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Development of an efficient regeneration protocol for pear rootstock Pyrodwarf and assessment of SSR variability in regenerating shoots

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Abstract — The use of biotechnological methods is of particular interest in fruit tree crops since conventional breeding programs require long time and high resources before having impact upon commercialization. To develop an efficient plant regeneration protocol for pear rootstock *Pyrodwarf*, different combinations of two hormones, naphthalene-1-acetic acid (NAA) and Thidiazuron (TDZ) were studied on regeneration process. Other factors were also studied, such as darkness period and the use of different hormone concentrations during induction and expression phase. Our experiments showed that the length of darkness did not affect *in vitro* regeneration. Different concentrations of TDZ have different effects on regeneration: 6 µM has been shown to be more effective than 2.5 µM. A higher level of TDZ during induction period was the only important factor to induce regeneration independently to the presence of hormones during expression phase. The two hormones showed to be uneffective to induce a sufficient number of regenerating shoots from leaf explants of another pear rootstock, *Fox11*. Variability during regeneration was assessed using SSR markers. An identical genetic profile was observed between *in vitro* explants and regenerating shoots. These results are useful to include *Pyrodwarf* in genetic engineering experiments for breeding purposes. On the contrary, other hormones have to be evaluated for their effectiveness to induce *in vitro* regeneration in *Fox11* leaf explants.

Key words: *Fox11*, NAA, *Pyrodwarf*, regeneration, SSR, TDZ.

INTRODUCTION

The development of an efficient regeneration protocol is a necessary step for the set-up of *in vitro* based plant improvement methods. The use of biotechnology-aided breeding is of particular interest with pear and other fruit tree crops since conventional breeding require long time and high resources before having impact upon commercial pear production. Shoot regeneration has been obtained from cultured leaves of Japanese pear (*Pyrus pyrifolia* [Burm.f.] Nakai) (Lane *et al*. 1998) and several pear (*Pyrus communis* L.) cultivars (Leblay *et al*. 1991; Predieri *et al*. 1989; Chevreau *et al*. 1989). Explants such as cotyledons (Turner and Singha 1988) and protoplasts of wild pear (*Pyrus communis* var *pyraster* L.) were also used (Ochatt and Caso 1986).

Interesting results in pear using *in vitro* regeneration approaches were obtained for relevant agronomic features, such as tolerance to iron-chlorosis (Palombi *et al*. 2007) and sodium chloride (Marino and Molendini 2005), while regeneration based genetic transformation approaches were also undertaken using sense or antisense cDNA encoding ACC oxidase (Gao *et al*. 2007), fungi resistance (Xiao *et al.* 2007) and *rolB* genes (Zhu *et al*. 2003).

Modern high density planting system requires dwarfing rootstocks having high compatibility with the scion cultivar. The pear rootstocks studied in this work *Pyrodwarf* and *Fox11* are dwarfing rootstocks selection obtained from *Pyrus communis*. The first one is a hybrid between "Old Home" and "Gute Luise" (obtained in Germany) varieties that do not undergo to iron-chlorosis in soil containing high calcium carbonate. This rootostock has a medium resistance to fire blight,

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high productivity and it is suitable for high-density orchard. The second one, *Fox11* has been selected at the university of Bologna (Italy) from *Pyrus communis*. It is characterized by a high rooting ability, good productivity, and compatibility with scion cultivars and it grow well in soils with high presence of calcium carbonate.

Although adventitious shoot regeneration has been also obtained for dwarfing pear rootstocks, such as *OHF333* and *BP10030* (ZHU and WELANDER 2000) no data are present in literature relating to regeneration process in *Pyrodwarf* and *Fox11.* Growth regulator and mineral composition of the regeneration medium, as well as the duration and light requirement for the induction and expression phases have been studied (LEBLAY *et al.* 1991) for optimize pear regeneration.

