Efficient Shoot Regeneration System of Pear Rootstock OHF 333 (*Pyrus communis* L.) Leaves

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Abstract

The pear rootstock OH × F (*P. communis* L.), bred in the USA, shows 10% more vigorous growth compared to BA29, but it has a slower growth than the seedlings of 'Williams'. The rootstock is characterized by high yield efficiency and moderate resistance to fire blight (*Erwinia amylovora*). Some clones of OH × F are difficult to propagate, that is why they are propagated in vitro. The aim of the present research was to develop an efficient system for shoot regeneration from pear rootstock OHF 333 leaf explants and to investigate the effect of plant growth regulators on the regeneration capacity. Studies were carried out with leaf segments of in vitro propagated plants of OHF 333 (*P. communis* L.) cultivar. Leaf segments of the source plants were cultivated on nutrient media for regeneration based on Murashige and Skoog (MS) with added TDZ (thidiazuron, 7.5 and 9μM), 2.46 μM indole-3-butyric acid (IBA), 10 g/L sucrose, 30 g/L sorbitol and different indole acetic acid (IAA) content – 5, 10, 15 and 20 μM. The explants were cultivated in darkness for 15 days, after which at a photoperiod of 16/8 hours (40 μmol $m^{-2}s^{-1}$ PPFD) at a temperature of 22±2°C for 20 days. The best efficiency of somatic organogenesis (over 80% regeneration and more than 5 regenerants per explant) was achieved on two nutrient media - with 7.5 μM TDZ, 2.46 μM IBA and 20 μM IAA or with 9 μM TDZ, 2.46 μM IBA and 10 μM IAA. All the regenerants obtained were cloned, propagated and would be tested for resistance to fire blight (*Erwinia amylovora*) after acclimatization.

INTRODUCTION

Pear (*Pyrus communis* L.) is a fruit of strong consumer demand and high commercial value. However, most of the pear rootstocks and cultivars were susceptible to diseases, caused by fungi, bacteria and viruses (Sun et al., 2006). One of the most devastating diseases is fire blight (*Erwinia amilovora* Burrill). Although quarantine measures have been taken, it continues to spread throughout Europe. The lack of efficient control procedures made breeding one of the most promising tools within the framework of an integrated control program (Dondini, 2002). Conventional breeding for disease resistance is difficult due to the fact that pear is highly heterozygotic and progenies from the cross breeding express a lot of characteristics different from those of the parents. The seedlings required a long juvenile period before fruiting (Predieri, 2002). New approaches to the problem are offered by molecular biology, somaclonal variation and genetic transformation.

The pear rootstock OHF 333 (*P. communis* L.) was bred in the USA and it was a part of OH × F ('Old Home' × 'Farmingdale') rootstock series. It was characterized by high yields and a moderate degree of resistance to fire blight (*Erwinia amylovora*) (Van der Zwet and Beer, 1995; Wertheim, 2002). The rootstock showed 10% more vigorous growth compared to BA29, but it had a slower growth than the seedlings of 'Williams' (Masseron, 1989). Its improvement by means of somaclonal variation and/or genetic transformation necessitated the development of methods for in vitro propagation and somatic regeneration.

Several studies were conducted on micropropagation of pear rootstocks and cultivars (Viseur, 1987; Sansavini, 1994; Predieri and Govoni, 1998; De Paoli et al.,

2002; Barros, 2005; Sotiropoulos et al., 2006a, b).

Adventitious shoot regeneration from leaf explants was successful for various pear cultivars and rootstocks (Leblay et al., 1991; Lane et al., 1998; Lebedev and Dolgov, 2002; Hennayake et al., 2003; Marino and Molendini, 2005; Abdollahi et al., 2006; Marino et al., 2008; Poudyal et al., 2008). The influence of the medium composition and plant growth regulators on adventitious shoot formation of the popular pear cultivars 'Old Home' (one of the parents of OHF 333) was studied (Sun et al., 2005, 2006). The regeneration capacity of the cultivar reached almost 100% when the leaf explants were subject to a two-phase cultural regime. As far as the information we have, until now there are no studies on regeneration from somatic tissues of OHF 333.

The aim of the present research was to develop an efficient system for shoot regeneration from pear rootstock OHF 333 leaf explants and to investigate the effect of plant growth regulators on the regeneration capacity.

