High frequency *in vitro* propagation of M × M60, a cherry rootstock: the effects of culture media and growth regulators

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Abstract
Effects of various culture media and growth regulators on micropropagation of cherry rootstock, M × M60 were investigated in the present study. Stem cuttings of the current season growth were taken from mature stockplants and cultured on the establishment medium after disinfection. After four weeks, elongated shoots were cut and subcultured on the proliferation media including MS, DKW, QL and ME supplemented with 0, 0.5, 0.7, 1 mg L^{-1} of BAP. As a result, shoot number, average shoot length node number and internode length (except leaf area) were significantly affected by the interaction of medium with BAP level. The largest number of shoots was obtained in DKW (10.3 shoots/explants), ME (9.1 shoots/explants) and QL (9.1 shoots/explants) containing 0.7 mg L^{-1} BAP. The results showed that DKW with 0.7 mg L^{-1} BAP had the highest productivity compared to other treatments. For rooting, *in vitro* proliferated and elongated shoots were planted on LS, MS and ½ MS medium containing 0, 0.5, 1 and 1.5 mg L^{-1} of IBA. High rooting percentage, root length and root number were obtained on ½ MS supplemented with 1 mg L^{-1} IBA. The *in vitro* plantlets were transferred into the soil and the survival rate *ex vitro* was 95%.

***Key words*:** M×M60, Cherry rootstock, Micropropagation, Rooting, BAP.

INTRODUCTION

Rootstocks are responsible for water and nutrient uptake, resistance to soil-borne pathogens and tolerance to environmental stresses. A variety of rootstocks are used for *Prunus* species on a worldwide basis (Rom and Ferree 1984), each having a particular set of advantages and limitations for adaptation to different geographical regions. The vegetative rootstock M × M60 is an interspecies cherry hybrid and perhaps one of the most widely used rootstocks for sweet and sour cherries, particularly due to strength, compatibility with different varieties and resistance to *Phytophthora megasperma* and *Ph. cambivora*. The sign "M × M" may indicate the cross between *Prunus mazzard* and *Prunus mahaleb*, which their offspring are probably the best known for tolerance of unfavorable soils, suitable rooting system and high resistance against pests and blights (Ganji moghaddam and Abdollahzadeh Gonabadi 2008, Hrotkó 2016). MAMA rootstocks are more efficient and smaller than Mahaleb seedling rootstocks (Hrotkó and Füzesséry 1993, Hrotkó and Simon 1993, Hrotkó *et al.*, 1996).

Although M × M60 is propagated from greenwood cuttings, some main problems encounter with this conventional cloning such as low rate of propagation,

high labor time and cost. In contrast, *in vitro* propagation or micropropagation is a time saving and cost effective approach. Many studies have successfully micropropagated *Prunus* species such as sour and sweet cherries (Borkowska 1984, Hammatt and Grant 1997, Matt and Jehle 2005, Canli and Tian 2008, Ružić and Vujović 2008). Protocols for micropropagation are presented in Gisela 5 (Vujović *et al.*, 2012), Gisela 6 (Vujovic *et al.*, 2009, Hossini *et al.*, 2010), PHL-A (Sedlak *et al.*, 2008, Mahdavian *et al*., 2011), sweet and sour cherry rootstock selections (Dradi *et al.*, 1993, Singh *et al.*, 2010).

