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Effects of media and plant growth regulators on micropropagation of a dwarfing cherry rootstock (PHL-A)

Morteza MAHDAVIAN[1], Naser BOUZARI[2,*] and Hamid ABDOLLAHI[2]

1. Science and Research Branch, Islamic Azad University of Tehran, Tehran, Iran, *E-mail:* mahdavian.morteza@gmail.com
2. Seed and Plant Improvement Research Institute, P.O. Box: 31585-4119, Karaj, Iran, *E-mail:* bouzari1111@yahoo.com
*Corresponding author, N. Bouzari, E-mail: bouzari1111@yahoo.com
Phone: +989121089192; Fax:+982616700908

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Abstract. PHL-A is a hybrid of *Prunus avium* × *Prunus cerasus*. Growth reduction reaches 70 percent for this rootstock, in comparison with seedling cherry rootstocks, resulting in higher harvest with lower production costs. In this research, the effects of different culture media and plant growth regulators in micropropagation of this rootstock were investigated. Shoot tips and axillary buds, derived from greenhouse-grown one-year-old trees, were used as explants and established in vitro. In proliferation stage, multiplication rate of about 5.37 was achieved over a 4 week period using MS medium with 0.5 mg l^{-1} benzyladenine. Rooting of shoots, with up to 100 percent efficiency, were also obtained within four weeks on DKW medium without growth regulators in vitro. Application of double-phase medium instead of agar-gelled medium resulted in better control of hyperhydricity. In the acclimatization stage, rooted plantlets were transferred to peatmoss: cocopeat: perlite mixture (2:2:1 v/v/v) that were fertilized with inorganic salts. Acclimatization was affected directly by rooting treatment and leaf development.

Keywords: PHL-A, *Prunus avium* × *Prunus cerasus*, plant growth regulators, micropropagation.

Introduction

Historically, the mazzard has been the rootstock of choice for growers in Iran. For most parts, our soils are moderately light loess soils, most of them being loamy in nature, with a range from sandy loam to clay loam. Most of our soils are deep and correspondingly vigorous. On these soils, mazzard trees grow very quickly, are extremely non-precocious, and are very big. On the other hand, due to critical role of rootstock in the rate of growth, early-maturity, functionality and tolerance against the diseases, the selection of rootstock will play an important role in the Orchard management programs. The main problem in cherry Orchards is the hypergrowth of trees. There are a number of reasons for this interest in dwarfing rootstocks in Iran. Thus the suitable rootstocks for fruit trees and their easy and comfortable propagation have always been of importance. However, prior to mass production, vegetative cherry rootstocks have to be comprehensively studied. Many of them can only be propagated by tissue culture. Since 1963, several series of cherry dwarfing rootstocks have been investigated in the Research and Breeding Institute of Pomology Holovousy Ltd. in Czech Republic. Three seedlings (numbered 84, 224 and 6) were selected from a collection of six seedlings (6, 4, 5, 84, 103 and 224). These were named PHL-A, PHL-B and PHL-C rootstocks, respectively. The PHL-Series rootstocks are believed to be derived from a cross between *P. avium* and *P. cerasus*. The growth reduction reaches 70% for PHL-A, 50% for PHL-B and 80% for PHL-C in comparison with seedling depending on the cultivar (Erbenova et al. 2001). The fruit size on these rootstocks was suitable, thus improving the productivity and precocity of cherry grafted onto them. Ten clonal dwarf or semi-dwarf rootstocks were evaluated in a trial that was established in the spring of 1999 at Holovousy. With Lapins cv. used as a scion tester for other rootstocks, higher yield efficiencies were calculated for PHL-A, G 195/20 and Tabel Edabriz (Blazkova & Hlusickova 2007). However, these cultivars are extremely difficult for direct rooting. That is why the sweet cherry rootstocks PHL-Series have been propagated commercially through in vitro culture (Erbenova et al. 2001).

Regeneration of plants from callus of the cherry rootstocks has been the subject of several studies (Webster 1980, James et al. 1984, Bhagwat & David Lane 2004, Durkovic 2006). More recently, different factors have been considered on the *Prunus* micropropagation such as: effect of carbohydrates (Harada & Murai 1996, Nowak et al. 2004), comparison of different iron sources in the culture medium (Molassiotis et al. 2003), effect of different combinations of growth regulators (Pruski et al. 2000; Sedlak & Paprstein 2008, Ruzic & Vujovic 2008), micropropagation and genetic markers analysis for cloned plants (Al-Ansary et al. 2007, Ning et al. 2007), and effect of different concentration of salts on growth of explants (Ruzic et al. 2003).

