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ARTICLE

Optimizing culture media for *in vitro* proliferation and rooting of Tetra (*Prunus empyrean* 3) rootstock

F. Sadeghi [a], **A. Yadollahi** [b,*], **M. Jafarkhani Kermani** [c], **M. Eftekhari** [b]

[a] *Department of Plant Breeding, Faculty of Agriculture, Tarbiat Modares University, Tehran, Islamic Republic of Iran*
[b] *Department of Horticultural Sciences, Faculty of Agriculture, Tarbiat Modares University, Tehran, Islamic Republic of Iran*
[c] *Department of Tissue Culture and Gene Transformation, Agricultural Biotechnology Research Institute of Iran, Mahdasht Road, Karaj, Islamic Republic of Iran*

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Abstract The enormous demand for new rootstock genotypes in *Prunus* spp. makes us to use micropropagation as an unavoidable propagation method. Therefore, the study on micropropagation of a new semi-dwarf vegetative rootstock namely Tetra (*Prunus empyrean* 3) was carried out to develop an optimized protocol. Culture establishment using nodal segments was enhanced using WPM (woody plant medium) medium lacking growth regulators. From various shoot multiplication treatments, the highest number of shoots per explant (30.4) was found on ME (Media created specifically) medium supplemented with 0.8 mg l^{-1} BAP and 0.05 mg l^{-1} IBA. 100% *in vitro* rooting was achieved on ½ strength MS medium with 0.5 mg l^{-1} IBA, 1.6 mg l^{-1} thiamine and 150 mg l^{-1} iron sequestrene.

1. Introduction

Tetra or Empyrean® 3 (*Prunus domestica*) is a semi-dwarf (80–90% of Lovell) vegetative rootstock which is resistant to root-knot nematode, phytophthora, waterlogged, and heavy soils [18] compatible with peach, plum, almond and apricot. It is native to Istituto Sperimentale per la Frutticoltura (ISF) di Roma, Italy. It is non-suckering and is easily propagated by hardwood cutting [using a quick-dip in 2000 mg l^{-1} indol-3-butric acid (IBA)] [18]. *In vitro* propagation is also a massive propagation strategy which can be used in response to the growing request for new commercial *Prunus* spp. Micropropagation of *P. domestica* as rootstock has been dealt with first by Boxus and Quoirin [3], Quoirin et al. [20] and Zuccherelli [30]. An effective *in vitro* culture system for mature stem segments of Chinese plum (*P. salicina* Lindl. cv. 'Gulf ruby') has been established [29]. They collected 1 cm nodal explants of newly emerged shoots, sterilized and established them *in vitro*. Successful culture establishment was achieved on a woody plant medium (WPM) supplemented with 0.05–0.1 mg l^{-1} IBA, 0.5–1.0 mg l^{-1} 6-Benzylaminon purine (BAP), 30 g l^{-1} glucose, 5 g l^{-1} agar and 1.0 g l^{-1} vitamin C (VC). They obtained the highest shoot multiplication rate on WPM with 0.05–

Abbreviations: BAP, 6-benzylamino purine; DW, dry weight; FW, fresh weight; IBA, indol-3-butric acid; LS, Linsmaier and Skoog (1965); ME, media created specifically (Cos et al., 2004); MS, Murashige and Skoog (1962); WPM, woody plant medium

* Corresponding author. Tel.: +98 2148292091; fax: +98 2148292200.
E-mail address: yadollah@modares.ac.ir (A. Yadollahi).

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0.1 mg l^{-1} IBA, 0.2 mg l^{-1} BA, 0.3 mg l^{-1} kinetin (KT) and 1.0 g l^{-1} casein hydrolysate. According their results, *in vitro* shoot elongation was facilitated on WPM with 0.05 mg l^{-1} IBA, 0.3 mg l^{-1} KT/BA and 1.0 g l^{-1} casein hydrolysate. They carried out *in vitro* rooting in half strength MS (½ MS) medium supplemented with 0.2–0.5 mg l^{-1} IBA, 15 g l^{-1} sucrose and 20–40 mg l^{-1} phloroglucinol.