In this work the role of hormonal and light factors were studied during induction and expression phase on regeneration efficiency of *Pyrodwarf* and *Fox11* rootstocks. Moreover, SSR (Simple Sequence Repeat) markers were considered to determine if variability at these loci might occur during the regeneration process.

MATERIAL AND METHOD

Plant material and micropropagation - *Pyrodwarf* and *Fox11* plantlets were maintained on QL medium (QUORIN *et al.* 1977) macro- and micro-elements, 40 mg l^{-1} FeNaEDTA, vitamins (1.0 mg l^{-1} thiamine-HCl, 100 mg l^{-1} myo-inositol, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine -HCl), 1 mg l^{-1} Benziladenina (BA), 0.1 mg l^{-1} Gibberellic Acid (GA_3) and 30 g l^{-1} sucrose, 7 g l^{-1} Agar. The pH was adjusted to 5.5 before autoclaving the medium at 121°C for 20 minutes. Plantlets were maintained in a climatic chamber at 24°C, under 12 µmol $m^{-2}s^{-1}$ PAR of light intensity and a 16/8 light/dark photoperiod and sub-cultured every 3 weeks.

Regeneration experiments - Regeneration studies were performed on the apical fully expanded leaves sampled from *in vitro* plantlets at 21th day after subculture. The explants were incubated in the dark at 24°C during the first 21 days and then left under continuous light at a photon flux density of 12 µmol $m^{-2}s^{-1}$. The young leaves were cut perpendicularly to the central vascular strands and then the explants, petiole included, were placed on Petri dishes containing the same basal medium used for the micropropagation supplemented with the following plant hormones and concentrations:

- for *Pyrodwarf*: TDZ (2.5 µM, 6 µM and 15 µM) and NAA (1 µM and 5.4 µM).
- for *Fox11*: TDZ (2.5 µM and 6 µM) and NAA (5.4 µM)

For *Pyrodwarf* explants, the following parameters were studied: use of different medium, a darkness period of 3 or 8 weeks and different induction and expression period length.

The explants were scored for total shoot number, shoot number/regenerating explants, and percentage of regenerating explants after 9 weeks from the beginning of the period under light. The regenerating shoots were excised and transferred in QL medium and the parameters were measured starting from the beginning of the experiment.

Statistical analysis - Experiments were repeated twice in order to control the source of variability due to age, physiological conditions of the explants and other environmental conditions.

Each treatment of one experiment was repeated in 5 Petri dishes (90 mm diam.) containing 20 leaves each and data were analysed using one-way ANOVA and Post-hoc LSD test was used (Significance Level=0.05) for mean comparison.

SSR analysis - Green full-expanded leaves and regenerating shoots were harvested from *in vitro* plantlets of *Pyrodwarf* and stored immediately at -80°C. The DNA extraction was performed following the extraction process of DNeasy Plant MiniKit (Qiagen). 3 specific primers were used, (KA 16, KA 14, BGT23b), according to the highest number of alleles detected (YAMAMOTO *et al.* 2002). Reaction mixes and PCR amplification profiles were performed as described by HOKANSON *et al.* (1998) and YAMAMOTO *et al.* (2001). The amplification products were loaded and run on the automatic ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

RESULTS

A preliminary experiment was performed for *Pyrodwarf* explants to compare two different growth media containing different combination of hormones (TDZ and NAA) and sugar (sorbitol or sucrose). These two media were chosen since they showed the highest percentage of regeneration in two pear rootstocks, *BP10030* and *OHF333* (ZHU and WELANDER 2000). However, only the one containing sucrose and 1 µM NAA + 15 µM TDZ showed to have a good effect on regeneration process whereas the other, containing sorbitol and lower concentrations of hormones

had no regeneration ability (Table 1). Considering these results, the first medium was used for following regeneration experiments starting with the combination of hormones used and studying additionally the effect of other combinations on regeneration process. In particular, we studied the effect of four different combinations of hormones: two level of TDZ, 6.0μM and 15.0μM and two levels of NAA, 1.0 μM or 5.4 μM to determine which combination induce the highest number of regenerating shoots. We repeated twice the experiments in order to increase the confidence on the results and controlling the source of variability due to the physiological conditions of the plant material and other sources of variability.