MATERIAL AND METHODS

Plant Material

All the experiments were carried out with in vitro propagated plants of pear rootstock OHF 333 (*P. communis* L.).

The source plants were cultured on the nutrient medium A1 – modified MS (Murashige and Skoog, 1962) with ½ concentration of NH_4NO_3 and $CaCl_2$ and 1000 mg/L $Ca(NO_3)_2$. It was supplemented with 2.5 μM 6-benzylaminopurine (BAP), 0.005 μM IBA, 30 g/L sucrose, 6.5 g/L agar, pH 5.6 (before autoclaving).

The plants were maintained in the growth room at an air temperature of 22-24°C with 16/8 hours photoperiod supplied by cool-white fluorescent lamps (OSRAM 40W; 40 μmol $m^{-2}s^{-1}$ PPFD).

Experiment 1

3-4 of the youngest, fully expanded leaves of 15-day old microplants were excised and stored in Petri dishes with sterile distilled water for adventitious shoot induction. The petiole with basal one-third of each leaf was dissected and the midrib was wounded. The explants were placed abaxial side up on a regeneration medium. Ten explants were placed in each of 9 cm Petri dishes.

The regeneration media consisted of MS macro- and microsalts with different content of plant growth regulators and carbohydrates (Table 1). The medium was solidified with 5.8 g/L agar (Fluka) and adjusted to pH 5.6 before autoclaving.

The number of regenerants per explant, callus and root formation were reported after 15-day cultivation in darkness and 20-day period at 16h photoperiod in a growth-room.

All the regenerants obtained were elongated on A2 medium – ½ MS macro- and microsalts supplemented with 1.3 μM BAP, 0.003 μM IBA, 30 g/L sucrose, 6.5 g/L agar, pH 5.6 (before autoclaving).

Experiment 2

The plant material for regeneration was prepared as described in Experiment 1. The regeneration media contained MS macro- and microsalts with added 7.5 μM TDZ, IAA - 5, 10, 15 and 20 μM, 10 g/L sucrose and 30 g/L sorbitol. The medium was solidified with 5.8 g/L agar (Fluka) and adjusted to pH 5.6 before autoclaving.

Data were obtained after 15-day cultivation in darkness and 20-day period at 16h photoperiod in a growth room. All the regenerants were elongated on A2 medium.

Experiment 3, Two Step Regeneration Procedure

1. First Step. The experimental procedure and the environmental conditions were the same as described above. The regeneration media consisted of MS macro- and microsalts supplemented with different concentrations of TDZ (7.5 μM and 9μM), IAA, and IBA

(Table 2), 10 g/L sucrose, 30 g/L sorbitol, 5.8 g/L agar, pH 5.6 (before autoclaving).

The number of adventitious shoots was determined after 15-day cultivation in darkness and 20-day period at 16h photoperiod.

2. Second Step. The leaf explants with regenerated adventitious shoots were plated on A2 medium for shoot elongation for 20 days.

The regeneration frequency, mean number of shoots per explant with regeneration and the shoot forming capacity (SFC) were evaluated after 15-day cultivation in dark, 20 days at 16h photoperiod and 20 days on A2 medium for shoot elongation.

The shoot forming capacity (SFC) index was calculated according to Martinez-Pulido et al. (1992), as follows:

SFC index = (% of explants with shoots) × (mean number of shoots per explant)/100.

Data Analysis

Data about the mean number of shoots per explant were determined by the analysis of variance and the means were separated using the Duncan's multiple range test ($P<0.05$).

RESULTS AND DISCUSSION

Experiment 1

The explants were fresh, vital and they formed yellow to light green loose callus on all the tested nutrient media. The regenerants were located mainly at the place of excision, in most cases on the callus tissue. The formation of both stem and root meristems was established, which developed after the transfer of the explants to light conditions. The results confirmed the conclusions from our previous experiments about the strong influence of the carbohydrate type and concentration on adventitious shoot formation (Gercheva et al., 2000, 2005a, b). The best regeneration rate was achieved on media 1/3 TIB with 10 g/L sucrose and 30 g/L sorbitol (Fig. 1).