As well, in order to develop a protocol for micropropagation of plum (*P. domestica* L.), Ruzic and Vujovic [23] regenerated shoots from *in vitro* nodal segments of plum cultivars ČačanskaRodna and Valjevka established in Murashige and Skoog [16] (MS) medium containing 2.0 mg l^{-1} BA, 0.5 mg l^{-1} IBA, 0.1 mg l^{-1} gibberellic acid (GA_3), 7 g l^{-1} agar and 20 g l^{-1} sucrose. Regenerated shoots were multiplied in MS medium supplemented with 1.0 mg l^{-1} BA, 0.1 mg l^{-1} IBA, 0.1 mg l^{-1} GA_3, 7 g l^{-1} agar and 20 g l^{-1} sucrose. The shoot regeneration medium consisted of MS supplemented with 30 g l^{-1} sucrose and 7 g l^{-1} agar. The growth regulators used to induce shoot regeneration from plum leaves were 5 mg l^{-1} BAP and 0.5 mg l^{-1} IBA. The basal rooting medium was ½ MS with 1% sucrose, proliferation medium organics and 0.5 mg l^{-1} IBA.

To our knowledge, this is the first comprehensive compiled study on the micropropagation of a new important *Prunus* rootstock viz. Tetra. The aim of the present investigation was to optimize *in vitro* propagation technique for Tetra (*P. domestica*) rootstock, develop a practical protocol for its commercial propagation. As well as study the effect of the chelated form of the iron salt of ethylene diamine di-o-hydroxyphenyl acetic acid (Fe-EDDHA) (6% Fe) on *in vitro* rooting of the Tetra rootstock.

2. Material and methods

2.1. Plant material sterilization and culture conditions

15–20 cm length shoots were cut of pot plants maintained in glasshouse of the Horticultural Department of Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran during September 2011 and transferred to laboratory.

Nodal segments (3 cm) were excised and were surface sterilized by agitating for 15 min in a solution containing 2 drops of Tween-20 in 100 ml of water and then washed under running tap water for 30 min. Explants were sterilized with 0.1% mercury chloride for 4 min. Later, they were washed twice with double distillated water containing 700 mg l^{-1} citric acid.

All explants were cultured on different types of media and transferred to growth room with light intensity of 2500–3000 lux, photoperiod of 16/8 h light/dark, relative humidity of 45% and constant temperature of 25 ± 1 °C.

2.2. Culture establishment

McCarthy glasses were used for culture establishment stage of explants. Woody Plant Medium (WPM) containing 7 g l^{-1} agar and 30 g l^{-1} sucrose (pH of all media was adjusted to 5.75 before autoclaving) was used to establish the explants and after 4 weeks, explants were transferred to shootlet proliferation media.

2.3. Shootlet proliferation

WPM and media created specifically [6] (Cos) containing 3 concentrations of BAP (0.4, 0.8 and 1.2 mg l^{-1}) and 30 g l^{-1} sucrose and 7.5 g l^{-1} agar were compared. Number and length of shoots were recorded after 4 weeks.

In the second experiment of proliferation optimization, the effect of different concentrations of IBA (0, 0.05, 0.1, 0.15 and 0.2 mg l^{-1}) in combination with 0.8 mg l^{-1} BAP in Cos medium was assessed.

2.4. Shoot elongation

The proliferated shoots were elongated on Cos medium supplemented with different concentrations of Naphthalene acetic acid (NAA) (0, 0.01 and 0.1 mg l^{-1}) and sucrose (20 and 30 g l^{-1}) and length of shoots was measured.

2.5. Rooting

In vitro shoots (2–3 cm) were transferred to ½ MS, LS [11] and Cos media supplemented with 20 g l^{-1} sucrose, 7.5 g l^{-1} agar, and different concentrations of IBA (0.5, 1, 1.5 and 2 mg l^{-1}).

All treatments were maintained in the dark for 1 week and then they were transferred to the light. Each treatment included 5 replications, each replicate included 2 explants. Rooting percentage, root number and root length (cm) were recorded after 40 days.

In order to optimize the rooting conditions, the effect of different combinations of Fe-EDDHA (100, 150 and 200 mg l^{-1}) and thiamine (0, 1.6, 2.8 and 4 mg l^{-1}) was considered.

2.6. Statistical analysis

The experiment was carried out based on completely randomized design (CRD) with factorial arrangement and 5 replications per treatment. Statistical analysis of the data was carried out using SPSS 18 software and obtained means were compared using Duncan's Multiple Range Test ($p \leqslant 0.05$).

3. Results and discussion

3.1. Proliferation

Based on the results obtained, the interaction effect of culture media and BAP concentration (Table 1) on both number and length of shoots were significant. The effect of Cos medium containing 0.4 mg l^{-1} BAP on number of shoot (5.6) was significantly more than the effect of the same medium containing 0.8 mg l^{-1} BAP (3.6) and WPM medium containing 0.4 mg l^{-1} BAP. In this respect, there were no significant differences among treatments.

Shoot length mean in Cos containing 0.8 mg l^{-1} BAP (2.03 cm) was significantly higher than ones in the rest treatments except WPM containing 0.4 mg l^{-1} BAP (1.99 cm). So it can be realized that in Cos medium enhancing shoot number and length will be achieved by 0.4 and 0.8 mg l^{-1} BAP, respectively. But in case of WPM medium it is suggested that in general, both 0.4 and 1.2 mg l^{-1} BAP concentrations could be

Table 1 Interaction of culture media and BA concentrations on *in vitro* proliferation of Tetra rootstock.

Medium	BA (mg l^{-1})	Number of shoot	Length of shoot (cm)
ME	0.4	5.6 a*	1.45 c
	0.8	3.6 b	2.03 a
	1.2	4.0 ab	1.43 c
WPM	0.4	3.4 b	1.99 ab
	0.8	3.7 ab	1.31 c
	1.2	4.6 ab	1.54 bc

* Different letters in each column show significant differences ($p < 0.05$).

Table 2 The effect of IBA concentrations on *in vitro* proliferation of Tetra rootstock.

Medium	IBA (mg l^{-1})	Number of shoot	Length of shoot (cm)
ME + 0.8 mg l^{-1} BAP	0.05	30.4 a*	2.042 a
	0.1	18.2 b	1.934 ab
	0.15	17.2 b	1.72 b
	0.2	12 b	1.296 c

* Different letters in each column show significant differences ($p < 0.05$).

Table 3 Interaction of NAA and sucrose concentrations on *in vitro* shoot elongation of Tetra rootstock.

Medium	NAA (mg l^{-1})	Sugar (g l^{-1})	Length of shoot (cm)
ME	0	20	2.12 a*
	0	30	1.76 bc
	0.01	20	1.61 bc
	0.01	30	1.8 abc
	0.1	20	1.94 ab
	0.1	30	1.47 c

* Different letters in each column show significant differences ($p < 0.05$).

appropriate. Cytokinins are essential factors for breaking the apex dormancy and inducing auxiliary shoot proliferation that influence the success of *in vitro* multiplication [25]. BAP cytokinin induces cell division, shoot multiplication and lateral bud development [27].

3.1.1. The effect of IBA concentration on shootlet proliferation

For inspecting the effect of IBA as an auxin along with BAP cytokinin on proliferation and achieve a protocol which could improve both number and length of shoot, different concentrations of IBA were added to Cos medium containing 0.8 mg l^{-1} BAP (attained as a good concentration to increase length of shoot). Our results showed that both the number (30.4) and length (2.04 cm) of shoots were enhanced by adding 0.05 mg l^{-1} IBA (Table 2). So, we propose Cos medium containing 0.8 mg l^{-1} BAP and 0.05 mg l^{-1} IBA as an applicable protocol for high proliferation of Tetra rootstock.

Previous studies have shown that cytokinins alone or along with auxins are essential for explant proliferation [13]. Attained results by Cos et al. [6] showed that the most responsive treatment in proliferation was achieved using 1.5 mg l^{-1} BAP and 0.1 mg l^{-1} IBA. Numerous studies have reported that integration of auxin and cytokinin is highly effective in proliferation of woody plants; however, auxin concentration should not be more than a significant level [7,4,14].

Another point found in this research is that subculture number effects on proliferation rate impressively. It is evident of this study results that proliferation rate raised considerably by increasing subculture times which is attributed to plant rejuvenation status. This experiment was conducted in subculture 5 and shoot number was enhanced of 6–30 (data not shown) which is in accordance with Alizadeh [1].

3.2. Shoot elongation

3.2.1. The interaction effect of NAA and sucrose concentrations

An experiment was designed to increase the length of studied *in vitro* plantlets in which the impact of various levels of NAA (0, 0.01 and 0.1 mg l^{-1}) and different amounts of sugar (20 and 30 g l^{-1}) was examined (Table 3). It can be revealed from the data shown in Table 3 that there is no need to use NAA in elongation phase as shoot length in 20 g l^{-1} sucrose treatment (2.12 cm) was not enhanced neither by adding 0.01 (1.61 cm) nor 0.1 (1.94) mg l^{-1} NAA (Table 3).

Table 4 Interaction of culture media and IBA concentrations on *in vitro* rooting of Tetra rootstock.

Medium	IBA (mg l^{-1})	Number of root	Length of root (cm)	Root fresh weight (mg)	Root dry weight (mg)	Percentage of rooting (%)
½ MS	0.5	11.75 abc*	5.08 a	502.5 a	62.5 ab	100 a
	1	15.63 abc	2.27 b	430 ab	57.5 b	100 a
	1.5	18.04 a	1.42 cd	525 a	70 a	100 a
	2	13.25 abc	1.24 d	207.5 c	30 de	100 a
LS	0.5	13.88 abc	1.97 bc	290 bc	37.5 cd	87.5 a
	1	17.75 ab	1.27 d	270 bc	40 c	100 a
	1.5	15.63 abc	1.13 d	225 c	43.75 c	50 b
	2	8 c	0.731 d	122.5 c	26.25 e	25 c
ME	0.5	8.125 c	2.12 b	162.5 c	27.5 e	12.5 c
	1	11.13 abc	1.12 d	97.5 c	26.25 e	50 b
	1.5	13.25 abc	0.89 d	150 c	27.25 e	50 b
	2	10 bc	0.82 d	107.5 c	25 e	25 c

* Different letters in each column show significant differences ($p < 0.05$).

Table 5 Interaction of different concentrations of Fe-EDDHA and thiamine on *in vitro* rooting of Tetra rootstock.

Medium	Fe-EDDHA (mg l^{-1})	Thiamine (mg l^{-1})	Number of root	Length of root (cm)	Fresh weight (mg)	Dry weight (mg)
½ MS + 0.5 mg l^{-1} IBA	100	0	5 b*	2.924 c	110.8 ef	16.48 de
	100	1.6	7.7 ab	2.262 cd	162.2 cdef	20.38 d
	100	2.8	6.5 ab	2.464 cd	141.4 def	17.04 de
	100	4	7.4 ab	2.32 cd	135.6 def	16.26 de
	150	0	7.6 ab	4.374 b	192.2 bcde	34.2 c
	150	1.6	9.4 a	5.046 a	428.6 a	73.8 a
	150	2.8	8.2 ab	4.332 b	232 bc	50 b
	150	4	8.2 ab	4.0556 b	366.4 a	68.8 a
	200	0	7.6 ab	3.848 b	208.6 bcd	31.6 c
	200	1.6	6.8 ab	2.354 cd	128.4 ef	13.8 de
	200	2.8	7.5 ab	2.7666 cd	254.8 b	29.8 c
	200	4	5.3 b	2.116 d	91.6 f	10.8 e

* Different letters in each column show significant differences ($p < 0.05$).