A successful micropropagation protocol depends on using optimal culture medium and growth regulator(s) (Šiško 2011, Bošnjak *et al.*, 2012). Different culture media have been used for micropropagation of cherries (Hammatt and Grant 1997, Hammatt and Grant 1998, Ďurkovič 2006). Murashige and Skoog (MS) (Murashige and Skoog 1962) medium with double-strength, half strength and quarter strength macro salt is widely used for micropropagation of sweet cherry rootstocks such as Gisela 5 and M × M14 (Sarropoulou *et al.*, 2013, Fallahpour *et al.*, 2015). However, DKW (Driver and Kuniyuki Walnut medium) (Driver and Kuniyuki 1984) and QL (Quoirin and Lepoivre medium) (Quoirin and Lepoivre 1977) media have been used for the micropropagation of Gisela 5 rootstock (Šiško 2011, Bošnjak *et al.*, 2012). Almehdi and Parfitt medium (AP) was found to be the best medium for the multiplication of M × M46, M × M2 and M × M60 (Zilkah *et al.*, 1991). Benzyladenine (BA) and indole-3-butyric acid (IBA) are the common growth regulators for propagation and rooting of cherry rootstocks (Šiško 2011, Bošnjak *et al.*, 2012). In Maxma-14, a semi-dwarfing cherry rootstock, high rate of multiplication was obtained on the MS medium containing 4.44 µM BA and 0.49 µM IBA (Muna *et al.*, 1999). Zilkah *et al.* (1991) used 0.2, 6 and 0.5 mg L^{-1} BA for the multiplication of M × M2, M × M46 and M × M60, respectively. Some authors recommended liquid medium to obtain high rate of multiplication, rooting and acclimatization rates (Muna *et al.*, 1999, Doric *et al.*, 2014). Aghaye *et al*. (2013) achieved 100% rooting in Gisela 6 rootstock using thiamine and Fe-EDDTA Application of melathonine improved adventitious rooting of Gisela 6, CAB-6P and M × M60 (Sarropoulou *et al.*, 2012). Half-strength MS medium containing 0.5 NAA mg L^{-1} was used for rooting of *in vitro* microshoots of M × M60 (Zilkah *et al.*, 1991).

The objective of the present study was to increase micropropagation efficiency of M × M60 and to investigate the effect of different culture media and growth regulators on multiplication rate and rooting.

MATERIAL AND METHODS

Surface sterilization and in vitro establishment

Actively growing shoot twigs with 4-5 axillary buds of mature M × M60 trees were used for *in vitro* establishment. In order to prevent internal contamination, stem cuttings with auxiliary buds were collected during growth season. Stem cuttings were washed for 20 min with tap water and then were sterilized in 0.1 % (v/v) mercuric chloride and Tween 20 for 3 minutes. The buds were further rinsed twice with autoclaved water containing citric acid (0.3 %) for three minutes. Then stem segments were trimmed with a sterile scalpel blade into smaller nodal segments (1-1.5 cm in length), each with one node and used as explants. The excised segments were cultured in 15 or 18 × 2.5 cm test tubes each with 10 ml of agar-gelled ME medium (Cos *et al.*, 2002) supplemented with 0.2 mg L^{-1} BAP. The explants were incubated in a culture room under a 16-h photoperiod at low-light intensity (15 to 20 µmol. m^{-2}. s^{-1}) provided with cool-white (40W) fluorescent tubes at 25 ± 1°C. After four weeks, elongated shoots were subcultured on the proliferation media.

Culture media for proliferation

Media in all experiments contained 0.05 mg L^{-1} IAA, 0.1 mg L^{-1} GA3 and 30 g L^{-1} sucrose and were solidified with 5.7 g L^{-1} agar. pH was adjusted to 5.8 with 0.1 N KOH. The media were sterilized in an autoclave for 20 min at 120°C.

ME medium (Cos *et al.*, 2002) supplemented with 0.2 mg L^{-1} BAP was used for the establishment stage. In the proliferation stage, three basal culture media including MS, ME, QL and DKW, with four concentrations of BAP, 0, 0.5, 0.7 and 1 mg L^{-1}, were tested.

Microshoots (1–1.5 cm in length) were obtained from the establishment cultures and placed in 250 ml jars containing 25 ml of each medium. Each treatment consisted of three replicates (jar) with 3 shoots per jar. The proliferation was evaluated six weeks after the beginning of the experiment. Number of shoots per explant, average shoot length, leaf area, internode length and node number were recorded.

Rooting and adaptation

For rooting stage, approximately 15 mm-long shoots derived from four week-old cultures were used. Elongated shoots were transferred to LS (Linsmaier and Skoog 1965), MS (Murashige and Skoog 1962), and ½ MS supplemented with 0, 0.5, 1 and 1.5 mg L^{-1} IBA.