Here, we studied micropropagation of PHL-A dwarfing cherry rootstock. The objective of this work was to investigate effects of different culture media and growth regulators on proliferation and rooting of a dwarfing cherry rootstock.

Materials and methods

Shoot apices and axillary buds were collected in summer from greenhouse-grown 1-year-old trees of cherry rootstock PHL-A. Nodal segments, 1-1.5 cm in length, without leaves were then excised from the shoots. For surface-sterilization, explants were disinfected with 5% sodium hypochlorite solution for 15 minutes followed by three rinses in sterile distilled water. Subsequently, the explants were placed in 15 or 18 × 2.5 cm test tubes each with 10 ml of agar-gelled QL medium without growth regulators. After about 2 weeks, sterile explants were transferred into glass culture vessels (400 ml) containing 40-50 ml culture media. Then, after 2-3 subcultures, shoots were separated routinely from each other every 4 weeks and sub-cultured into shoot proliferation medium. Six different shoot proliferation media were: MS and DKW basal medium and modified QL containing 30 g l^{-1} sucrose, 0.6% agar and other growth regulators (Tables 1 and 2). Double-phase medium, composed of a liquid medium with a perlite substrate was used to support the shoots. Liquid media used to test the hyperhydricity included: MS + 1 mg l^{-1} BA, MS + 0.5 mg l^{-1} BA, modified QL + 0.5 mg l^{-1} BA + 0.5

mg l^{-1} GA_3 + 0.01 mg l^{-1} NAA (QL1), modified QL containing 2mg l^{-1} BA and 0.01 mg l^{-1} IBA (QL2) and the modified QL medium with no growth regulators (QL3).

Table 1. Composition of modified QL medium.

NH_4NO_3	0.4 mg l^{-1}
Ca (NO_3). $4H_2O$	1.2 mg l^{-1}
$MgSO_4$. $7H_2O$	0.36 mg l^{-1}
KNO_3	2.1 mg l^{-1}
KH_2PO_4	0.27 mg l^{-1}
$MnSO_4$. H_2O	16.90 / mg l^{-1}
$CuSO_4$. $5H_2O$	0.025 mg l^{-1}
$CoCl_2$. $6H_2O$	0.025 mg l^{-1}
$ZnSO_4$. $7H_2O$	8.60 mg l^{-1}
H_3BO_3	6.20 mg l^{-1}
Na_2MoO_4. $2H_2O$	0.25 mg l^{-1}
KI	0.83 mg l^{-1}
FeNaEDTA	20 mg l^{-1}
Nicotinic acid	0.50 mg l^{-1}
Pyridoxin	0.50 mg l^{-1}
Thiamine	0.15 mg l^{-1}
Glycine	1 mg l^{-1}
Sucrose	30 g l^{-1}

Table 2. Types of media used for shoot proliferation experiments.

Media	Plant Growth Regulators
DKW	0.5 mg l^{-1} BA
MS	0.5 mg l^{-1} BA
MS	1mg l^{-1} BA
Modified QL1	0.5 mg l^{-1} BA, 0.5 mg l^{-1} GA_3 and 0.01mg l^{-1} NAA
Modified QL2	2mg l^{-1} BA and 0.01 mg l^{-1} IBA
Modified QL3	no plant growth regulators

All proliferation media were adjusted to pH 5.75 with 1 M KOH or 1 M HCl before dispensing in test tubes and autoclaving at 121°C at 1.2 - 1.3 kg cm^{-2} for 20 min. prior to adding to the vessels. Vessels were maintained at 22°C, under a 16h photoperiod with a light intensity of 45 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps located 45 cm above the shelf. For root induction, axillary shoots (2-3 cm long) with approximately three leaves, were excised from shoot proliferating cultures and transferred into glass culture vessels (400 ml) containing 40-50 ml of agar-gelled modified QL, DKW and MS-based rooting media, at half or full strength, and supplemented with different concentrations of growth regulators at pH of 5.75 and 6.2 (Table 3). All media were autoclaved together. Vessels were maintained at 22°C, under a 16h photoperiod with a light intensity of 45 µmol m^{-2} s^{-1}.

