

Impacts of plant growth regulators and culture media on *in vitro* propagation of three apple (*Malus domestica* Borkh.) rootstocks

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ABSTRACT

Apple is one of the most important temperate-zone fruit trees in the world. Micropropagation of apple scion and rootstocks is an important method of rapid production of healthy and disease-free plants. In this research, *in vitro* propagation of three apple rootstocks including Azayesh-Esfahan, Morabbaee-Mashhad and M9 was investigated. Shoot proliferation carried out in two basal media (MS and WPM) containing three concentrations of BA (0.5, 1 and 1.5 mgL⁻¹) and also, rooting of microshoots were investigated in two basal media (MS and ½ MS) with three concentrations of IBA (0.5, 1 and 1.5 mgL⁻¹). The results showed that all studied factors including cultivars, media, BAP, IBA concentrations and interaction among them had significant effects on both shoot proliferation and rooting of rootstocks. Regardless of rootstocks and media, the maximum and minimum shoot proliferation rates were obtained in the media containing 1.5 mgL⁻¹ and 0.5 mgL⁻¹ BA, respectively. The MS medium was more effective on shoot proliferation than WPM medium. The Azayesh-Esfahan and Morabbaee-Mashhad showed the maximum and minimum (4.46 and 3.66 shoots/explants) shoot proliferation values, respectively. However, all rootstocks had the maximum rooting in the ½MS media containing 1.5 mgL⁻¹ IBA. In overall, Azayesh-Esfahan showed the maximum shoot proliferation (5.11 shoots/explants) and rooting (48.33 %) among rootstocks.

Key words: Apple, Azayesh-Esfahan, Morabbaee-Mashhad, PGR, Proliferation, Rooting.

INTRODUCTION

Apple (*Malus domestica* Borkh.) is one of the most important fruits in temperate-zones. It is the third most important fruit tree in the world and Iran is ranked 5th among producing countries (FAO, 2013). Vegetative propagation via budding or grafting is the conventional

apple propagation method which cannot ensure disease-free and healthy plants (Dobrąnszki *et al.*, 2010). Micropropagation provides the rapid propagation of new varieties, breeding lines or mutants in apple breeding because it is the most necessary stage in the regeneration of transgenic lines and determines the effectiveness of a transformation protocol (Aldwinckle and Malnoy, 2009). Recently in apple, many reliable methods have been developed for both propagation of rootstocks and scions using *in vitro* techniques. Successful micropropagation of apple using microcuttings or single node cuttings is influenced by several internal and external factors, including genotype, physiological state of sampling, *in vitro* media constituents and their ratio, light, temperature and other factors (George, 1996b; Zandrea *et al.*, 2006; Dobrąnszki and Silva, 2010).

Lane and McDougald (1982) investigated the shoot proliferation of four apple rootstocks and cultivars, including M.9, M.26, M.27, and Macspur and found that genotypes differed in their response to the concentration of BA in the medium. They reported that optimum concentration of BA for shoot proliferation varied between cultivars (5 µM for M.26 and Macspur and 10 µM for M.9 and M.27). Webster and Jones (1991) found differences in shoot production of four apple rootstocks. Shoot production was readily achieved with P.22 and Ottawa3, but it was more difficult with P.2 and B.9. Yepes and Aldwinckle (1994) studied on *in vitro* proliferation and rooting of thirteen apple cultivars and rootstocks. They tested four proliferation media and concluded that proliferation rates varied depending on the genotype and medium used. The highest proliferation rate was obtained for a rootstock that produced 11.6 ± 2.5 shoots per tube per month. Rooting was induced with IBA and optimal IBA concentration was genotype dependent (0.1 to 1.0 mg L⁻¹ IBA). Sotiropoulos *et al.* (2006) reported the effects of sucrose and sorbitol on shoot growth and proliferation, peroxidase and catalase isoenzymes, nutritional status

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of phosphorous in M9 and MM106 *in vitro*. Liu *et al.* (2009) studied the potential regulation of apple *in vitro* shoot growth via modulation of cellular polyamine contents.