Table 1. Analysis of variance for various shoot proliferation parameters of M × M60 rootstock.

Source of variations	df	Means of Squares (MS)				
		Shoot number	Average Shoot length (cm)	Node number	Internode length	Leaf area
Medium	3	347.176**	4.567**	96.806**	0.162**	2.192**
BAP level	3	512.004**	1.593**	11.927**	.015^{ns}	1.806**
Medium × BAP	9	56.169**	0.374**	6.519**	0.022**	0.33^{ns}
Error		3.878	0.125	0.906	0.008	0.251
Coefficient of variation (%)		6.4	6.1	7.6	8.1	5.2

** Means are significantly different at p<0.01.; ns means are not significantly different.

After 30 to 35 days, rooting percentage, root length and average number of roots per explants were recorded.

Rooted plantlets were washed under tap water and dipped in Captan fungicide (1000 ppm). They were then transplanted in pots containing peat moss. Established plants were transferred to the greenhouse and placed in plastic containers with transparent covers. After one week, the covers were opened gradually over a 48 hours period. A balanced liquid fertilizer (1 g L^{-1}) was applied after 10 days. Acclimatized plantlets (30-35 cm) were potted to a heavier soil mixture after about two months.

Statistical analysis

Data were subjected to the analysis of variance using a completely randomised design. The means of treatments were compared to Duncan's multiple range test (DMRT) to distinguish differences between treatment means at the probability level of 0.01 and 0.05 using SAS software.

RESULTS

Effect of different culture media on proliferation

Medium type significantly affected the number of shoots, average shoot length, node number, internode length and leaf area. Moreover, BAP level significantly affected the number of shoots, average shoot length, node number and leaf area except internode length. Number of shoots, average shoot length, node number and internode length were significantly affected by the interaction of medium with BAP level, whereas leaf area was independent of the interaction between culture media and BAP level (Table 1).

The longest shoots were obtained in DKW (2.9 cm) and ME (2.73 cm) media containing 0.7 mg L^{-1} BAP (Figure 1). However, in all media the smallest shoots were obtained in 1 mg L^{-1} BAP. As a result, small shoots were induced by 1 mg L^{-1} of BAP in all media.

The highest number of shoots was obtained in the DKW (10.3 shoots/explants), ME (9.1 shoots/explants) and QL (9.1 shoots/explants) containing 0.7 mg L^{-1} BAP (Figure 2 and 3). MS medium was not suitable for the multiplication stage. Generally, 1 mg L^{-1} BAP inhibited shoot proliferation in all media.

The number of nodes per shoot was affected by interaction between medium and BAP level. The highest number of nodes was produced in the MS medium containing 0.5 and 0.7 mg L^{-1} BAP (6.7 and 7.4, respectively) and ME with 0.7 mg L^{-1} BA (6.5) (Figure 4). As a result, 1 mg L^{-1} BAP produced the lowest number of nodes in all media. According to Figure 5, the longest internodes were produced on free MS medium.

Although leaf area was not affected by the interaction between BAP level and medium, it was reduced by the addition of BAP to the proliferation medium. However, leaf area was small in QL medium compared to three other media.

Rooting and adaptation

Medium had significant effects on length and rooting percentage but not root number. The highest root length (2.06 cm) was obtained in ½ MS (Table 2 and Figure 6).

IBA significantly affected root length, root number and rooting percentage. The largest root number was produced with 1 and 1.5 mg L^{-1} IBA. Long roots and high rooting percentages were obtained at 0.5, 1 and 1.5 mg L^{-1} IBA and there was no significant difference between them. Root number and root length were not affected by the interaction between IBA levels and medium.

