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A Reliable Protocol for Adventitious Shoot Regeneration in GF677 and Rabi Cultivar, a Late Flowering Almond

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Abstract
In vitro leaf explants of GF677 hybrid rootstock and Rabi cultivar were placed on MQL medium supplemented with BAP at 0, 1, 2, 3 and 4 mg/L and NAA at 0, 0.15 and 0.3 mg/L concentrations. The results revealed that BAP, NAA, genotype and their interactions had significant effect on both percentage and mean number of the regenerated shoots. For GF677, the highest percentage of shoot regeneration (44 ± 1.74) and mean number of shoots (2.64 ± 0.10) were obtained using 3 mg/L BAP without NAA. However, there were no significant differences among treatment combinations of BAP and NAA with regard to the Rabi cultivar. Furthermore, for GF677 and Rabi genotypes, using NAA at 1 mg/L produced the highest rooting rate (60 and 75%, respectively) and number of roots per shoot (1.85 ± 0.47 and 2.4 ± 0.46, respectively) when the shoots were maintained in the dark on the rooting medium for 10 days before transferring to a 16/8 h photoperiod. In total, 90% of the plantlets survived during acclimatization phase in the greenhouse.

Key words: Direct shoot regeneration; GF677; Leaf explants; Rabi cultivar; Rooting and acclimatization

Abbreviations: BAP: 6-benzylaminopurine; NAA: α-naphthaleneacetic acid; GNH-medium: Garoosi, Nezami and Haddad medium. PGRs: Plant growth regulators; AC: Activated charcoal; CH: Casein hydrolysis; AVG: Aminoethoxyvinylglycine; MQL-medium: Modified Quoirin and Lepoivre-medium; RIM-medium: Root induction medium; REM-medium: Root elongation medium; SEM-medium: Shoot elongation medium

Introduction

Improvement of genetic transformation system for fruit crops would help increase availability and commercialization of the selected genotypes carrying the desired traits. To accelerate the breeding process and broaden germplasm sources, there is a great interest in genetic modification to produce trees with herbicide tolerance and disease and pest resistance as well as reproductive manipulation for commercial plantations (Ainsley *et al*. 2000; Ahuja 2000). GF677 hybrid rootstock is an economically important rootstock for almond and peach with the ability of adaption to poor soil fertility and drought conditions (Monticelli *et al*. 2000). In addition, Rabi cultivar is one of the Iranian native almond cultivars with high productivity and late flowering date (Sahragard 2007). There is an interest in increasing the density planting system of GF677 (Hasan *et al*. 2010**)**. However, the hurdle is that both genotypes suffer from some fungal diseases like verticillium (Sahragard 2007).

Shoot regeneration in *Prunus* genus has been reported from leaves in apricot (Escalettes and Dosba 1993), *P. serotina* and *P. avium* (Hammatt and Grant 1998), almond (Miguel *et al.* 1996), *P. lannesiana* (Matsuta *et al.* 1993) and from embryonic tissues and mature explants in peach

(*Prunus persica*) (Pooler and Scorza 1995; Gentile *et al.* 2002). However, there are a few reports on shoot regeneration in GF677. Apparently, the micro-propagation of GF677 using conventional culture media has encountered some problems such as vitrification and shoot necrosis in a long term culturing period. These may be the main limitations in the successful adventitious shoot regeneration of this genotype (Tsipouridis and Thomidis 2003; Hasan *et al.* 2010). The above mentioned issues could be solved by introducing a novel medium called GNH-medium. In addition, using this medium, newly grown leaves had a suitable response to shoot regeneration. Consequently, it would allow for introducing a desirable protocol for GF677 hybrid rootstock's shoot regeneration with high regeneration percentage, particularly for inducing adventitious shoots from leaves, *in vitro* rooting and acclimatization of plantlets. To our best knowledge, there are no reports on the regeneration of Rabi cultivar. Here, a successful system was reported for the regeneration of GF677 rootstock and Rabi genotype to facilitate genetic modification of the stone fruits.

Materials and Methods

Plant material

As explants, buds from GF677 and seeds from Rabi, an Iranian native late flowering almond cultivar, were used. Shoot cultures of GF677 hybrid rootstock (*Prunus amygdalus* × *P. persica*) were established *in vitro* from buds of 14-year-old trees obtained from Sahand Research Center, East Azarbaijan, Iran. Open-pollinated almond fruits from 'Rabi cultivar' were collected from orchard-grown trees at the Chaharmahal and Bakhtiari Agriculture and Natural Resources Research Center, Iran, 100-115 days after full blooming. Then, the halls and shells were removed.