Rooting responses were evaluated after 4 weeks culture. Rooted plantlets were washed thoroughly in running tap water to remove all medium attached to the roots and then transplanted into plastic pots containing a mixture of peatmoss: cocopeat: perlite (2:2:1 v/v/v) treated with fungicide and liquid fertilizer. Plantlets were maintained in a controlled growth chamber at 23°C with 16h photoperiod under cool-white fluorescent light and yellow gaseous lamp (55 µmol m^{-2} s^{-1}). Pots were kept in transparent plastic containers to maintain high humidity and gradually hardened off after 10 days. Bags were removed completely after about 8 weeks. Subsequently, acclimatized plantlets were transplanted to other pots containing a mixture of Orchard soil: peat moss: sand: perlite (2:2:1:1 v/v/v/v) kept in a greenhouse and maintained at 19°C, 70% humidity under a 14h photoperiod with a light intensity of 50 µmol m^{-2} s^{-1}.

All the experiments were conducted as a completely randomized design, with four replicates of 4 explants in each treatment that repeated three times. The response variable had 1 to 5 values. One-Way ANOVA and a Duncan's multiple range tests were performed to analyze the results. All statistical analyses were done using SAS software.

Results

Fungi polluting factors will appear one week after cultivation if they have not been demolished, but the bacterial factors need more time to appear depending on the type of bacteria. The pollution signs can be detected by white spots (milk white spots) on the media or on the end part of the explants. Based on visual observations, contamination rates were about 4% for primary explants establishment, less than 1% for subcultures and 4% for the explants that were obtained from branches of field-grown trees at early March.

After successful culture establishment, shoot clumps were separated and treated the various media and growth regulator combinations. 1-2 cm-long tips from proliferating axillary shoots were cultured on different media. Then, at the end of the fourth week, the total number and height of shoots, which were produced per explants, were recorded. Axillary shoot formation was observed on all media. Callus formation was also induced at the bases of shoots and the new shoots were differentiated from these calli. Proliferation of shoots was also influenced by type and concentration of growth regulators, particularly cytokinin and type of medium. Explants cultured on medium consist of MS + 0.5 mg l^{-1} BA resulted in a proliferation of 5.37 new shoots per explant (Fig. 1). A multiplication rate of about 5.31 was achieved on medium composed of DKW + 0.5 mg l^{-1} BA. In QL3 medium, multiplication rate was 2.81. The QL1 medium resulted in rate of multiplication of 4.125. QL2 medium resulted in rate of multiplication of 3.93 shoots per explant (Table 4). Therefore, results indicate that the best media for shoot proliferation of this rootstock are MS + 0.5 mg l^{-1} BA and DKW + 0.5 mg l^{-1} BA. Overall, there were significant differences ($P < 0.05$) in shoot number and shoot height and ($P < 0.01$) leaf development between the media (Table 5).

Table 3. Characteristics of rooting media.

pH	Growth regulator	Culture media	Name of media
5.75	hormone-free	MS	MS (H-free)
6.2	hormone-free	Modified QL	QLM (6.2)
5.75	hormone-free	DKW	DKW (H-free)
5.75	0.1 mg l^{-1} BA + 0.1 mg l^{-1} NAA	MS ½	MS (1/2)
5.75	0.1 mg l^{-1} BA + 0.1 mg l^{-1} NAA	DKW	DKW (5.7)
6.2	0.1 mg l^{-1} BA + 0.1 mg l^{-1} NAA	DKW	DKW (6.2)
5.75	1 mg l^{-1} IBA	MS	MS-1 IBA
5.75	0.5 mg l^{-1} IBA	MS	MS-0.5 IBA
5.75	1 mg l^{-1} NAA	MS	MS- 1NAA
5.75	hormone-free	Modified QL	QL.M3
5.75	hormone-free	QL	QL (5.7)

Explants cultured on double-phase medium produced greater fresh plant mass, more leaves and longer roots. Application of double-phase medium instead of agar-gelled medium resulted in reduced hyperhydricity. There were significant differences ($P < 0.05$) in leaf development and hyperhydricity between the media (Table 6).

Optimum root formation (100%) was observed on DKW medium without growth regulators (Fig. 2). Rooting response was 81 percent when MS medium (containing 0.5 mg l^{-1} IBA) was used. However, using three media consisting of MS medium with no growth regulators, MS +1mg l^{-1} IBA and QL3, rooting was 75 percent (Table 4). The highest number of roots was observed on DKW medium without growth regulators in comparison with other media (6.5 roots per explant). But QL3 medium had the best effects on leaf development and increased the vigour of plantlets in comparison with other media (Fig. 3). However, there were significant differences ($P < 0.05$) in root number and ($P < 0.01$) rooted plantlets and root length between the media (Table 7). Explants cultured on QL3 medium produced 2.81 axillary shoots per explants. However these plantlets were healthy, vigorous and gave the best leaf development among the double phase and agar-gelled media (Table 5 & 6). Thus, the QL3 medium produced better results for acclimatization. Best survival rate was observed when plantlets were transferred to pots one week after root formation. Plantlets with older and browning roots were less successful than those vigorous plantlets with white and fully roots. Higher survival rates (more than 75%) were obtained after acclimatization of rooted plants under greenhouse conditions. Increase of pH from 5.75 to 6.2 resulted in development of very weak plantlets with no root induction in rooting media.