The first experiment showed a significative higher shoot number only when TDZ was 15 μM and NAA at 5.4 μM (Table 2). The second experiment partly confirmed the previous results showing that a significative increase of regeneration occurs not only at highest levels of the two hormones but it is sufficient that one of them is high (Table 2). Considering these two experiments together, we concluded that the combination with lowest levels for NAA (1.0μM) and TDZ (6.0 μM) was not able to induce a good regeneration. The two hormones at high level efficiently induce regeneration of shoots in both the two experiments.

Different induction period at darkness and hormone combinations were studied for understand if this factor was effective to induce a different percentage of regeneration or shoot number/explants (Table 3). After the incubation period, the explants were transferred in expression medium including TDZ at 2.5 μM. After 9 weeks since the beginning of the experiment, we obtained regeneration with a similar percentage of regeneration and shoot number/explants at NAA 5.4 μM and TDZ 6.0 μM independently to the incubation length whereas no regeneration was obtained when TDZ was at 2.5 μM.

The effects of different levels of NAA and TDZ during incubation period and expression phase was also studied. In particular, four different combinations of hormones were used: two of them were maintained also during expression phase and the others were used only in incubation period and then removed in expression phase

Table 1 — Regeneration percentage and shoot number per regenerating explants for *Pyrodwarf* explants in two Induction Medium. Explants were incubated in darkness for three weeks on induction medium (induction phase) plus hormones, after that phase hormones were removed (expression phase).

Sugar Source (30 g l^{-1})	Hormones Type and Concentrations in the Induction Medium during the first 3 weeks	Regeneration Percentage (%)	Shoot number per regenerating explants
Sucrose	1 μM NAA + 15 μM TDZ	65	2.1
Sorbitol	0.1 μM NAA + 5 μM TDZ	0	0

Table 2 — Shoot number, shoot number per explants, and regeneration percentage after 63 days in two independent (A and B) *Pyrodwarf* regeneration experiments. The effect of two different concentrations of TDZ (6.0μM or 15.0μM) and NAA (1.0μM or 5.4μM) were studied.

Experiment	Hormones (μM)		Shoot number	Shoot number per explants	Regeneration (%)
	TDZ	NAA			
A	6.0	1.0	12b	1.90b	5a
		5.4	19b	1.15b	12a
	15.0	1.0	16b	1.40b	6a
		5.4	43a	2.07a	17a
B	6.0	1.0	5b	0.63b	7b
		5.4	57a	2.66a	49a
	15.0	1.0	29a	1.63ab	34a
		5.4	27a	2.05ab	39a

Regeneration percentages were previously subjected to arcsin transformation. Numbers in column for the same experiment followed by the same letter are not significantly different using ANOVA and Post-hoc LSD test (Significance Level=0.05).

Table 3 — Shoot number per explants and regeneration percentage after 63 days in *Pyrodwarf* regeneration experiments. The effect of a different darkness induction period and concentrations of TDZ are shown. NAA concentration was maintained at 5.4 µM.

Darkness Induction Period (weeks)	TDZ (µM)	Shoot number per explants	Regeneration (%)
8	2.5	0	0
	6	1.7	12.5
3	2.5	0	0
	6	2.5	15

Table 4 — Shoot number per explants and regeneration percentage after 63 days in *Pyrodwarf* regeneration experiments. The effect of different concentrations of TDZ and NAA during induction and expression phase was studied.

Hormones (µM)				Regeneration (%)	Shoot number per explants
Induction Medium		Expression Medium			
NAA	TDZ	NAA	TDZ		
1	2.5	1	2.5	0	0
	6		6	0	0
5.4	2.5	No Hormones		0	0
	6			20.8	1.8

Table 5 — Shoot number per regenerating explants and regeneration percentage in *Fox11* in two independent (A and B) experiments. The induction and expression medium have different hormone concentrations.