GF677 buds and seeds of Rabi which were free of shells were rinsed by tap water for 2 h and then sterilized by 96% (v/v) ethanol for 3-4 sec, sodium hypochlorite (1% active chlorine) for 10 min. and ultimately by sterilized distilled water three times; then, they were placed on GNH medium (Table 1) (Nezami *et al.* 2010) supplemented with 3% sucrose, 0.7% plant agar (Duchefa, Netherlands). pH was adjusted to 5.7 before autoclaving (Carolina *et al.* 2006). The cultures were grown at 24 ± 2°C in a 16/8-h photoperiod at light intensity of 65 µmol/s/m^2 provided by white fluorescent tubes and sub-cultured into a fresh medium every three weeks.

Adventitious shoot induction from leaf explants

The expanded leaves with petioles (Figure 1) were excised using a stereo-microscope/dissecting microscope from 3-week-old *in vitro* grown shoots of GF677 and Rabi and, after being wounded by 3 cuts transversally to the midrib, they were firmly placed on the abaxial side up in the 100 × 15 mm Petri dishes containing 25 to 30 ml MQL medium (Table 1). The medium was supplemented with 0.0, 1, 2, 3, 4 mg/L BAP in combination with 0.0, 0.15, 0.3 mg/L NAA and solidified by 0.7% plant agar. For each genotype, five Petri dishes (replications) each containing 10 leaf pieces were used. According to Ainsley *et al.* (2000), the explants were maintained 21 days in the dark at 24 ± 2°C and then adventitious bud clusters or explants with the developed callus

were transferred to SEM-medium (Table 1) and maintained in the 16/8 photoperiod. The number of adventitious shoots per explant was recorded 40 days after beginning of the experiment. For micro-propagation, adventitious shoots were sub-cultured on GNH medium every three weeks. The shoots were maintained for the root induction experiments (Figure 1).

Rooting adventitious shoots from leaf explants

The elongated adventitious shoots (0.5 to 1 cm in length) were randomly placed on RIM-medium (Table 1) to root induction. The treatments with more adventitious shoots production were used for the rooting induction. The shoots were placed on

Table 1. Salts and hormonal contents of proliferation and regeneration media used in different experiments

Compounds	GNH**-medium (mg/L)	MQL***-medium (mg/L)	RIM-medium (mg/L)	S / REM-medium (mg/L)
Macronutrient				
NH_4NO_3	1650	800	825	1650
$Ca(NO_{3)2}.4H_2O$	800	832.84	400	800
KNO_3	25	1200	12.5	25
KH_2PO_4	300	270	150	300
$NaH_2PO_4.H_2O$	50	-	25	50
$MgSO_4.7H_2O$	540	360	270	540
Micronutrient	MS*	QL***	1/2MS	MS
$FeSO_4.7H_2O$	MS	-	MS	MS
Na_2-EDTA.$2H_2O$	MS	-	MS	MS
FeNaEDTA	-	36.7	-	-
Organics and Vitamins	MS	QL	MS	MS
BAP	0.5	-	-	-
IBA	0.1	-	-	-
Casein hydrolysate	-	100	-	-
Myo-inositol	100	100	100	-
Ascorbic acid	-	10	-	-
Citric acid	-	10	-	-
Silver nitrate	-	-	0.42	-
Cobalt chloride	-	-	0.95	-
Activated charcoal	-	-	25	-
Plant Agar	7000	7000	6000	6000 (REM-medium) / 7000 (SEM-medium)
pH	5.7	5.7	5.7	5.7