Bolar *et al.* (1998) reported an efficient method for rooting and acclimatization of micropropagated apple cultivars. Druart (1997) introduced an optimized *in vitro* culture medium for rooting of Compact Spartan apple cultivar. Magyar-Tábori *et al.* (2009) found factors affecting the rooting ability of *in vitro* apple shoots. Modgil *et al.* (2009) reported a commercially feasible protocol for rooting and acclimatization of micropropagated apple rootstocks. Bommineni *et al.* (2001) formulated a new method for rapid *in vitro* propagation of some apples and pears. Isutsa *et al.* (1998) reported a protocol for rooting and growing apple rootstock microshoots.

Many genotypes and cultivars of apples have been successfully cultured *in vitro*, and many studies have been carried out on different aspects of apple micropropagation. Magyar-Tábori *et al.* (2001b, 2001c and 2002b) reported effects of cytokinins, auxins and activated charcoal on the proliferation and rooting ability of apple shoots grown *in vitro*. Dobránszki *et al.* (2000a and b) compared the response of *in vitro* shoot multiplication of three apple rootstocks and some scions to cytokinin and auxin and they established a model experiment for micrografting in apple (Dobránszki *et al.*, 2010). Furthermore, micropropagation of apple rootstock M27 and Golden Delicious was studied by Al-Rihani *et al.* (2005) and Al-Tiawni *et al.* (2009), respectively.

In this research, micropropagation of three apple rootstocks including Azayesh-Esfahan and Morabbaeei-Mashhad (Iranian indigenous) and M9 was investigated on two culture media (MS and WPM) containing three concentrations of BA for shoot proliferation and two media (MS and ½MS) enriched with three concentrations of IBA (0.5, 1 and 1.5 mgL⁻¹) for rooting, and finally selecting the best condition for shoot proliferation and rooting in three cultivars specially in Iranian indigenous rootstocks.

MATERIALS AND METHODS

Plant materials

Plant materials were collected from the growing trees during spring (almost fast growing times) and sometimes in the dormant seasons (winter and autumn) of 2010 and 2011, in collection of Horticultural Crops Research Department of SPII, Karaj, Iran. Single node cuttings were selected as explants from three apple rootstocks including; Azayesh-Esfahan and Morab-

baeei-Mashhad (Iranian native cultivars) and M9 as dwarf rootstocks.

Sterilization and Stabilization

Explants were washed with tap water and soap for 30-60 min and then by immersion in fungicides solution, Benomile and Ridomile (2 gL⁻¹ w/v) for 20 min for reducing contamination. Single node explants were surface sterilized by immersion in 70% (v/v) ethanol for 60 seconds and then rinsed three times with sterile distilled water, followed by immersion in 4% (v/v) sodium hypochlorite (NaClO) for 10 to 18 min depending on the diameter of shoots. Then rinsed three times with sterile distilled water. Finally, explants were further sterilized by mercuric chloride (HgCl₂) (100 mg HgCl₂ in 100 ml distilled water) for 2 min, followed by three times of rinsing with sterile distilled water. For the stabilization they were cultured on the MS basal medium enriched with GA3 (1 mgL⁻¹), BA (0.5 mgL⁻¹) and IAA (0.5 mgL⁻¹). Cultures were placed under of 25 ± 2 °C and 16/8 hour day length conditions. After three sub-cultures on the stabilization medium, the newly grown shoots were used as the source of explants for shoot proliferation and rooting.

Shoot proliferation and rooting media

Single node explants were obtained from sterilized shoots grown *in-vitro* and cultured on two basal media, Murashige and Skoog (MS) and Woody Plant Medium (WPM) supplemented with 30 grL⁻¹ sucrose, 8 grL⁻¹ agar and three concentrations of BA (0.5, 1 and 1.5 mgL⁻¹). The pH of all media was adjusted to 5.7 before adding the gelling agent. Media were autoclaved for 15 min in 1.2 k Pa pressure at 121 °C. Cultures were placed at 25 ± 2 °C and 16/8 h light/dark day length conditions in a culture room. The cultures were grown for three 20-d sub-cultures, transferring the entire cultures into the fresh medium each time.

Also, two basal media (MS and ½ MS) were used for rooting of microshoots as indicated by Han *et al.* (2009). Seven-week-old shoots, 2-3 cm long, were cultured on half strength MS and MS salts with 30 grL⁻¹ sucrose and 0.7% (w/v) agar. pH was adjusted to 5.7. The media containing three concentrations of IBA (0.5, 1 and 1.5 mgL⁻¹) were placed in the dark at 25 ± 2 °C for 2 days, and then transferred to a 16 h photoperiod.

