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In vitro multiplication and rooting of 'F12-1' (*Prunus avium* L.) and 'Maxma 14' (*Prunus mahaleb* L. × *P. avium* L.) rootstocks

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ABSTRACT

A study was carried out to establish efficient and reliable *in vitro* multiplication protocols for 'F12-1' (*Prunus avium* L.) and 'Maxma 14' (*Prunus mahaleb* L. × *P. avium* L.) rootstocks. Shoot tips of 'F12-1' and 'Maxma 14' were collected in the spring and cultured on MS medium. After the *in vitro* cultures were sufficiently established, experiments were conducted to assess the influence of different levels of benzyladenine (BA) and thidiazuron (TDZ) on multiplication of these rootstocks. After six weeks, data were collected on percent multiplication, shoot number, shoot length, percent rooting, root number and callus formation. The best multiplication rates (90 and 86.6%) for 'F12-1' rootstock were obtained with 20 µM BA and 2.27 µM TDZ concentrations. 'Maxma 14' rootstock multiplied the best (93.3%) on medium supplemented with 20 µM BA and 9.08 µM TDZ. Both rootstocks rooted the best (70 and 60%) with 9.84 µM IBA. The plantlets were transferred to glasshouse for hardening.

Key words: Benzyladenine, cherry rootstocks, *in vitro* propagation, thidiazuron.

INTRODUCTION

Almost all commercial *Prunus* fruit trees are either budded or grafted on recommended rootstocks. *Prunus* rootstocks are commercially produced through stem seeds or cuttings. The production of rootstocks through seeds result in segregation and therefore uniform plants cannot be obtained and the mother plant characteristics cannot be maintained. Alternatively, rootstocks can be produced by cuttings and this clonal propagation method is the favoured in many parts of the world, because it allows the production of uniform propagules. However, propagation by cuttings is difficult in some *Prunus* genotypes due to the low rooting potential (Fachinello, 5) and propagation of *Prunus* spp. by cutting does not guarantee healthy and disease-free plants (Holtz *et al*., 7). In these circumstances, *in vitro* propagation of rootstocks emerge as a viable alternative way of propagation because it is not dependent on season, provides clean, disease and virus-free planting material.

'F 12/1' is a clonally propagated selection of 'Mazzard' and favoured over to 'Mazzard' due to its uniformity and resistance to bacterial canker. 'Maxma 14' (*P. mahaleb* × *P. avium*) is a clonal selection of *P. mahaleb* and originated from an open-pollinated 'Mahaleb' tree. It has been widely used in France due to its semi-dwarfing nature, precocity, and resistance to chlorosis (Long and Kaiser, 9). Several studies were carried out in *Prunus* species using different levels of BA (varying from 0.1 to 6.0 mg l^{-1}), which confirmed the efficiency of cytokinins in *in vitro* propagation. The optimum type and concentration of cytokinins needed for *in vitro* propagation varies according to species or cultivar (Teixeira *et al*., 13).

Prunus avium L. is especially difficult-to-root both *in vitro* and *in vivo*. Externally applied auxin is one of the factors affecting the plant growth regulator balance in the cuttings and thus root formation (Dimassi-Theriou, 2). The response of cuttings to external auxin treatments is dependent on various external and internal factors. The type and the concentration of auxins and the sensitivity of treated plant section to these substances may be restricting factors (Dolezelova *et al*., 3). In *Prunus*, some species present *in vitro* propagation problems, generating a vast variability and sometimes not permitting reproducible results (Radmann *et al*., 12). Therefore, the aim of this study was to determine the effects of benzyladenine (BA) and (TDZ) on multiplication, optimize rooting and establish efficient and reliable *in vitro* propagation protocols for 'F12-1'and 'Maxma 14' rootstocks.

MATERIALS AND METHODS

Shoot tips of 'Maxma 14' and 'F12-1' plants were collected in spring from the Egirdir Horticultural Research Station and were excised into about 4 cm segments. Shoot tip segments were first agitated in tap water and sterilized with 0.78% sodium hypochlorite for 15 min. Thereafter, they were rinsed with sterile double-distilled water, stem segments were further excised into about 1-2 cm explants having one or two buds. These were then cultured on MS medium containing 30 g/l

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sucrose, 7 g/l agar, 0.54 µM NAA and 3 µM BA. After adjusting the pH to 5.5, the medium was autoclaved at 18 atm at 121°C for 30 min. and dispersed into 9 × 25 × 150 mm culture tubes. The cultures were placed in a culture room maintained at 24 ± 1°C and a 16/8 h photoperiod supplied by fluorescent lambs (131 µM $m^{-2}s^{-1}$). After sprouting of the shoots, newly formed shoots were excised from the original explant tissue and explanted into glass jars containing the same medium as described above. These shoot cultures were sub-cultured every 4-5 weeks.