Rooting percentage was dependent on the interaction between IBA levels and medium (Figure 7). LS

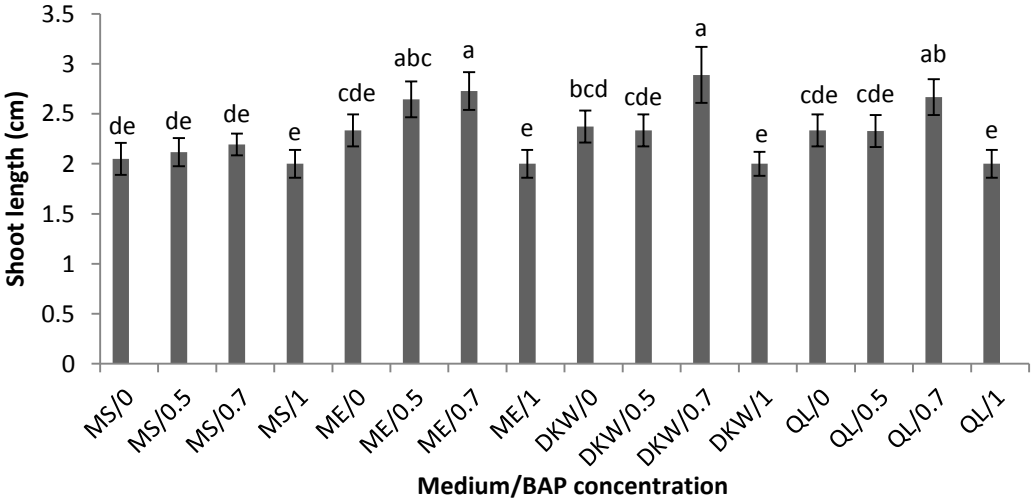

Figure 1. Effect of various culture media and different concentrations of BAP on length of M × M60 shoots.

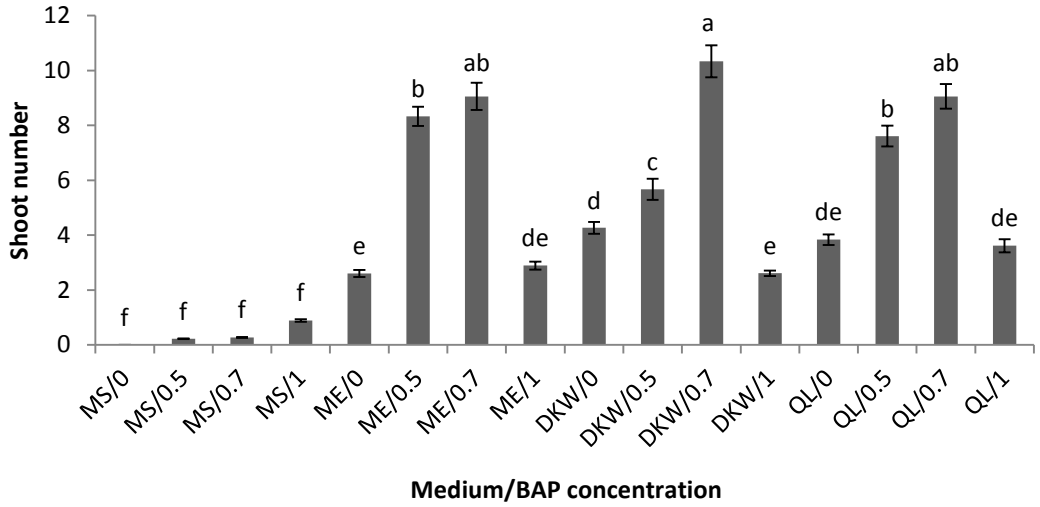

Figure 2. Effect of various culture media and different concentrations of BAP on the number of M × M60 shoots.

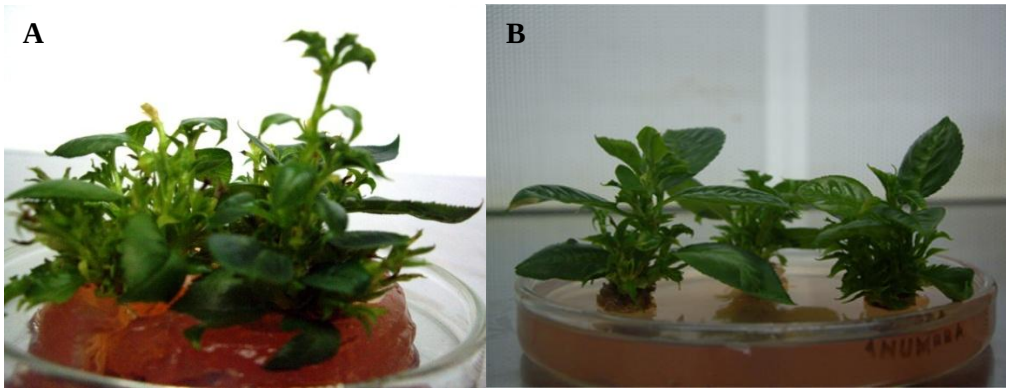

Figure 3. Multiplied shoots of M × M60 after culture on DKW supplemented with 0.7 mg L^{-1} BA **(A)** and on ME supplemented with 0.7 mg L^{-1} BA **(B)**. Bar:1cm.

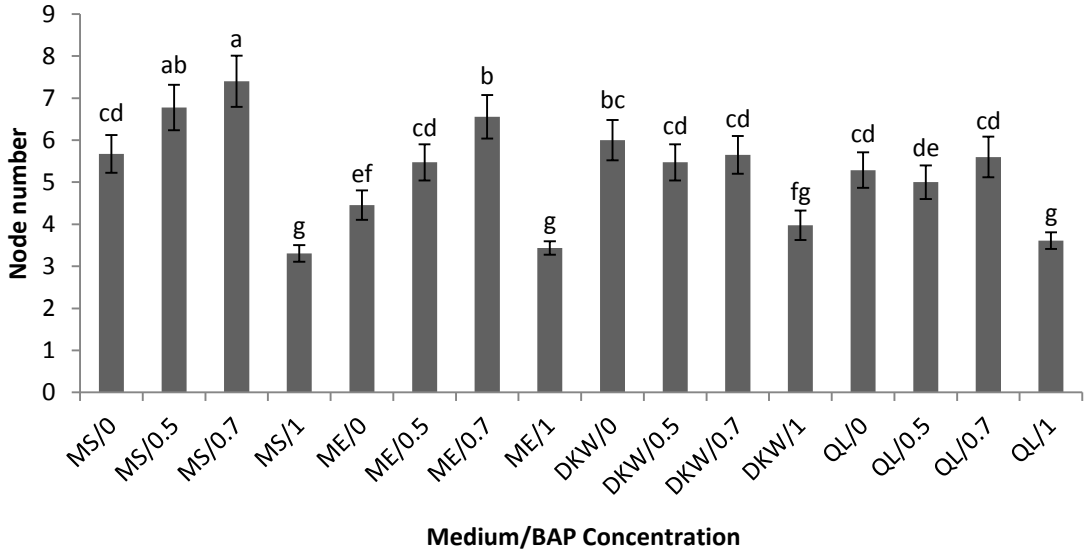

Figure 4. Effect of various culture media and different concentrations of BAP on the number of nodes in M × M60 shoots.

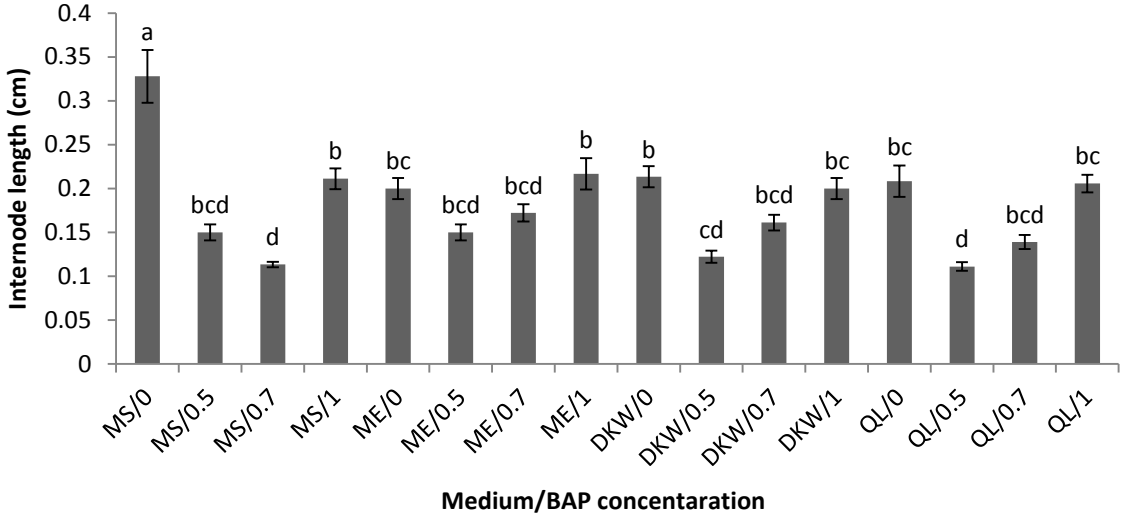

Figure 5. Effect of various culture media and different concentrations of BAP on internodes of M × M60 shoots.

Table 2. Effect of different media and IBA levels on *in vitro* rooting properties of M × M60 shoots.

Parameters	Rooting media			IBA levels (mg L^{-1})			
	1/2MS	LS	MS	0	0.5	1	1.5
Average number of roots	1.55^{a}± 0.22	1.42^{a}± 0.41	1.34^{a}± 0.32	0.78^{c}± 0.07	1.37^{b}± 0.17	1.74^{a}± 0.28	1.8^{a}± 0.32
Average root length (cm)	2.06^{a}± 0.52	1.56^{b}± 0.39	1.62^{b}± 0.44	1^{b}± 0.45	2.03^{a}± 0.54	2.03^{a}± 0.46	1.88^{a}± 0.48

Means with the same letters are not significantly different at p≤0.01.

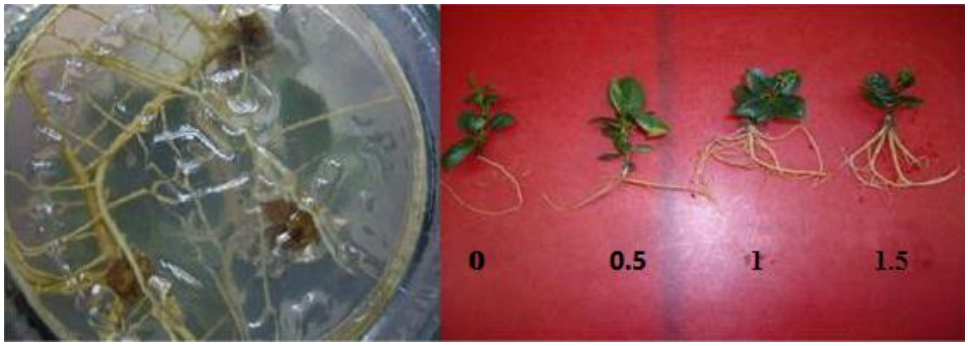

Figure 6. Root number and root length of M × M60 shoots after subculture on ½ MS supplemented with 0, 0.5, 1 and 1.5 mg L^{-1} IBA. Bar:1 cm.