Experimental design and statistical analysis

Factorial experiment was arranged based on complete random design for both shoot proliferation and rooting steps, separately. For shoot proliferation, the experiment was carried out with three replications (three jars,

each jar containing 4 explants) including three cultivars, two basal media (MS and WPM) and three concentrations of BA (0.5, 1 and 1.5 mgL⁻¹) and for rooting, designed on three replications with three cultivars, two basal media (MS and ½ MS) and three levels of IBA (0.5, 1 and 1.5 mgL⁻¹). For shoot proliferation, data were recorded according to the number of formed lateral shoots after 50 days and after 40 days for rooting percentage of microshoots. All data were analyzed by SPSS and MSTAT-C software and comparison of means were carried out using Duncan's Multiple Range Test. Differences were regarded as significant at P < 0.05 and P < 0.01.

RESULTS

The effect of different BA concentrations and media on shoot proliferation

The Analysis of Variance showed that BA concentrations, the type of medium, rootstock, and their interactions were significant at 1 and 5 % probability levels. The exception was the interaction between BA and media (Table 1). The results showed that the media type and rootstocks were independent, 1.5 mg L⁻¹ BA showed the maximum shoot proliferation rate with an average of 5.45 shoots per explants. Also, 0.5 mg L⁻¹ BA produced the minimum shoot number with an average of 2.89 shoots per explants. Shoots were multiplied at 50 days intervals (Figure 6). Also, regardless of BA concentration and media, the results showed that Azayesh-Esfahan rootstock had the highest shoot proliferation with a mean of 5.11 shoot and Morabbaeei-Mashhad rootstock produced the lowest (2.97) shoot number per explant. However, the maximum shoot proliferation rate of all studied rootstocks was obtained in the MS compared to the WPM basal medium.

The interaction between rootstocks and BA concentration indicated that maximum shoot proliferation of rootstocks was observed in M9 and Azayesh-Esfahan rootstocks in 1.5 mgL⁻¹ BA and the minimum shoot proliferation rate was obtained in Morabbaeei-Mashhad in 0.5 mgL⁻¹ BA (Figure 1).

The interaction between rootstocks and media on shoot proliferation of rootstocks showed a significant difference in M9 only and the maximum shoot proliferation rate of M9 was observed in the MS medium. In Iranian rootstocks (Azayesh-Esfahan and Morabbaeei-Mashhad) a significant difference was not observed between MS and WPM media even though, the maximum and minimum shoot proliferation rates of roots-

stocks were obtained in Azayesh-Esfahan and Morabbaeei-Mashhad in WPM, respectively (Figure 2).

The interactions between rootstocks, media and BA concentration on shoot proliferation of rootstocks showed the maximum shoot proliferation rate in the MS medium containing 1.5 mgL⁻¹ BA in M9 and its minimum rate was observed in Morabbaeei-Mashhad in the WPM basal medium containing 0.5 mgL⁻¹ BA (Figure 3).

The effect of different IBA concentrations and media on the rooting of rootstocks

The analysis of variance results for rooting showed that IBA concentrations, The type of medium, rootstock, interaction between IBA and rootstocks was significant at 1% level, but the interaction between media and rootstocks was significant at 5% level (Table 2). The results showed that microshoot explants in the ½MS basal medium containing 1.5 mgL⁻¹ IBA had the maximum rate of rooting. However, Azayesh-Esfahan rootstock showed the highest rooting (Figure 7) rate. The interaction between media and IBA concentration indicated that there was not a significant difference between MS and ½ MS medium. However, the maximum rooting rate of rootstocks was observed in the ½ MS containing 1.5 mgL⁻¹ IBA and the minimum rooting rate was observed in the MS medium containing 0.5 mgL⁻¹ IBA. The interaction between rootstocks and IBA on rooting of rootstocks indicated that there was a significant difference at 1% probability level. Maximum rooting was observed in Azayesh-Esfahan in 1.5 mgL⁻¹ IBA and the minimum rooting was observed in Morabbaeei-Mashhad in 0.5 mgL⁻¹ IBA (Figure 4). level (Table 2). The results showed that microshoot explants in the ½MS basal medium containing 1.5 mgL⁻¹ IBA had the maximum rate of rooting. However, Azayesh-Esfahan rootstock showed the highest rooting (Figure 7) rate. The interaction between media and IBA concentration indicated that there was not a significant difference between MS and ½ MS medium. However, the maximum rooting rate of rootstocks was observed in the ½ MS containing 1.5 mgL⁻¹ IBA and the minimum rooting rate was observed in the MS medium containing 0.5 mgL⁻¹ IBA. The interaction between rootstocks and IBA on rooting of rootstocks indicated that there was a significant difference at 1% probability level. Maximum rooting was observed in Azayesh-Esfahan in 1.5 mgL⁻¹ IBA and the minimum rooting was observed in Morabbaeei-Mashhad in 0.5 mgL⁻¹ IBA (Figure 4).

Table 1. Analysis of variance for the effect of different BA, media and their interaction on shoot proliferation rate.

Source	df	MS
BA	2	30.129**
Media	1	8.640**
BA* Media	2	0.961 ns
Rootstocks	2	20.725**
Rootstocks * BA	4	4.978**
Rootstocks * media	2	9.929**
Rootstocks * media * BA	4	2.470 *
Error	34	0.711

**; Significant at 1% probability, *, significant at 5% probability, ns; non significant.

Table 2. Analysis of variance for the effect of different levels of IBA, media and their interaction on rooting of three apple rootstocks.

source	df	MS
IBA	2	**3859.722
Media	1	**1157.407
IBA* Media	2	92.130 ns
Rootstocks	2	**6693.056
Rootstocks * IBA	4	**311.111
Rootstocks * medium	2	*144.907
Rootstocks * medium * IBA	4	33.796 ns
Error	34	42.892

**; Significant at 1% probability, *, Significant at 5% probability, ns; non significant.

The interaction between rootstocks and media on rooting of rootstocks showed that there was a significant difference between Azayesh-Esfahan and Morabbaeei-Mashhad but in M9 a significant difference was not observed between MS and ½ MS. The maximum rooting was obtained in Azayesh-Esfahan in ½MS and the minimum rooting was obtained in Morabbaeei-Mashhad in ½ MS medium (Figure 5).

DISCUSSION

In the present study, it was shown that Azayesh-Esfahan as the Iranian native rootstock had a higher micropropagation potential among three rootstocks.

Different effects of genotype, media component, growth regulator concentrations and other factors on shoot multiplication of apple have been described by several authors (Dobránszki., 2010).

According to our results, many researchers have reported that the multiplication of newly developed shoots and rooting were also strongly influenced by the genotype and plant growth regulators (Baraldi *et al.*, 1991; Marin *et al.*, 1993; Dobránszki *et al.*, 2000a, b; Sharma *et al.*, 2000; Kaushal *et al.*, 2005).

Shoot proliferation of apple as well as many other woody plants is based on media containing cytokinins as the major PGR, in low concentrations also auxins and in some cases gibberellin. The effect of different

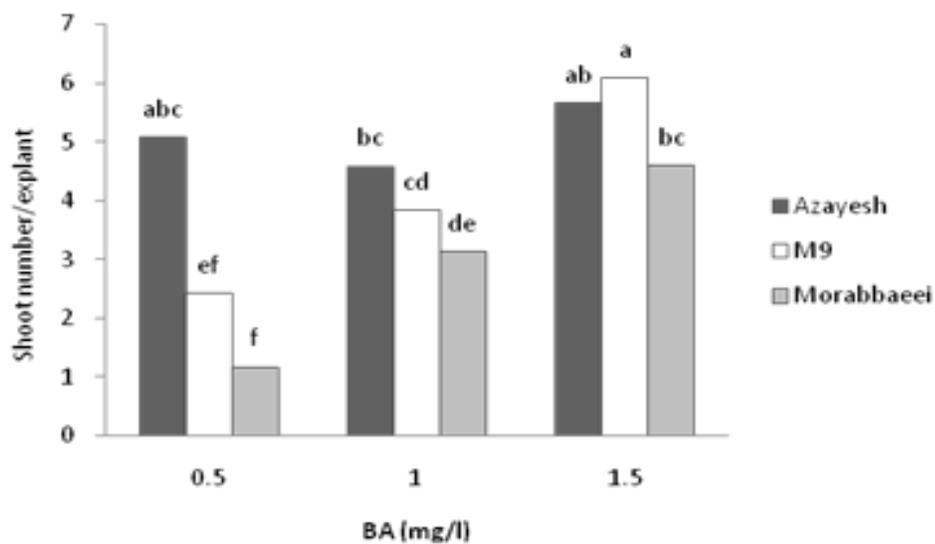


Figure 1. Interaction between rootstocks and BA on shoot proliferation on both media (MS and WPM).

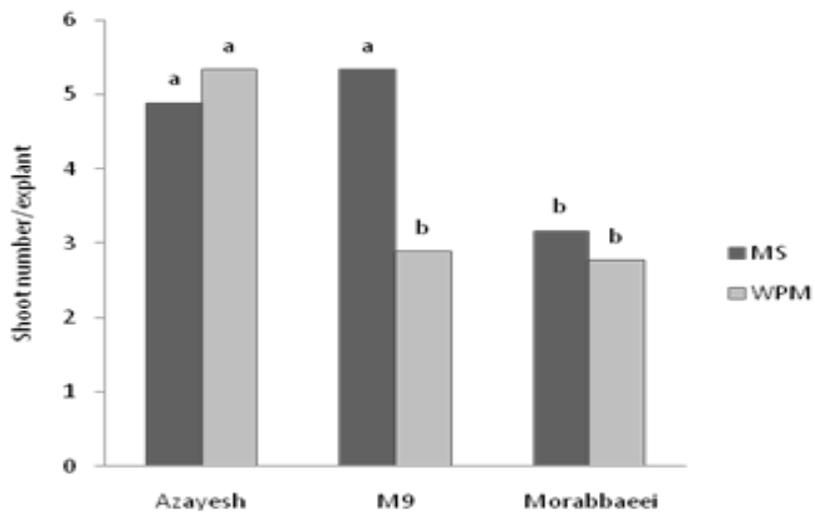


Figure 2. Interaction between rootstocks and media on shoot with different concentrations of BA.

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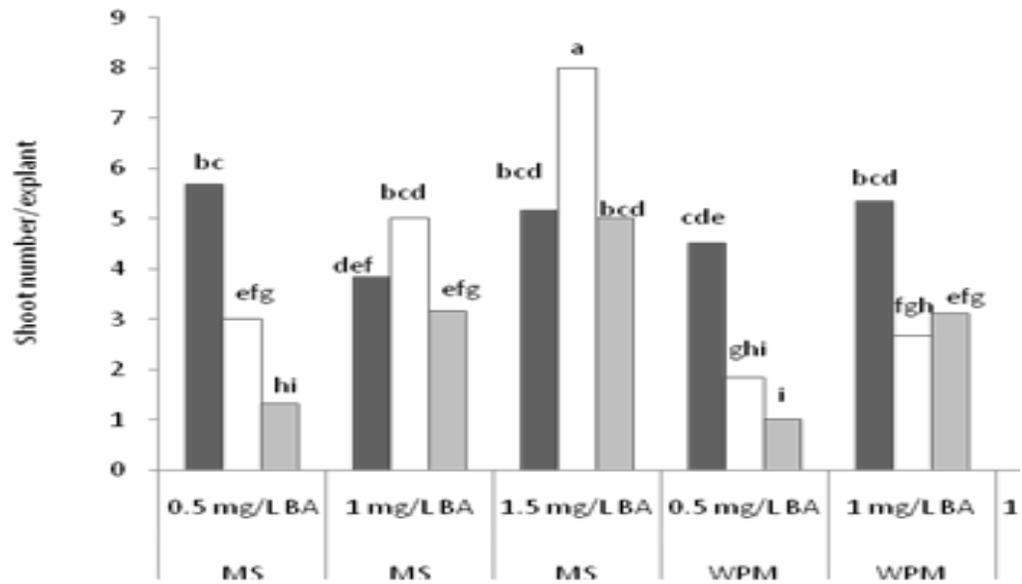


Figure 3. Interactions between rootstocks, media and BA concentration on shoot proliferation.

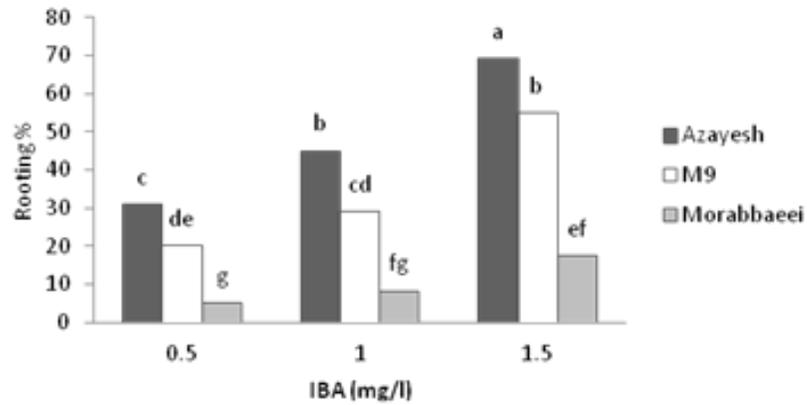


Figure 4. Interactions between rootstocks and IBA on rooting of three rootstocks on ½ MS and MS Media.

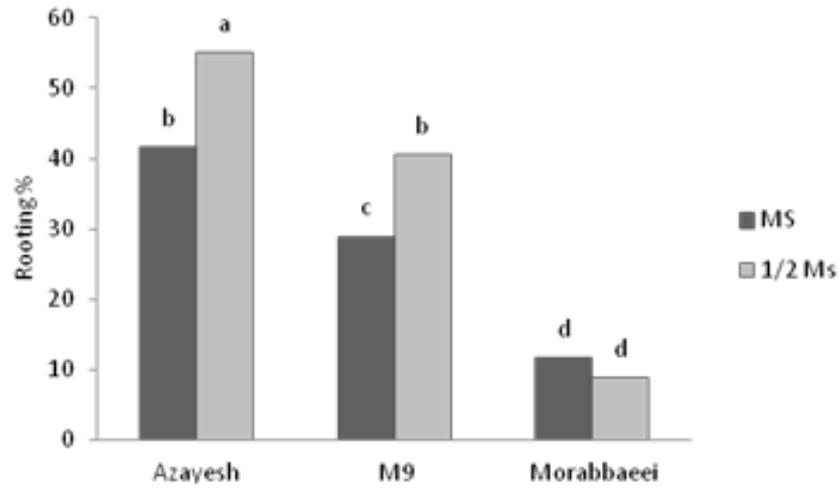


Figure 5. Interactions between rootstocks and media on rooting of three rootstocks with IBA.