Experiment	Hormones (µM) in the Induction Medium	Hormones (µM) in the Expression Medium	Regeneration (%)	Shoot number per regenerating explants
A	5.4 NAA + 2.5 TDZ	2.5 TDZ	0	0
	5.4 NAA + 6.0 TDZ		20	3
B	5.4 NAA + 2.5 TDZ	No hormones	0	0
	5.4 NAA + 6.0 TDZ		0	0

(absence of hormones in the medium) (Table 4). The results showed that regeneration occurred only for NAA at 5.4 and TDZ at 6.0. In addition the absence of hormones in expression medium did not influence the regeneration process and a period of 3 weeks of incubation was sufficient to produce regeneration. The factor that determined regeneration was the presence at highest levels used for NAA and TDZ in our experiment (respectively 5.4 µM and 6 µM).

Relating to *Fox11* a preliminary experiment was performed in order to determine the tendency or regeneration capacity for this rootstock using two concentrations of TDZ (2.5 µM and 6 µM). During the expression phase, the use of TDZ (2.5 µM) in comparison with absence of hormone was also studied. Anyway *Fox11* showed to be more difficult to regenerate than *Pyrodwarf* using TDZ and NAA. The only combination that showed the presence of regenerating shoots was when NAA was at 5.4 µM and TDZ at 6.0 µM (Table 5) confirming the results obtained in *Pyrodwarf*. However the presence of TDZ in the expression medium seemed to be beneficial to induce shoot regeneration.

Regenerating shoots of *Pyrodwarf* were also analysed using SSR analysis to determine an eventual variability at these locus during the regeneration process. This analysis was performed using 3 SSR primers (KA 16, KA 14, BGT23b) (YAMAMOTO *et*

al. 2002) and choosing 4 regenerating shoots for each hormone combination from the experiment A (Table 6). All the regenerating shoots showed the same profile previously known for *Pyrodwarf* leaf discs cultured *in vitro*. In particular the sizes of the amplification fragments (bp) were the following: 184, 125 – 132, 196 – 205. Although a higher number of primers need to be used in future, this evidence showed that most likely no variation occurred at SSR locus.

DISCUSSION

Regeneration of adventitious buds from mature leaf explants has been reported for several times for pear cultivars and rootstocks (Abdollahi *et al.* 2006; LEBLAY *et al.* 1991; PREDIERI *et al.* 1989; CHEVREAU *et al.* 1989).

Different combinations and levels of hormones have been successfully used to obtain regenerating shoots from *in vitro* cultured explants of pear, such as NAA and BA (PALOMBI *et al.* 2007; CABONI *et al.* 2002), NAA and TDZ (MARINO and MOLENDINI 2005), NAA and BA and TDZ (CABONI *et al.* 1999), TDZ and Gibberellic acid (LANE *et al.* 1998). It is known that it is generally difficult to regenerate adult material from woody species and that regeneration frequency is very much genotype-dependent. Relating to pear cultivars, such as *Comice*, *Passe-Crassane*, *Williams* and *Conference*, highest regeneration frequencies were obtained from 60% to 97% under TDZ at 2.5-5 µM and NAA at 1.0-5.4 µM (LEBLAY *et al.* 1991).

Regeneration experiments have been performed using leaves of two pear rootstock: *OHF333* and *BP10030* (ZHU and WELANDER 2000). For *BP10030*, 98% of regeneration have been obtained on the medium containing 1µM NAA and 15µM TDZ. Differences were observed using sucrose or sorbitol in the medium. For *OHF333*, 66% of regeneration was obtained using 0.1µM NAA and 1µM TDZ (ZHU and WELANDER 2000). Additionally, a combination of NAA (5.4µM) and TDZ (4.5µM) has been successfully used to induce somaclones resistant to different NaCl concentrations (MARINO and MOLENDINI 2005).