Two media with a low concentration of auxin (0.49 μM IAA) were tested with the aim of reducing callus formation. Adventitious shoot regeneration was not observed on those cultural media (Fig. 1). That confirmed the assumption that under the conditions of our experiment, the high auxin concentration and dedifferentiation of the plant cells was a precondition for successful regeneration.

Experiment 2

The aim of experiment 2 was to study the possibility of using IAA – a natural auxin which can be deactivated in the plants, for somatic regeneration from leaf segments of the pear rootstock OHF 333.

The results demonstrated that on all the tested media, the explants formed yellow-green soft callus, root and shoot meristems, mostly on the wounded surfaces. The regeneration rate of the leaf segments increased with the increase of the IAA concentration (Fig. 2).

Experiment 3

1. First Step. All the cultural media stimulated callus formation on the wounded margins of the leaf explants, from 50% to 80% of the leaf surface or the entire explant being covered by callus. Clusters of bud and root primordia regenerated from the callus or from the midrib. The differences in the plant growth regulators used had a little effect on the regeneration rate – between 80% and 100% on all the media tested (Fig. 3). At the same time the combination of two auxins (IAA and IBA) in the cultural media stimulated the formation of more than 5 bud primordia in 50-80% of the leaf explants. On cultural media I and II, which had one auxin (IAA or IBA), only 10-20% of the explants had more than 5 regenerants (Fig. 3).

2. Second Step. Data analysis after 20 days of elongation showed that the content of the nutrient medium for regeneration had a significant effect on the survival and development

of the regenerants.

The stimulating effect of the increased concentrations of IAA auxin in combination with IBA was obviously expressed, especially in media of lower concentrations of TDZ (7.5 μM). For example, on media I, II, III and IV the percentage of regeneration decreased from the reported 80-90% in the first step to 40-50% in the second step (Fig. 3, Table 2). At the same time, the percentage of regeneration in variants with high concentrations of auxin (VI, VII, VIII, IX and X) changed from 90-100% before elongation to 80-90% after that. It means that almost all the stem primordia reported at step I initiated vital and normally developed regenerants.

An important characteristic showing the regeneration success, is the number of plants obtained from one segment. After elongation, the best results in that aspect were reported for variant VI - 7.5 μM TDZ, 2.46 μM IBA and 20 μM IAA.

The highest SFC values (a coefficient showing both the percentage of regeneration and the mean number of regenerants per explant) were obtained in variants VI, VII, VIII and X (Fig. 4).

Keeping in mind all the reported characteristics – percentage of regeneration, high SFC and good development of the regenerants at the stage of elongation, we concluded that the most suitable media for induction of somatic organogenesis in OHF 333 pear rootstock (*P. communis* L.) were variants VI (7.5 μM TDZ, 2.46 μM IBA and 20 μM IAA) and VIII (9 μM TDZ, 2.46 μM IBA and 10 μM IAA).

In result of the experiments carried out, more than 500 regenerants were obtained, which were successfully cloned and used in the breeding programmes for in vitro and in vivo breeding for resistance to fire blight (*Erwinia amylovora*).

CONCLUSIONS

- The carbohydrate type and concentration in the cultural media significantly influenced the percentage of adventitious shoot formation. The best results were achieved on media 1/3 TIB (10 g/L sucrose and 30 g/L sorbitol), which was used as control in the next experiments.
- The regeneration rate of the leaf segments of OHF 333 increased with the increase of the IAA concentration.
- The high concentrations of IAA auxin in combination with IBA in the media for regeneration had a stimulating effect on the survival and development of the regenerants at the stage of elongation.
- The best efficiency of somatic organogenesis (over 80% regeneration and more than 5 regenerants per explant), high SFC and good development of regenerants on the elongation medium were achieved on two nutrient media – with 7.5 μM TDZ, 2.46 μM IBA and 20 μM IAA or with 9 μM TDZ, 2.46 μM IBA and 10 μM IAA.

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Tables

Table 1. The regeneration media used in Experiment 1 and Experiment 2: MS macro- and microsalts with different content of plant growth regulators and carbohydrates.