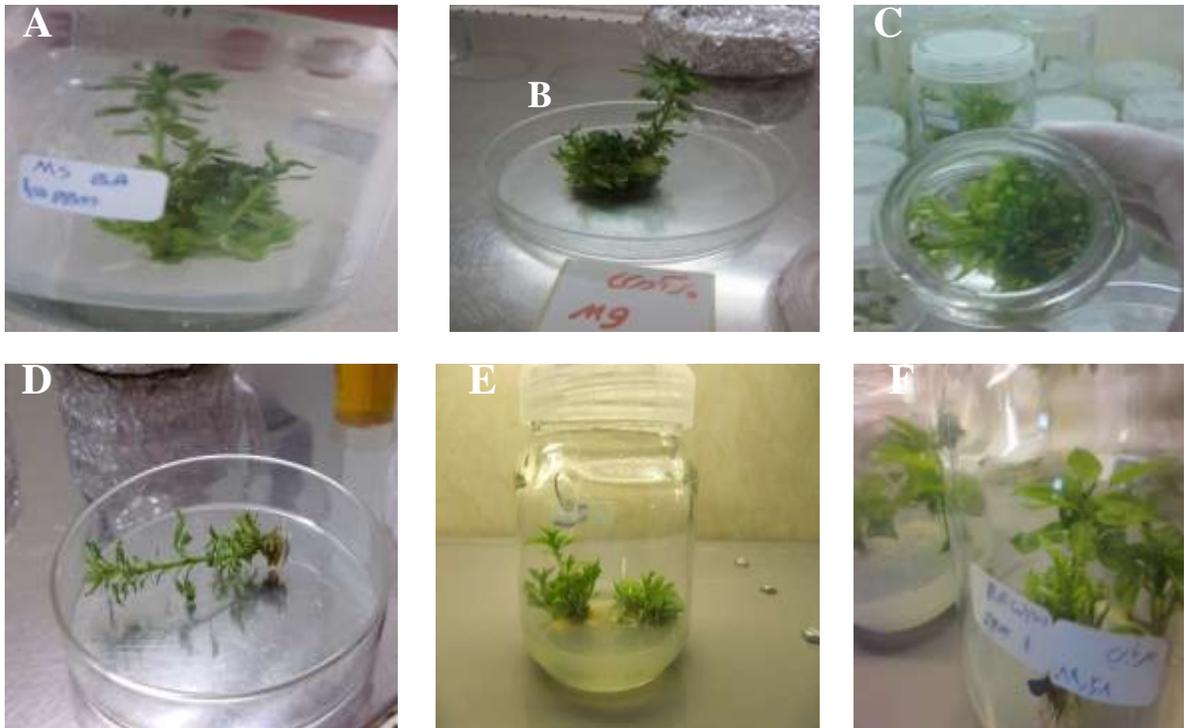


Figure 6. Shoot proliferation of three apple rootstocks, A and B): Shoot proliferation of M9 rootstock on the MS basal medium supplemented with 1.5 mg L⁻¹ BA C and D): Shoot proliferation of Azaysh-Esfahan on the MS basal medium supplemented with 1.5 mg L⁻¹ BA .E):Shoot proliferation of Morabbaeei-Mashhad on MS basal medium containing 1.5 mg L⁻¹ BA .F): Shoot proliferation of Morabbaeei-Mashhad on the WPM basal medium supplemented with 1 mg L⁻¹ BA

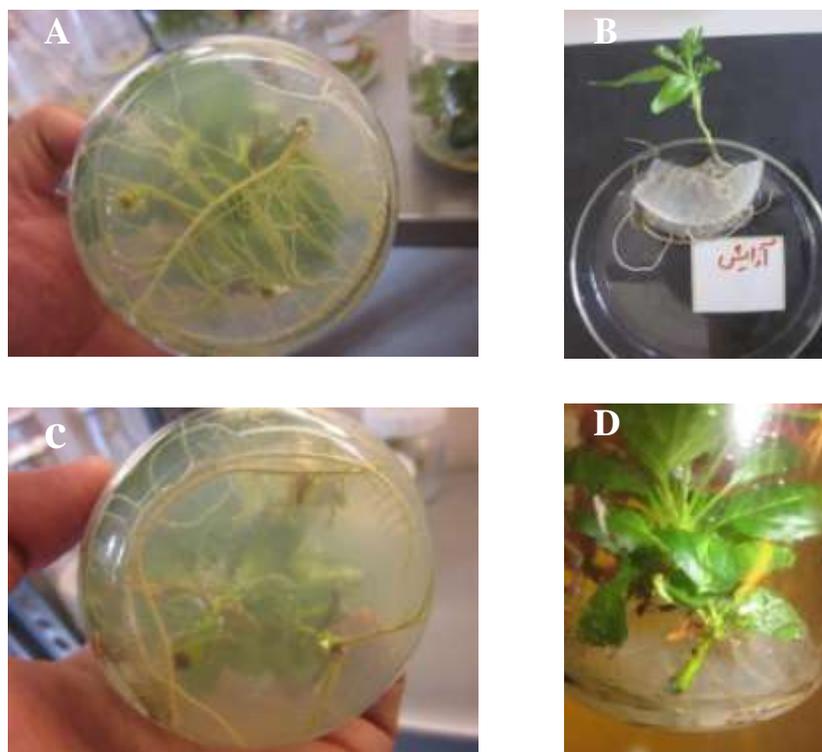


Figure 7. Rooting of two Iranian native apple rootstocks; A and B): Rooted microshoots of Azaysh-Esfahan rootstock on $\frac{1}{2}$ MS with 1.5 mg L^{-1} BA. C and D): Rooted microshoots of Morabbaeei-Mashhad rootstock on $\frac{1}{2}$ MS with 1.5 mg L^{-1} BA.

PGRs is highly genotype dependent (Dobrąnszki., 2010).

In most cases, in shoot multiplication of apple, BA was used as the cytokinin source mainly in a concentration ranging between 0.5 and 2 mg L^{-1} (Sharma *et al.*, 2000). Lane and McDougald (1982) investigated the response of apple shoot proliferation to different concentrations of BA (1 to $10 \text{ }\mu\text{M}$) in different rootstocks and cultivars including M27, M9, M26, MM111 and Macspur. They found that a low concentration of BA ($1 \text{ }\mu\text{M}$) resulted in fewer shoots and also BA concentration at $10 \text{ }\mu\text{M}$ decreased shoot proliferations in M26 and in Macspur. Optimal concentrations of BA for the production of maximum number of shoots differed with the genotype. They also compared the response of four mentioned cultivars to different concentrations of NAA and concluded that the best level of NAA for rooting differed between them. M27, M.26 and Macspur rooted best at $1 \text{ }\mu\text{M}$ while MM.111 rooted when $3.3 \text{ }\mu\text{M}$ NAA was used. They found that in the same NAA concen

tration, rootstocks rooted (84–85%) better than scion Macspur (58%). We also, obtained similar results among three apple rootstocks.