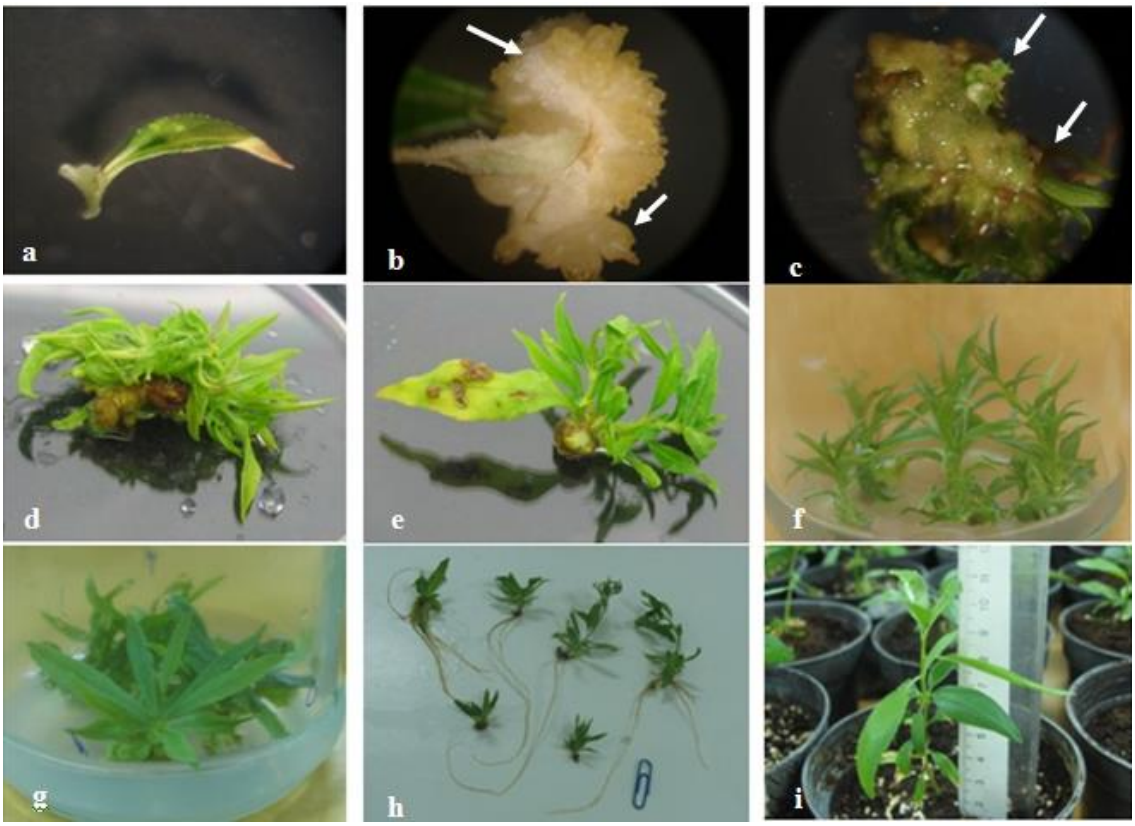

Figure 1. Adventitious shoot regeneration from *in vitro* cultured leaves, micro-propagation, *in vitro* rooting and acclimatization of GF677 and Rabi plantlets; a) *in vitro* of GF677 leaf; b) and c) adventitious bud primordial regenerated from callus (petiole as an explants); d) and e) development of the regenerated buds. Adventitious shoots on the elongation medium in the process of differentiation and elongation; f) and g) micro-propagation of the regenerated shoots in GF677 and Rabi, respectively; h) Rooting stage; i) acclimatization stage. Arrow indicates bud primordial.

the rooting induction medium with IBA and NAA at 0.0, 0.5, 1.0, 2.0, 3.0 mg/L and maintained in the dark at 24 ±2°C for 10 days, followed by transferring to the REM medium (Table 1) with the light intensity of 65 µmol/s/m^2, provided by white-cool florescent tubes in the same conditions reported for the multiplication phase. Each treatment was replicated four times with five shootlets per replication. The percentage, mean number and length of the roots per shootlet were recorded 30 days after beginning of the experiment.

Acclimatization of rooted plants

The rooted plantlets were transplanted into 9 × 10 cm plastic pots containing peat moss: perlit mixture (1:3 v/v) and were capped with a transparent polyethylene glass (for one week) and placed in a growth chamber with 96% humidity (humidity was gradually decreased to 60% during acclimatization) under a 16/8 h photoperiod (60 µmol/m^2s) and 25 ± 2°C. The plantlets were watered weekly to maintain soil moisture and acclimatized gradually to the room temperature over a period of 4 to 5 weeks. Subsequently, the

plants were transferred to the greenhouse.

Data analysis

The number of shoots and roots were presented as means (± standard error of the mean) of the shoot and regenerated root per explants. Regeneration and rooting rates were expressed as the average percentage (± standard error of the mean (SEM)) of both leaves, differentiated into shoots and roots over the total leaf and shoot number. Since the data did not follow a normal distribution, the number of regenerated shoots was normalized by the reciprocal transformation and shoot and root regeneration percentage was analyzed after arcsine transformation. For the shoot induction, the experiment was conducted as the split plot design with five replications. Genotypes were arranged in the main plots by using completely randomized design and the combinations of NAA and BAP were regarded as sub-plots. However, for the root induction, the experiment was arranged as factorial for the same factors on the basis of completely randomized design with four replications. Analysis of variance was carried out using GLM procedure of SAS Software (version 9.1) and the means were compared by the Duncan's Multiple Range Test.