Table 4. Comparison of effects of various culture media on growth of axillary shoots and rooted plantlets.

Proliferation media			Rooting media		
media	Number of axillary shoots	Leaf development	media	Percent of rooted plantlets	Number of roots per plantlet
DKW+0.5 BA	5.31 A	3.5 C	MS (H-free)	75 B	2 BC
MS+1 BA	4.5 A	3 D	MS-0.5 IBA	81 AB	3 B
MS+0.5 BA	5.37 A	4 B	DKW (H-free)	100 A	6.5 A
Modified QL1	4.12 AB	3 D	MS (1/2)	16 CD	0.5 C
Modified QL2	3.92 AB	3 D	DKW (5.7)	7 D	0.2 C
Modified QL3	2.81 B	5 A	Modified QL3	75 B	5.5 A
			MS-1 IBA	75 B	3.3 B
			QL (5.7)	16 CD	0.9 C
			MS-1 NAA	32 C	1.9 BC
			DKW (6.2)	0	0
			QLM (6.2)	0	0

Means are reported at 5% level of probability for number of axillary shoots and roots per plantlet and 1% for leaf development and percent of rooted plantlets (DMRT). Mean numbers with similar letter are not statistically different.

Table 5. Analysis of variance of proliferation stage.

S.O.V	df	MS					
		Shoot number	Shoot height	Vigorous	Leaf development	Hyperhydricity	Long internode
Media	5	3.667*	0.37*	0.344ns	2.57**	0.9ns	1.37ns
Error	18	0.999	0.147	0.219	0.012	0.42	0.486
Total	23	4.666	0.517	0.563	2.582	1.32	1.856
C.V (%)		22.95	13.7	11.87	1	22.5	18.5

ns = not significant, ** = significant at 1% probability level, * = significant at 5% probability level.
df = degree of freedom, S.O.V = source of variation, C.V (%) = coefficient of variation.

Table 6. Analysis of variance between double-phase and agar-gelled media.

S.O.V	df	MS			
		Shoot number	Leaf development	Hyperhydricity	Root length
Media	4	3.11 ns	4.92*	2.33*	5.08*
Error	24	0.85	1.25	1.13	0.9
Total	28	3.96	6.17	3.46	5.98
CV (%)		16.61	18.21	16.07	22.79

ns = not significant, ** = significant at 1% probability level, * = significant at 5% probability level.
df = degree of freedom, S.O.V = source of variation, C.V (%) = coefficient of variation.

Table 7. Analysis of variance of rooting stage.

S.O.V	df.	MS				
		Rooted plantlets	Vegetative growth	Root length	Root thickness	Root number
Media	10	9.491**	9.991**	7.08**	13.027**	37.286*
Error	33	0.371	0.21	0.987	1.043	2.83
Total	43	0.371	0.21	8.067	14.07	40.116
C.V (%)		15.74	14.63	22.79	21.18	23.86

ns = not significant, ** = significant at 1% probability level, * = significant at 5% probability level.
df = degree of freedom, S.O.V = source of variation, C.V (%) = coefficient of variation.

Figure 1. Proliferation medium containing MS + 0.5 mg l^{-1} BA after 4 weeks.

Figure 2. Effect of DKW medium without growth regulators on root formation after 4 weeks.

Figure 3. Modified QL medium without growth regulator after 4 weeks which resulted in strong leaf development.

Figure 4. Effect of MS + 1mg l^{-1} BA medium on hyperhydricity.

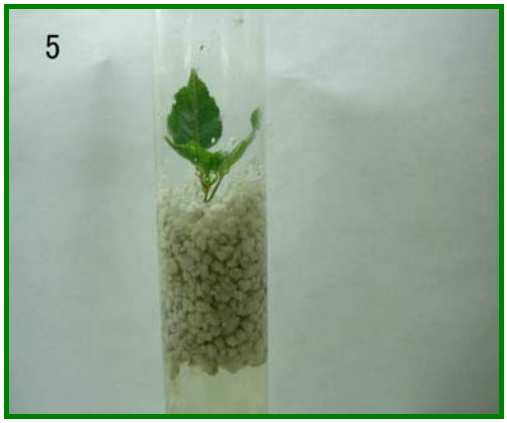

Figure 5. Application of double-phase medium containing MS + 1mg l^{-1} BA resulted in control of hyperhydricity.