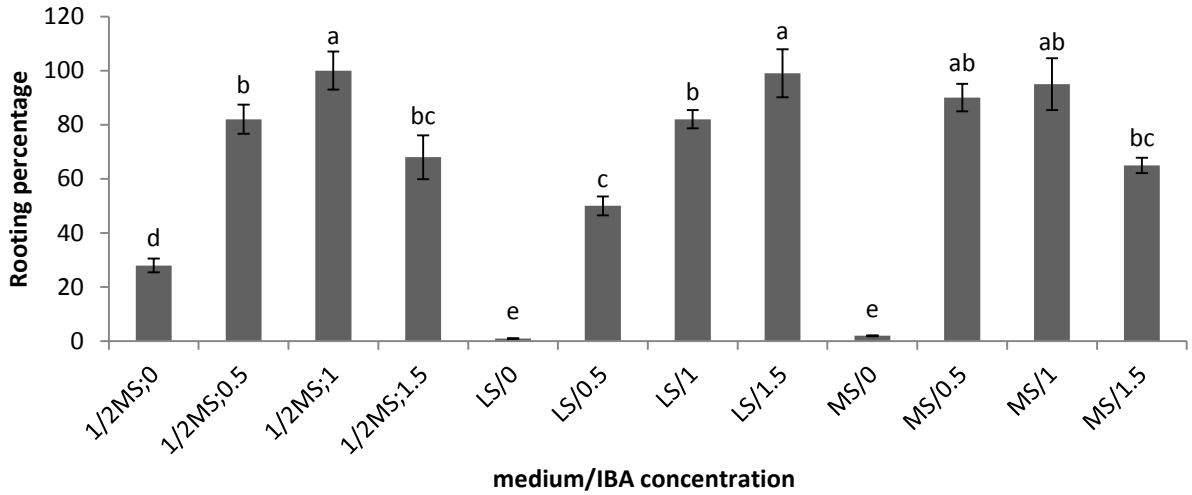

Figure 7. Effect of different media and IBA levels on rooting percentage of M × M60 *in vitro* shoots.

containing 1.5 mg L^{-1} IBA and ½ MS containing 1 mg L^{-1} IBA showed 100% rooting. However, ½ MS containing 0.5 mg L^{-1} IBA produced the least amount of callus at the base of the shoots.

No contamination was observed after transplanting explants to the soil. The rate of adaptation was 95%. Adaptation of the plants was considerable after three weeks (Figure 8).

DISCUSSION

The purpose of this study was to improve micropropagation of M × M60 rootstock. In our study, a high rate of multiplication (10.3 shoots/explants) in the DKW medium containing 0.7 mg L^{-1} BAP has been observed. This result was significant in comparison with previous reports. Zilkh *et al*. (1991) reported a

Figure 8. Acclimatized M × M60 plants in greenhouse.

multiplication rate of 4-5 shoots per month for M × M60, M × M2 and M × M46. They used AP medium containing 0.5 BA mg L^{-1}. In Gisela 6, a multiplication rate of 4 shoots has been reported (Doric *et al.*, 2014). Šiško (2011) obtained 4.2 shoots per explants by micropropagation of Gisela 5 on WPM medium (Šiško, 2011).

Several culture media were investigated for the proliferation stage. As a result, DKW was found more productive than MS. Productivity comprises shoot number and shoot length. DKW has previously been used for cherry propagation (Matt and Jehle, 2005, Ďurkovič, 2006). DKW contains less amount of ammonium nitrate compared to MS and ME media. The importance of the nitrate/ammonium ratio for various purposes has been discussed comprehensively (George *et al.*, 2008). Generally, for shoot cultures or root growth, this ratio is dependent on genotype or species, physiological age of tissue and the degree of differentiation of the tissue (George *et al.*, 2008). DKW was more efficient than MS for the proliferation of Gisela 5 (Bošnjak *et al.*, 2012, Fallahpour *et al.*, 2015). Dorić *et al*. (2014) successfully used DKW medium for the micropropagation of several sweet and sour cherry rootstocks. ME medium has a high ratio of NO_3^- to NH_4^+ and high concentration of Ca^{++}, causing shoot elongation. QL is a low ammonium medium. In addition, QL uses calcium nitrate as a nitrogen source. Therefore, it has a higher calcium concentration while chlorine ions are almost eliminated. In our experiment, QL was also better than MS, which is in agreement with Bošnjak *et al*. (2012) findings.