Figure 1 Different stages of Tetra micropropagation: (a) establishment; (b) shootlet proliferation; (c) shoot elongation; (d) rooting; (e) and (f) hardening.

Positive effect of low sucrose amount (20 mg l^{-1} to 30 mg l^{-1}) on elongation could be attributed to the medium high water potential which makes water more available for explant and subsequently improves elongation [22].

3.3. Rooting

3.3.1. The interaction effect of culture media and IBA concentration on rooting

For rooting experiments, different media (½ MS, LS and Cos) containing various concentrations of IBA (0.5, 1, 1.5 and 2 mg l^{-1}) were tested (Table 4). Half strength of MS with 0.5 mg l^{-1} IBA (100%) was found as the best medium for *in vitro* rooting of Tetra proliferated shoots as it produced higher root number (11.75), root length (5.08 cm), fresh weight (502.5 mg), dry weight (62.5 mg), and rooting percent (100%) (Table 4).

Use of auxins for *in vitro* rooting stage has also been reported by many other researches; in a study on GF677, the highest rooting percent was found on medium containing 0.5 mg l^{-1} IBA [28]. It has also been reported that IBA induced lateral rooting better than indole acetic acid (IAA) [26,21,8,12]. Furthermore, IBA is more stable and less sensitive to auxin degrading enzymes [19,9,21]. It has been stated that IAA is metabolized by peroxidase enzymes rapidly [5,17].

3.3.2. The interaction effect of Fe-EDDHA and thiamine on rooting

Because of low quality of emerged roots (thick and brittle) and the positive role of thiamine vitamin [10] and iron sequestrene [15] in increasing quality and quantity of *in vitro* fruit trees' roots, an experiment was performed to evaluate the effects of thiamine and Fe-EDDHA on rooting of Tetra. 2.5–3 cm explants were transferred to ½ MS medium containing 0.5 mg l^{-1} (the treatment resulted the best in the previous experiment). Statistical analysis illustrated higher number (9.4), length (5.046 cm), fresh weight (428.6 mg) and dry weight (73.8 mg) of roots by treatments of 150 mg l^{-1} Fe-EDDHA and 1.6 mg 1^{-1} thiamine in ½ MS medium containing 0.5 mg l^{-1} IBA (Table 5). Different stages of Tetra micropropagation are shown in Fig. 1.

In the MS culture medium iron is added in the form of Fe-EDTA, however, many problems are attributed to the use of Fe-EDTA, such as precipitation, decrease of Fe availability and production of toxic compounds [2]. It was reported that in GF-677 explants treated with Fe-EDTA (common MS medium) rooting was not initiated, whereas when explants were treated with Fe-EDDHA rooting occurred [15]. It was shown that interaction of thiamine and IBA had significant effects on *in vitro* rooting percentage of GF 677 [24].

In the present investigation, medium containing 1.6 mg l^{-1} thiamin and 150 mg l^{-1} iron sequestrene provided not only more and longer roots but also more qualitative roots. Emerged roots from this treatment were narrow, flexible and also as cluster. Such long and qualitative roots may result in better acclimatization and less plantlet losses at hardening under glasshouse conditions.

It seems that thiamin affects on root quality indirectly. The effect of thiamin on shootlet rooting formation has also been studied by some researchers so that it was shown that the highest rooting rate was obtained when explants were exposed to LS medium supplemented with 0.3 mg l^{-1} NAA and 1.6 mg l^{-1} thiamin under a 7 day darkness period.

In conclusion, ME culture medium supplemented with 0.8 mg l^{-1} BAP + 0.05 mg l^{-1} IBA and ½ MS culture medium supplemented with 0.5 mg l^{-1} IBA + 1.6 mg l^{-1} thiamin and 150 mg l^{-1} iron sequestrene are suggested for *in vitro* proliferation and rooting of Tetra rootstock, respectively.

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