Figure 6. Acclimatized plants in greenhouse were kept at 70% humidity to maintain high humidity and gradually hardened.

Discussion

There are many reports in which cherry explants were successfully surface-sterilized using mercuric chloride solution (Muna et al. 1999, Andreu & Marin 2005, Durkovic 2006). On the contrary, 44% (Karešova cv.) and 52% (Rivan cv.) of initial explants did not develop shoots after the sterilization procedure. It was probably due to the toxicity of the mercuric chloride concentration used (0.15% solution for 1 min.) (Sedlak & Paprštein 2008). Furthermore, use of dichloroisocyanuric acid Na_2 salt (DICA) for 15 minutes for surface-sterilization resulted in production of more vigorous plants in micropropagation of cherry rootstock (Osterc et al. 2004). Also several reports indicate successful surface-sterilization by using sodium hypochlorite (Hammatt & Grant 1997, Pruski et al. 2000, Pruski et al. 2005, Kalinina & Brown 2007). We used sodium hypochlorite and all of treatments were successful in surface-sterilization and resulted in very low visual contamination.

Hyperhydricity was observed in four media, namely MS + 1mg l^{-1} BA, MS + 0.5 mg l^{-1} BA, QL1 and the QL2 (Fig. 4). Hyperhydricity of tissues by TDZ were reported on *Pyrus pyrifolia* and compared with kinetin and BA (Kadota & Nimi 2003). Medium containing a high cytokinin level can induce

hyperhydricity (Prknová 2007). Higher concentration of BA causes better multiplication but results in the shoots to be more vitrified (Dziedzic & Malodobry 2006). Application of double-phase medium instead of agar-gelled medium resulted in better control of hyperhydricity (Fig. 5). Induction of axillary shoots was directly induced from the excised explants. Callus formed at the bases of these explants and some lateral shoots differentiated from them. Axillary branching in the axil of leaves was also observed. This method is a better approach than the callus method where calli often produce cytologically abnormal plants. Analysis of the effect of four types of cytokinin (BA, 2iP, TDZ and kinetin) indicated that BA was better for the proliferation phase of Lapins cherry (Ruzic & Vujovic 2008). In the presence of BA, high rates of shoot proliferation occurred at the base of stem. These lateral shoots are both axillary and adventitious (Muna et al. 1999). The function of the continuous BA supply is thus to break the apical dominance and stimulate growth of new shoots. Another obvious effect of the presence of BA in the medium is the complete or partial inhibition of root formation. Increase of BA concentration in medium (MS + 0.5 mg l^{-1} BA compared with MS + 1 mg l^{-1} BA) resulted in increase of the shoot number and decrease of the shoot length (Pruski et al. 2005). During the proliferation phase, use of 0.5 mg l^{-1} GA_3 in modified QL containing 0.5 mg l^{-1} BA and 0.01 mg l^{-1} NAA gave better rooting after 6 weeks compared to that of same modified QL containing 2 mg l^{-1} BA and 0.01 mg l^{-1} IBA without GA_3. Such a result was probably due to the low concentration of BA or effect of GA_3. Positive effect of GA_3 on rooting of F12/1 rootstock was reported by Hammatt and Grant, (1997). However, the highest number of roots on DKW medium without growth regulators and greater leaf, shoot and root growth were achieved by using double-phase medium containing modified QL with no growth regulators. It seems, this vigour and high length is due to the optimum conditions for biosynthesis of endogenous hormones, in plantlets.

Acclimatization was affected directly by rooting treatment and Leaf development. Maintenance on rooting media gave rise to longer roots but resulted in poor survival. Overall, media which induce vigour, leaf development and rooting resulted in better acclimatization and higher survival rate.

Conclusions

A protocol was developed to propagate a valuable dwarfing cherry rootstock. Overall, MS medium with 0.5 mg l^{-1} benzyladenine was optimal for shoot proliferation (5.37 shoots per explant) and the DKW medium without growth regulators and QL3 medium were the best media for rooting. Rootstock plantlet acclimatization and vigorous plants were successfully achieved using above mentioned medium (Fig. 6). More importantly, application of the double-phase media, composed of liquid medium with perlite, instead of commonly used agar-gelled medium gave better support for the shoots and resulted in better control of hyperhydricity, greater development of fresh mass and leaf and longer roots.

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