Kaushal *et al.* (2005) investigated *in vitro* clonal multiplication of an apple rootstock by culture of shoot apices and axillary buds and reported similar results to ours. In accordance with our findings, Kwon *et al.* (2004) studied the effects of growth regulators and culture conditions on *ex vitro* rooting and acclimatization of apple rootstock *in vitro* propagated and also, Ali Bacha *et al.* (2009b) reported similar results for *in vitro* propagation of the apple local cultivar Sukari. Naija *et al.* (2008) investigated anatomical and biochemical changes during adventitious rooting of apple rootstocks 'MM106' cultured in similar media with minor differences.

In another research, Dobrąnszki *et al.* (2000b) reported that shoot proliferation increased by adding two cytokinins, 1.0 mg L^{-1} BA + 1.0 mg L^{-1} Kin in Prima (8.1 shoots/explant) and in Galaxy (10.4 shoots/explant) or 0.5 mg L^{-1} BA + 1.5 mg L^{-1}

Kin in Galaxy (10.9 shoots/explant) compared to when a single cytokinin (1.0 mgL^{-1} BA) was used. However, the application of two cytokinins was not favorable for the shoot multiplication of Jonagold. When Kin was combined with BAR the multiplication rate of Jonagold decreased from 6.5 to 4.0 shoots/explants (Dobrąnszki *et al.*, 2000b). It could be pointed out that shoot proliferation depends on the initiation and activity of axillary meristems, which are controlled mainly by cytokinins; however, they act in interaction with auxins (Ward and Leyser., 2004).

The effect of some media such as MS, QL (Quirin and Lepoivre., 1977), WPM (Lloyd and McCown., 1981) and DKW (Driver and Kuniyuki., 1984) was investigated on the shoot proliferation in *M. sieboldii* and 10 *M. sieboldii* derived hybrid genotypes (Ciccoti *et al.*, 2008, 2009). They found a strong relationship between the kind of media salt composition and genotypes. In this research the best shoot proliferation rates were obtained using MS medium for two genotypes (*M. sieboldii* and C1907), QL medium for D2212 and H0801 genotypes and in DKW medium the best proliferation was achieved in genotype 4608.

In a study carried out by George *et al.*, (2008) the minimum shoot height and chloroses were observed on the WPM medium, because the medium probably has a low nitrogen content and the highest proliferation rate was observed on the MS medium. It is known that the ratio of cytokinin /auxin is very important in plant propagation and in tissue culture. When concentration of auxin is high and cytokinin is low or nil, adventitious roots or somatic embryos will form, when cytokinin concentration is high, adventitious shoots will develop, and when there are moderate to high levels of both, callus will form. The plant growth regulators are the most important signals for cell changing, or becoming determined. It is clearly known that different genotypes have often shown different responses when cultured on the same medium. So, studies on the medium optimization for different species or different cultivars are required. During a plant growth and development, different genes are expressed differentially in different cells. Therefore, this differential gene expression (epigenetic variation) can affect plant micropropagation (George *et al.*, 2008).

Furthermore, in apple micropropagation there are two main problems, explant browning and contamination

that are associated with field grown trees. In this research, we observed that the bacterial contamination (internal) and browning of explants were less and growth was faster if they were collected in spring as compared to the explants collected in winter or autumn. Similar observations were recorded earlier (Webster and Jones., 1991; Modgil M., 1999).

Conclusion

It was concluded that studied rootstocks showed different micropropagation reactions to different media based on their genotypes. Iranian rootstock Azayesh-Esfahan showed higher micropropagation potential among three rootstocks. However, all rootstocks showed maximum rooting and shoot proliferation in the media contained 1.5 mg L^{-1} IBA and 1.5 mgL^{-1} BA, respectively. Moreover, shoot proliferation in MS was higher than WPM. Also, the $\frac{1}{2}$ MS medium was the best medium for rooting.

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