After shoot cultures had reached the necessary proliferation, multiplication and rooting experiments were conducted. All of the experiments had six replicates and each jar having 5 explants.

To evaluate the effects of different levels of BA on multiplication of 'F12-1'and 'Maxma 14' rootstocks, about 1 cm long stem segments were cultured on MS medium containing 0.54 µM NAA and different levels of BA (0, 1.25, 2.5, 5, 10 and 20 µM). To evaluate the effects of TDZ on multiplication of 'F12-1' and 'Maxma 14' rootstocks, about 1 cm long young shootlets were cultured on MS medium containing NAA (0.54 µM) and different levels of TDZ (0, 0.57, 1.35, 2.27, 4.54, 6.81 and 9.08 µM). Rooting experiment was conducted to evaluate the effects of IBA on rooting of 'F12-1' and 'Maxma 14' rootstocks. About 1 cm long young stem pieces were explanted on the MS medium containing different levels of IBA (0, 0.23, 0.46, 2.46, 4.92 and 9.84 µM).

After culture for six weeks on the multiplication medium, shootlets were rooted on MS medium containing 9.08 µM IBA and were transferred to pots (8 × 8 cm) containing a mix of 1:1 perlite and peat moss. The potted plantlets were covered with a transparent lid to provide humidity for the first 10 days. After a month of acclimatization, plantlets were transferred to the greenhouse conditions.

All experiments were arranged in a completely randomized design. Data were analyzed using ANOVA and means were separated by LSD (SAS 9.1, SAS institute, Cary, NC).

RESULTS AND DISCUSSION

All BA levels gave significantly higher multiplication and shoot numbers than control in 'F12-1' and the highest multiplication percentage and shoot number were obtained at the highest BA concentration (20 µM) (Table 1). BA levels 2.5 and 5.0 µM had significantly higher shoot lengths than control in 'F12-1' (Table 1). The highest rooting and number of roots per explant were obtained at the control (Table 1). All BA levels stimulated callus formation, except the control (Table 1). The highest callus numbers were obtained at low and intermediate levels of BA (1.5, 2.5 and 5 µM) (Table 1).

In 'Maxma14' rootstock, all BA levels lead to significantly higher multiplication percentages and shoot numbers than control and the highest values were obtained at the highest BA concentration (20 µM) (Table 2). Longer shoots were obtained from 1.5, 2.5, 5.0, and 10 µM BA concentrations. The highest rooting percentage and mean root number were obtained from the control. All BA levels and the control induced callus formation and there were no significant differences among the treatments (Table 2).

The highest multiplication rate and highest numbers of shoots per explant were obtained at 10 and 20 µM BA levels for both 'F12-1' and 'Maxma 14' rootstocks; revealing that the higher levels of BA are necessary to break the apical dominance and to stimulate shoot proliferation. These results agreed with those obtained by Mansseri- Lamrioui *et al*. (10) and Ďurkovič (4), who reported the best shoot multiplication with 8.8 µM BAP.

Thidiazuron (TDZ) significantly affected multiplication percentage, shoot number, shoot

Table 1. Effects of different levels of BA on multiplication 'F12-1' rootstock.

BA (µM)	Multiplication (%)	No. of shoots	Shoot length (mm)	Rooting (%)	No. of roots	Callus formation (%)	Callus number[x]
0.0	16.6c[z]	2.24c	1.4c	26.6a	0.5a	0.00c	0.00d
1.25	66.6b	2.94 b	3.8b	0.0b	0.0b	100a	0.86a
2.5	76.0ab	3.08ab	6.2a	0.0b	0.0b	100a	0.80a
5.0	80.0ab	3.36ab	7.1a	0.0b	0.0b	100a	0.82a
10.0	84.0ab	3.20ab	3.4b	0.0b	0.0b	96.6a	0.60b
20.0	90.0a	3.40a	3.5b	0.0b	0.0b	76.6b	0.37c

[z]Different letters in the same column represent significant differences at $P \leq 0.05$ by LSD test

[y]Not the main explant but the longest shootlet was measured

[x]Callus smaller than 3 mm in diameter were not included

Table 2. Effects of different levels of BA on multiplication 'Maxma14' rootstock.