Results

Adventitious shoot induction from leaf explants

White callus with subsequent regeneration of clusters of shoot bud primordia (yellowish appearance) began to form on the petioles (Figure 1) and wounded midribs of the leaf explants as early as 4-10 days after the explants were placed on the media supplemented with BAP and NAA. Clusters of shoot bud primordial are usually regenerated from the petioles of the leaf explants. After 3 weeks, the calli on the explants which were maintained in the dark had yellowish appearance and the shoot bud primordia were etiolated. At the same time, the explants with callus or with shoot bud primordia were transferred to the REM, the callus turned brown, shoot bud primordial turned green and the shoots started to differentiate and elongate (Figure 1). No adventitious shoot regeneration was observed when leaf explants from GF677 and Rabi were placed on the hormone free medium. The adventitious shoots were phenotypically identical to the original shoot explants.

Table 2. Analysis of variance for the mean number of adventitious shoots, and percentage of regeneration for GF677 and Rabi.

Source of Variation	df	Shoots (N0.)		Regeneration (%)	
		Mean squares	F	Mean squares	F
Genotype	1	0.2266	13.68**	3.2698	16.28**
Error (a)	8	0.0165		0.2008	
NAA	2	0.1321	13.61**	1.3128	16.77**
BAP	4	0.4087	42.09**	8.5374	109.03**
BAP × NAA	8	0.0667	6.88**	0.6625	8.46**
Genotype × BAP	4	0.0650	6.70**	0.9682	12.37**
Genotype × NAA	2	0.0872	8.98**	0.6754	8.63**
Genotype × NAA × BAP	8	0.0388	4.00**	0.3145	4.02**
Error (b)	112	0.0097	0.64	0.0784	0.24
Leaf error (b´)	1350	0.0152		0.3224	
Total	1449				

*, ** Significant at P<0.05 and 0.01 respectively; ns: non-significant

Table 3. *In vitro* adventitious shoot regeneration for GF677 and Rabi

PGRs		GF677 Hybrid rootstock		Rabi cultivar	
NAA (mg/L)	BAP (mg/L)	Shoot Regeneration (%)± SEM	Shoot N0. ± SEM	Shoot Regeneration (%)± SEM	Shoot N0. ± SEM
0	0	0.0^{d}	0.0^{b}	0.0	0.0
	1	6.0±0.63^{c}	1.0±0.06^{ab}	2.0±0.4	1.2±0.24
	2	10±0.488^{bc}	1.2±0.09^{ab}	4.0±0.48	2.2±0.48
	3	44±1.74^{a}	2.64±0.10^{a}	4.0±0.48	1.8±0.22
	4	22±1.16^{b}	2.53±0.08^{a}	0.0	0.0
0.15	0	0.0^{b}	0.0^{b}	0.0	0.0
	1	2.0±0.4^{ab}	0.2±0.04^{ab}	6.0±0.8	0.7±0.1
	2	8.0±0.74^{a}	0.6±0.05^{a}	0.0	0.0
	3	4.0±0.48^{ab}	0.4±0.05^{ab}	0.0	0.0
	4	4.0±0.48^{ab}	0.4±0.05^{ab}	2.0	0.4±0.08
0.3	0	0.0±0.0^{c}	0.0±0.0^{b}	0.0	0.0
	1	4.0±0.48^{bc}	0.4±0.04^{b}	0.0	0.0
	2	8.0±0.52^{ab}	2.2±0.16^{a}	0.0	0.0
	3	12±0.4^{a}	2.1±0.06^{a}	0.0	0.0
	4	4.0±0.48^{bc}	0.8±0.01^{ab}	2.0±0.4	0.4±0.08

*Values with the same letters in the same column are not significantly different at α=0.05 using Duncan's Multiple Range Test, respectively.

Analysis of variance indicated that the effect of main factors including genotype, NAA and BAP and all their interactions with each other were significant (P<0.01) on the number of regenerated shoots and shoot regeneration percentage (Table 2). Comparing the means of treatment combinations revealed that the greatest percentage of regeneration (44 ± 1.74) as well as the mean number of shoot regeneration (2.64 ± 0.10) for GF677 was obtained when the cultures were maintained on 3.0 mg/L BAP without NAA compared to the other levels of NAA. Despite this, there were no significant differences between various PGRs concentrations on the studied characters for the Rabi genotype, with adventitious shoot regeneration percentage of less than 10%, suggesting Rabi as a recalcitrant almond cultivar (Table 3).

Rooting of adventitious shoots

The roots were induced as early as two weeks after culturing on the rooting induction medium and most of the roots were regenerated after three weeks. Analysis of variance showed variable significant effects of main factors and their interactions on the studied characters (Table 4). Hence, for each genotype, the means of different combinations of IBA and NAA were compared, separately, to avoid confusing interactions of studied factors. For instance, IBA had no significant effect on the rooting percentage for GF677; however, using up to 2 mg/L IBA increased the rooting percentage and mean number of roots per shoot with a suppressive effect at a higher concentration (3 mg/L). