblay *et al.*, 1991; Bacha *et al.,* 2021; Liu *et al.*, 2023), shoot regeneration was observed in a wide range of TDZ and NAA concentrations (from 0.5 to 5 microns and from 2.5 to 13 microns, respectively) depending on the genotype (Poudyal *et al.*, 2008; Sun *et al.*, 2011; Ricci *et al.*, 2023).

Carbohydrate sources: The main source of carbohydrates added to the regeneration medium is sucrose.

Some researchers have reported an improvement in the ability to regenerate the rootstock of the semi-dwarf pear OHF333 and the 'Spadona' cultivar by adding sorbitol (Zhu, 2000; Yancheva *et al.*, 2006).

Sorbitol is the main transport sugar and a reserve carbohydrate in the *Rosaceae* family. The natural sorbitol/sucrose ratio varies depending on the age of the plant and environmental conditions and ranges from 2:1 to 5:1 in *Rosaceae* species (Escobar-Gutienez & Gaudillere, 1994). The positive effect of sorbitol and sucrose has also been shown in relation to the proliferation of micro-propagating apricot (Marino *et al.*, 1993), the formation of adventitious shoots in the apple rootstock Jork9 (Pawlicki & Welander, 1994) and other commercial apple cultivars, as well as plums (Song *et al.*, 2011). which leads to an increase in the number of shoots and the rate of regeneration and makes it promising for use in pear protocols.

Gelling agents: The main gelling agent used in regeneration media is agar.

In the articles of Morgues (Mourgues *et al.,* 1996) with co-authors, it was reported on the effectiveness of using 2.5 $g \cdot l^{-1}$ gelrite for hardening the regeneration medium.

Researchers (Chevreau *et al*., 1997) demonstrated that the solidification of the regeneration medium (Abdollahi *et al*., 2006) with gellan gum (Phytagel™) instead of agar had a positive effect on the differentiation of accessory buds. This gelling agent induced faster cell division than agar, thus more callus was formed on the wound sites. Incubation on a gellan gum medium during the first 20 days after the appearance of the buds was enough to cause a stimulating effect on the regeneration and limit the formation of hyperhydrate buds. (Marino *et al.,* 2013).

Sensitivity of plant tissues to antibiotics: For pear cultivars it is necessary to select an effective concentration of antibiotics used for the deactivation of agrobacteria after co-cultivation and at the stages of regeneration, in which there will be no inhibition of explants and negative effects on regeneration.

Kanamycin is the most common selective antibiotic that is used after infection with agrobacteria. Mourgues and co-authors (Mourgues *et al.,* 1996), describing a gene transfer method mediated by *A. tumefaciens*, optimized a selection medium containing kanamycin at a concentration of 207 mg l^{-1}. Other researchers obtained transgenic lines of many pear cultivars, using in the selection medium concentrations of kanamycin from 52 to 165 mg l^{-1} (Matsuda *et al.*, 2005; Sun *et al.*, 2011). Scientific works have demonstrated how wide the range of kanamycin concentrations used in the protocols can be, but it should not exceed 207 mg l^{-1}.

However, in studies by Ricci *et al*., (Ricci *et al.,* 2023), it is reported that for the Conference pear cultivar, even when using kanamycin in extremely low concentrations, the regeneration of shoots decreased sharply compared with the corresponding control samples without the use of an antibiotic. Which indicates that this cultivar is rather sensitive to kanamycin as a selecting antibiotic. The detection of such strong sensitivity to kanamycin may require the use of delayed selection, that is, the inclusion of a selective antibiotic in the regeneration medium a few days/weeks after infection of this cultivar with Agrobacterium (Ricci *et al.,* 2023).

Cefotaxime is a semi–synthetic analogue of cephalosporin, a third-generation antibiotic. Cefotaxime is widely used for the elimination of *Agrobacterium tumifaciens* in the experiments on genetic transformation.

Table 1. The main parameters of effective *In vitro* regeneration protocols for commercially significant pear cultivars.