BA (µM)	Multiplication (%)	No. of shoots	Shoot length (mm)	Rooting (%)	No. of roots	Callus formation (%)	Callus number[x]
0	1.66c	2.00cd	1.7c	36.6a	0.36a	66.6b	0.24a
1.25	53.3b[x]	2.60c	4.1a	0.00b	0.00 b	80ab	0.28a
2.5	73.3ab	2.86bc	4.1a	0.00b	0.00 b	70.0b	0.26a
5	86.6a	2.90b	3.3a	0.00b	0.00 b	73.3ab	0.27a
10	90.0a	3.10ab	4.2a	0.00b	0.00 b	83.3ab	0.25a
20	93.3a	3.26a	2.8b	0.00 b	0.00 b	90.0ab, 3ab	0.31a

[z]Different letters in the same column represent significant differences at $P \leq 0.05$ by LSD test
[y]Not the main explant but the longest shootlet was measured
[x]Callus smaller than 3 mm in diameter were not included

length (Fig. 1-a), and callus formation in 'F12-1' rootstock (Table 3). The effect of TDZ on multiplication percentage, shoot number, shoot length (Fig. 1-b), rooting percentage, root number, and callus number of 'Maxma14' rootstock were also significant (Table 4). Intermediate levels of TDZ (2.27 and 4.54 µM) had significantly higher percentages of multiplication and shoot numbers than control in 'F12-1' (Table 3). The highest shoot length was obtained at 1.35 µM TDZ concentration. The highest callus numbers were observed in the intermediate and high levels of TDZ (2.27, 4.54, 6.81 and 9.08 µM). Callus number per explant decreased as the TDZ concentration decreased.

The percentages of shoot multiplication and mean shoot length were highest for 6.81 and 9.08 µM TDZ concentrations in 'Maxma14' (Table 4). The highest mean plant height was obtained at 9.08 µM TDZ. Only a small percentage of plants rooted in control and no rooting was observed in the presence of TDZ. 'Maxma14' plantlets formed callus in all treatments including the control. The high levels of TDZ (4.54, 6.81 and 9.08 µM) had significantly higher callus formation percentages than the control. The mean callus number per explant was highest for 9.08 µM TDZ, which decreased as the concentration was decreased.

The type and concentration of cytokinin influenced the percentage of multiplication and the number of shoots produced per explant. The shoot length of both 'F12-1' (*Prunus avium* L.) and 'Maxma 14' (*Prunus mahaleb* L. × *P. avium* L.) rootstocks were the lowest in the absence of cytokinins. This indicates that they have a positive and stimulating effect on shoot growth and shoot length. Our results are in agreement with those of Mansseri-Lamrioui *et al*. (10), and Ďurkovič (4).

Thidiazuron at concentrations of 1.35 and 2.27 µM yielded the highest number of shoots per explant and was most suitable for the *in vitro* propagation of 'F12-1' rootstock. This is in agreement with the results of Huettemann and Preece (8). Usually, extremely low concentrations of TDZ are needed to stimulate axillary shoot proliferation (Huettemann and Preece, 8).

Table 3. Effects of different levels of TDZ on multiplication 'F12-1' rootstock.

TDZ (µM)	Multiplication (%)	No. of shoots	Shoot length (mm)	Rooting (%)	Callus formation (%)	Callus number[x]
0	36.6c[z]	2.36d	2.9c	0	93.3ab	0.42c
0.57	63.3abc	3.03abc	3.6bc	0	96.6a	0.62b
1.35	68.0ab	3.52a	7.2a	0	88.0ab	0.64b
2.27	86.6a	3.16ab	5.6ab	0	96.6a	0.84a
4.54	83.3a	3.03bc	3.4bc	0	100.0a a	0.84a
6.81	56.6bc	2.90bc	3.8bc	0	83.3b	0.85a
9.08	64.0abc	2.64cd	2.6c	0	88.0ab	0.82a