BA is one of the most practical and commonly used cytokinin, which eliminates apical dominance and has been used for shoot multiplication of cherries (Mansseri-Lamrioui *et al.*, 2013). Ďurkovič (2006) and Mansseri-Lamrioui *et al*. (2009) reported that BA was necessary for the development of *Prunus avium* L. Saponari *et al*. (1999) successfully micropropagated rootstocks of *Prunus mahaleb* using 1 mg L^{-1} BA. BA was also recommended for the proliferation for several other *Prunus* species, including, *P. avium* L. (Hammatt and Grant, 1997), *P. insititia* L. (Loreti *et al.*, 1987) and *P. cerasus* (Borkowska, 1984). BA concentrations between 8.87–12.82 µM were optimal for shoot proliferation in chokecherry 'Garrington' (Pruski *et al.*, 2000). BA concentration of 4.44 µM gave optimal shoot proliferation in pincherry (Pruski *et al.*, 2000). The 8.87 µM BA has been reported to be optimal for shoot proliferation of the *Prunus cerasifera* × *Prunus munsoniana* rootstocks (Dalzotto and Docampo, 1997). Increase in BA concentration in the medium (MS + 0.5 mg l^{-1} BA compared with MS + 1 mg l^{-1} BA) resulted in an increase in the shoot number and a decrease in shoot length in Mongolian cherry (*Prunus fruticosa* L.) and Nanking cherry (*Prunus tomentosa* L.) (Pruski, 2007).

Rooting of M × M60 was dependent on IBA level. Rooting was induced over the entire range of IBA concentrations and ½ MS containing 1 mg L^{-1} of IBA was determined as the optimal rooting medium. Root growth is often promoted by the low ratio of NH^{+4}: NO^-_3. Therefore, root culture media contain no NH^{+4} or very little (George *et al.*, 2008). IBA is the most commonly used auxin for rooting initiation (George *et al.*, 2008). Mansseri-Lamrioui *et al*. (2013) carried out a study on the effect of different auxins and their concentrations on *in vitro* rooting of wild cherry. They found that 1 mg L^{-1} IBA improved rooting percentage, number of roots per explants and root length in wild cherry MJ1. Nissen and Sutter (1990) as well as Hausman (1993) reported that IBA is oxidized slowly and delayed degradation and slow movement may be the primary reason for better performance of IBA as compared to other auxins. Van der Krieken (1993) showed that IBA may also enhance rooting *via* increased internal free IBA or may synergistically modify the action of endogenous synthesis of IAA. Previous studies demonstrated that 1 mg L^{-1} of IBA is the most effective concentration (Gorst *et al.*, 1983, Drew *et al.*, 1991, Kalinina and Brown, 2007). Fuernkranz *et al.* (1990) also showed that the concentration of 4.65 µM is most efficient for rooting of *Prunus serotina* micro cuttings. Ahmad and Laghari (2004) obtained the maximum number of roots with 0.4 mg L^{-1} IBA in peach rootstock GF677. The high rooting (84%) was reported with IBA/NAA (9.80/2.69 µM) in chokecherry and pincherry cultivars (Pruski *et al.*, 2000). *In vitro* rooting of plantlets in auxin containing media usually produces shorter plantlets having roots with poorly developed root hairs (Pruski *et al.*, 2000). Maene and Debergh (1982) stated that auxin concentrations optimal for root initiation can be inhibitory for root elongation. In Gisela 6, a rooting percentage of 80% was reported in previous studies (Vujovic *et al.*, 2009, Hossini *et al.*, 2010, Aghaye *et al.*, 2013, Sarropoulou *et al.*, 2013). In a previous study, more than 90% of rooting efficiency was reported on half strength MS medium containing 0.5 mgL^{-1} in M × M46, M × M2 and M × M60 (Zilkah *et al.*, 1991).

CONCLUSION

The present work was the report of *in vitro* proliferation and rooting of M × M60 rootstock. The results obtained in the present study can be used as guidelines for improving *in vitro* propagation of M × M60, a suitable

rootstock for cherry. Furthermore, the results demonstrated the optimized stage for root induction. Modification of macroelements in DKW medium can be suggested for future studies of M × M60 propagation.

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