Genotype	Medium composition and optimal concentration of hormones for		Source of carbohydrates	Gelling agent	Reference
	Reproduction	Regeneration			
Abate Fétel	QL, pH 5,8 8,9 μM BA 0,1 μM NAA	½ M S basal medium, pH 5,89 μM TDZ 1 μM NAA	30 g·l^{-1} sucrose	6,5 g·l^{-1} agar	Ricci *et al*., 2023
Bartlett	MS, pH 5.6 1.0 mg·l^{-1} BA 0.1 mg·l^{-1} BA 0.5 mg·l^{-1} GA_3	MS pH 5.8 6,0 mg·l^{-1} BA 0,1 mg·l^{-1} NAA	30 g·l^{-1} sucrose	7 g·l^{-1} micro agar	Tang *et al.,* 2008
Conference	QL, pH 5,8 6,6 μM BA, 1,5 μM IBA	½ M S basal medium, pH 5,8 13.5 μM TDZ 1 μM NAA	30 g·l^{-1} sucrose	6,5 g·l^{-1} agar	Ricci *et al.*, 2023
La France	MS, pH 5,8. 0,5 mg·l^{-1} BA,	NN 10 μM TDZ 1 μM NAA	30 g·l^{-1} sucrose	0,8% agar	Gao *et al.,* 2007
Old Home	MS, pH 5,8 1,0 mg·l^{-1} BA, 0,2 mg·l^{-1} IBA, 3% sucrose and 6 g·l^{-1} agar	NN 4 mg·l^{-1} TDZ 0,3 mg·l^{-1} NAA	sucrose	Liquid medium without a gelling agent	Sun *et al.,* 2011
Packham's Triumph	MS 1.0 mg·l^{-1} BA 0.1 mg·l^{-1}IBA	MS 2,0 mg·l^{-1} TDZ 1.0 mg·l^{-1} NAA	30 g·l^{-1} sucrose	6,0 g·l^{-1} agar	Poudyal *et al.*, 2008
Spadona	AP (Almehdi & Parfitt, 1986), pH 5,7 25 mg·l^{-1} myinositol, 2 mg·l^{-1} pyridoxine HCl, 0,5 mg·l^{-1} BA, 0,01 mg·l^{-1} IBA, 0,2 mg·l^{-1} GA3, 3% sucrose, 0,35% bactoagar, 0,1% of gerlit.	AP, pH 5,7 5,0 mg·l^{-1} TDZ, 0,2 mg·l^{-1}NAA 160 mgl^{-1} adeningemisulfate	3% sucrose and 1, 5% sorbitol	0,75% agar	Yancheva *et al.,* 2006
Williams	MS, pH 5,7 4,4 μM BAP, 0.5 μM NAA 3% sucrose, 0,5% (w/v) agar, 0,5% pectin	MS 5 μM TDZ 2,7 mg·l^{-1}NAA	30 g·l^{-1} sucrose	6,5 g·l^{-1} agar	Leblay, *et al.*, 1991; Abdol-lahi *et al.,* 2006

* Abbreviations
BA, BAP: 6-benzylaminopurine
IBA: indole-3-butyric acid
NAA: a-naphthaleneacetic acid
TDZ: thidiazuron (N-phenyl-N'-1,2,3-thidiazol-5-ylurea)
GA_3: gibberellin A_3

Despite the neutral or beneficial effect of cefotaxime on the regeneration of shoots (Ricci *et al.*, 2020a, b), this antibiotic should be considered as a toxic compound for the organogenesis of a1cessory shoots, starting with the leaves of pears, at least when used in concentrations equal to or exceeding 630 mg l^{-1} (Schmitt *et al.*, 1997); lower efficiency in callogenesis when using cefotaxime (as well as vancomycin) in high concentrations may be caused by an increase in DNA methylation.

Basic protocol parameters for the creation of effective *In vitro* regeneration: Thus, regeneration protocols are available for various pear cultivars, some of which demonstrate high efficiency. Having analyzed the main factors influencing the organogenesis of pear leaves, Table 1 presents the main parameters of effective and reproducible protocols for optimizing the *In vitro* regeneration process for commercially significant pear cultivars

Conclusion

The contribution of biotechnological and molecular genetic methods to pear breeding research has increased significantly over the past decade. Currently, new opportunities have emerged for targeted plant transformation. The least expensive and most effective method among them is the clustering of the Cas9 nuclease system associated with short palindromic repeats (CRISPR). CRISPR / Cas9 has been a major breakthrough method in gene research over the past decade. The effectiveness of the protocols for targeted plant transformation, including this one, on the effective delivery of genes and the productive regeneration of treated explants. And to create effective *In vitro* regeneration systems, optimization of protocols specifically designed for the cultivar of interest. This review was aimed at systematizing the parameters of effective *In vitro* regeneration protocols for commercially significant pear cultivars in order to increase the frequency of transformation mediated by *Agrobacterium* for further solving important problems of these cultivars. For this reason, the influence of various factors on the efficiency of regeneration of pear genotypes was evaluated, such as: the choice of the initial explant, the use of plant growth regulators (especially cytokinins) and their concentrations, the basic salt composition of the medium for the induction of shoots, gelling agents, type of carbohydrates and genotype. For the first time, optimal protocol parameters have been collected to create effective *In vitro* regeneration systems for the main commercially important pear cultivars. The summarized results of this review will be useful in the development of future genetic transformation trials for commercially significant cultivars and for the application of basic NGTs for the genetic improvement of pears. In particular, cultivars such as ‘Conference’ and ‘Abate Fétel’ require further genetic improvement, mainly due to their high susceptibility to brown spot caused by *Stemphylium vesicarium*, an anamorphic fungus that becomes resistant to the most common fungicides.

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