[z]Different letters in the same column represent significant differences at $P \leq 0.05$ by LSD test
[y]Not the main explant but the longest shootlet was measured
[x]Callus smaller than 3 mm in diameter were not included

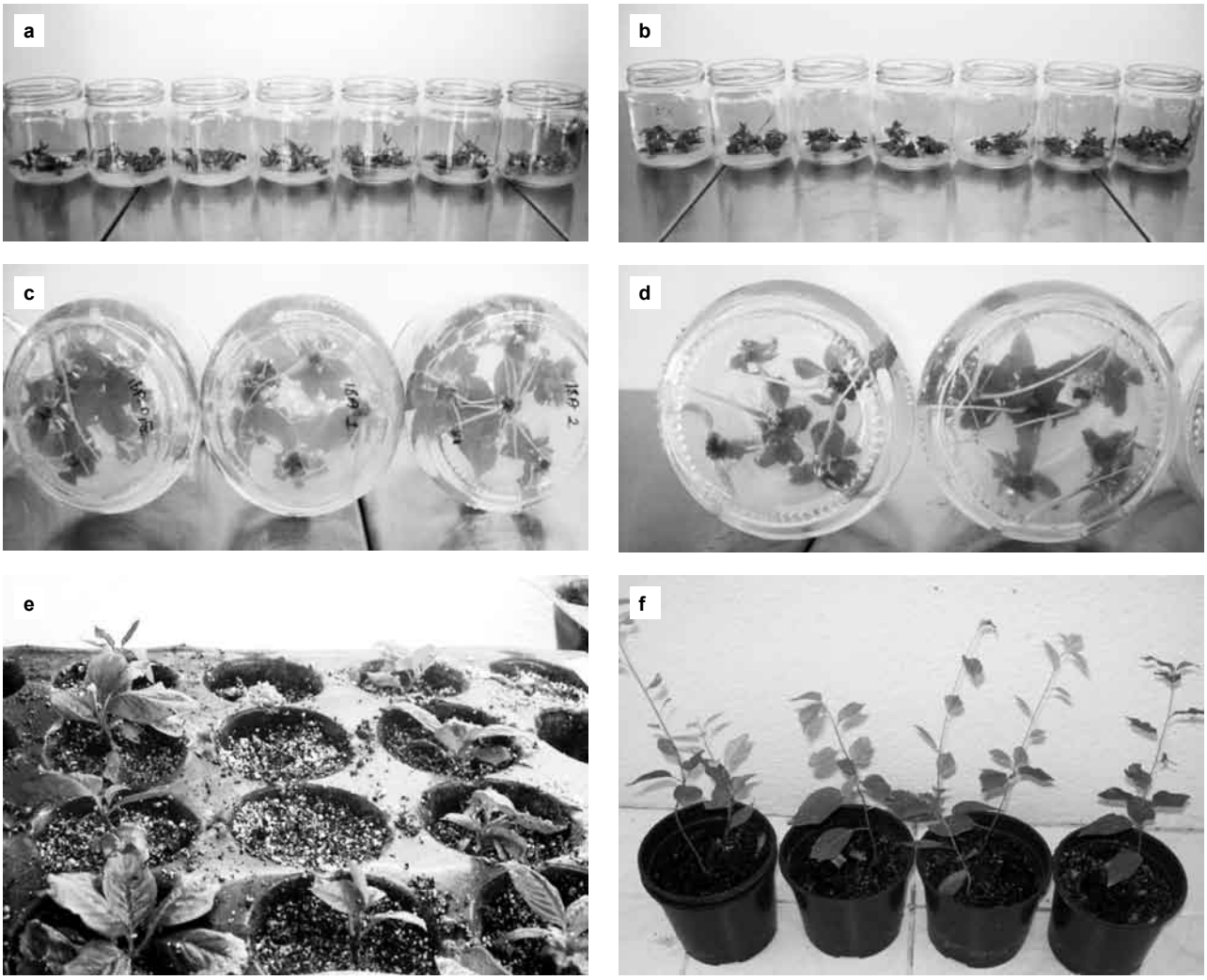

Fig. 1. *In vitro* propagation of 'F12-1' (*Prunus avium* L.) and 'Maxma 14' (*Prunus mahaleb* L. × *P. avium* L.) rootstocks; (A) Effect of TDZ (left to right: 0, 0.57, 1.35, 2.27, 4.54, 6.81 and 9.08 µM) on multiplication of 'F12-1'; (B) Effect of TDZ (left to right: 0, 0.57, 1.35, 2.27, 4.54, 6.81 and 9.08 µM) on 'Maxma 14'; (C) Rooting of 'F12-1'; (D) Rooting of 'Maxma 14'; (E) Acclimatization of regenerants of 'F12-1'; (F) Acclimatized plants of 'F12-1' in the greenhouse.