Variants	TDZ (μM)	IAA (μM)	IBA (μM)	sucrose (g/L)	sorbitol (g/L)
Experiment 1					
1/3 TIB	7.5		2.5	10	30
1/2 TIB	7.5		2.5	10	20
2/2 TIB	7.5		2.5	20	20
5 TIA	6.8	0.49	0	30	0
6 TIA	6.8	0.49	0	10	30
Experiment 2					
TIA 5 μM IAA	7.5	5	0	10	30
TIA 10 μM IAA	7.5	10	0	10	30
TIA 15 μM IAA	7.5	15	0	10	30
TIA 20 μM IAA	7.5	20	0	10	30

Table 2. Effect of types and concentrations of plant growth regulators on regeneration percentage, number of shoots per regenerating explant and SFC of pear rootstock OHF 333 (Experiment 3).

Variants	TDZ (μM)	IAA (μM)	IBA (μM)	Shoot regeneration (%)	Mean number of shoots/explant (mean±SE)*	Shoot forming capacity (%)	Root regeneration (%)
I	7.5	15	0	42.5	3.47±0.43 c	1.48	0
II	7.5	0	2.46	40	2.33±0.62 b	0.93	0
III	7.5	5	2.46	50	2.05±0.25 b	1.03	0
IV	7.5	10	2.46	7.5	1.33±0.41 a	0.01	35
V	7.5	15	2.46	47.62	2.40±0.39 b	1.14	19
VI	7.5	20	2.46	82.5	4.06±0.26 c	3.35	2.5
VII	9	5	2.46	87.5	2.54±0.25 b	2.22	12.5
VIII	9	10	2.46	92.5	2.29±0.28 b	2.12	15
IX	9	15	2.46	95	1.33±0.19 a	1.27	20
X	9	20	2.46	95	2.21±0.19 b	2.1	25

*Different letters indicates significant difference (P<0.05) by DMRT.

Figures

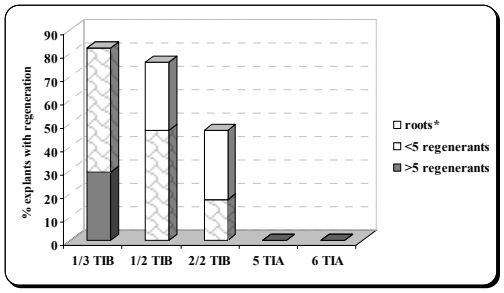

Fig. 1. Effect of different media on adventitious shoot regeneration from in vitro derived leaves of pear rootstock OHF 333. * Explants with adventitious roots.

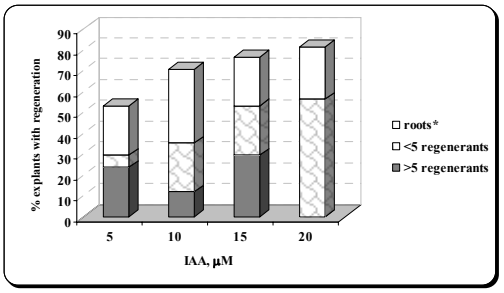

Fig. 2. Effects of IAA concentrations on shoot production from leaf explants of pear rootstock OHF 333 after 15 days cultivation in darkness and 20 days at light – Experiment 2. * Explants with adventitious roots.

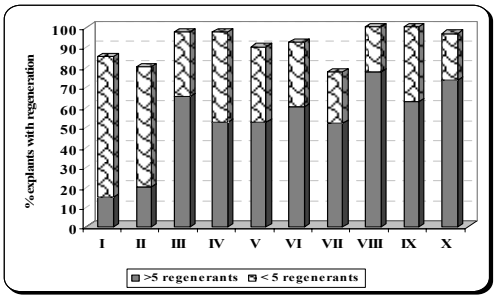

Fig. 3. Effect of types and concentrations of plant growth regulators on regeneration from leaf explants of pear rootstock OHF 333 (Experiment 3, First step).

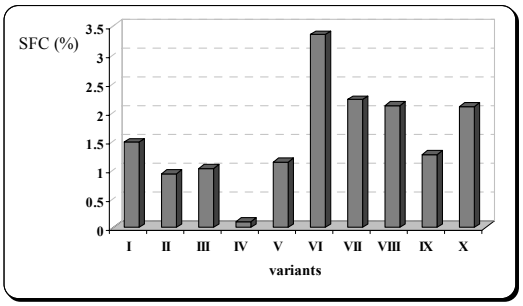

Fig. 4. Effect of types and concentrations of plant growth regulators on the shoot forming capacity (SFC) index after 20 days on elongation medium (Experiment 3, Second step).