In this work, we decided to study media successfully used in previously published studies (ZHU and WELANDER 2000; ABDOLLAHI *et al.* 2006). We determined a good level of regeneration in the one containing sucrose and NAA 1µM and TDZ at 15µM while the other medium containing sorbitol and NAA 0.1µM and 5µM TDZ did not seem to be effective for regeneration. However it has to be demonstrated if the higher regeneration was due to the higher levels of these hormones than to the presence of sucrose instead of sorbitol. Using the composition of this medium, we decided to investigate the effect of other hormone combinations of NAA and TDZ on regeneration. Whereas the two intermediate combinations (Table 2) gave contrasting results probably due to natural physiological variability of the explants, the presence of the hormones at highest levels (5.4µM for NAA and 15µM for TDZ) determined a significant increase of the regeneration percentage and regenerating number of shoots/explants in both the experiments performed.

Differences between the two experiments were probably due to heterogeneity between explants in a particular experiment and to a change in the physiological state of the mother cultures in time with repeated subcultures. However the high variability among replicates of the same experiment and among experiments repeated at different dates was also observed by LEBLAY *et al.* (1991). For this reason, the effects of mother plant pre-treatment and explant choice on regeneration have been investigated using *in vitro* pear leaves (LEBLAY *et al.* 1991). In *Pyrodwarf*, 8 or 3 weeks of darkness period for *in vitro* explants seemed to not vary shoot regeneration. Although a period of 3 weeks is shown to be beneficial for shoot regeneration, darkness period is a factor that is mostly genotype-specific as shown in pear, apricot and apple cultivars (PREDIERI *et al.* 1989; LEBLAY *et al.* 1991; PEREZ-TORNERO *et al.* 2000). In our experiments we demonstrated that the length of darkness did not affect regeneration in *Pyrodwarf in vitro* explants. On the contrary, the concentration of TDZ used was essential to obtain regeneration. In fact, TDZ at 6 µM when used in combination with NAA at 5.4 µM, induced regenerating shoots. On the contrary TDZ at 2.5 µM did not induce any regenerating shoots.

Subdividing the regeneration process into two phases (induction and expression) has rarely been applied to fruit species. This approach is similar to the one developed on *Convolvulus* by CHRISTIANSON and WARNICK (1988) and then used for pear cultivars (LEBLAY *et al.* 1991). In our work, we showed that in Pyrodwarf the highest level of TDZ (6µM instead of 2.5µM) during induction period was the only important factor to induce regeneration independently to the presence of hormones during expression phase.

Relating to *Fox11*, no data are present in literature regarding regeneration experiments for any explants. In this work, a preliminary experiment

was performed in order to determine the regeneration efficiency of this pear rootstock. NAA at 5.4 µM was used in combination with TDZ at two different concentrations (2.5 µM and 6 µM). Poor regeneration capacity was observed: only when TDZ was present in the induction media (5.4 µM) and also in the expression media (2.5 µM) few regenerating shoots were obtained. Other experiments should be performed in future to understand if the presence of TDZ in the expression phase is beneficial for regeneration. However these results were useful to use other concentrations or include other hormones for the development of an efficient regeneration protocol for *Fox11.*

SSR analysis has been used to assess genetic and epigenetic stability during *in vitro* shoot culture in peas (Smykal *et al*. 2007), in derived plantlets in cork oak (Lopes *et al*. 2006) and micropropagated plants in *Camellia sinensis* (Devarumath *et al*. 2002). In addition SSR or inter-SSR techniques have been successfully used to assess mutation rates that often occurred in plants regenerated from *in vitro* culture, giving rise to the production of genetic chimeras (Lopez *et al*. 2004) and for detecting somaclonal variation among leaf-culture-regenerated plants in horseradish (Rostiana *et al*. 1999). Although somaclonal variability cannot be excluded and an higher number of SSR primers should be used, in this article the genetic profile of the regenerating shoots at the three SSR locus were identical.

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