Table 4. Effects of different TDZ levels on multiplication 'Maxma14' rootstock.

TDZ (µM)	Multiplication (%)	No. of shoots	Shoot length (mm)	Rooting (%)	No. of roots	Callus formation (%)	Callus number[x]
0.00	1.66c[z]	2.00bc	0.0d	3.30a	0.03a	83.3b	0.27d
0.57	64.0 bc	2.73b	3.0c	0.00 b	0.00 b	90.0ab	0.38c
1.35	70.0 b	2.76b	3.8bc	0.00 b	0.00 b	90.0ab	0.38c
2.27	73.3b	2.76b	3.2bc	0.00 b	0.00 b	93.3ab	0.39c
4.54	83.3 ab	2.96ab	4.3b	0.00 b	0.00 b	100a	0.47b
6.81	90.0 a	3.03a	4.3b	0.00 b	0.00 b	100a	0.48b
9.08	93.3 abbca	3.20a	6.7a	0.00 b	0.00 b	100a	0.60a

[z]Different letters in the same column represent significant differences at $P \leq 0.05$ by LSD test
[y]Not the main explant but the longest shootlet was measured
[x]Calluses smaller than 3 mm in diameter were not included

Table 5. Effects of different levels IBA on rooting of 'F12-1' and 'Maxma 14' rootstocks.

IBA (µM)	'F12-1'			'Maxma 14'		
	Rooting (%)	No. of roots	Root length (mm)	Rooting (%)	No. of roots	Root length (mm)
0.00	33.3d[z]	0.70c	11.3c	20.0c[z]	0.20b	15.6cd
0.23	40.0cd	0.86c	16.2bc	26.6bc	0.26b	11.6d
0.46	46.6 bcd	1.13bc	14.8bc	30 bc	0.43b	19.8bcd
2.46	56.6abc	2.00ab	33.0a	36.6b	0.53ab	39.9a
4.92	63.3ab	2.06ab	26.9ab	56.6a	0.90a	34.2ab
9.84	70.0a	2.34a	14.0bc	60.0a	0.90a	33.1abc

[z]Different letters in the same column represent significant differences at $P \leq 0.05$ by LSD test
[y]Length of the longest roots for each explant was measured

Auxin IBA significantly affected rooting percentage, root number, and root length of both 'F12-1' (Fig. 1-c) (Table 5) and Maxma14' rootstocks (Fig. 1-d). In 'F12-1' the percentage of rooted plants was highest at the highest level of IBA. The highest number of roots per explant was also obtained at the highest IBA concentration (9.84 µM). The highest mean root length was obtained at 2.46 µM IBA. In 'Maxma 14', the percentage of rooted explants and mean root number were highest for high levels of IBA (4.92 and 9.84 µM). IBA levels 4.92 and 9.84 µM had higher mean root lengths than control. *In vitro* derived plantlets were successfully acclimatized (Fig. 1-e) and transferred to the pots (Fig. 1-f).

The concentration of IBA significantly influenced the percentage of root formation, root number and root length in both rootstocks. The best rooting percentage and root number were obtained at 9.84 µM IBA for both 'F12-1' and 'Maxma 14' rootstocks. Our results agreed with those obtained by Mansseri-Lamrioui *et al*. (10) in wild cherry and Hassanen and Mahdia (6) in *Pyrus betulaefolia*. Indeed *in vivo* root induction by application of 9.8 µM IBA has been reported for some *Prunus* spp. such as *Prunus tomentosa*, *Prunus fructicosa*, *Prunus verginiaca* and *Prunus pensylvanica* (Pruski *et al*., 11).

These results indicate that the cytokinin type and concentration suitable for *in vitro* propagation of two rootstocks are probably genotype dependent. The protocol developed can be used for *in vitro* mass propagation of 'F12-1' and 'Maxma 14' rootstocks.

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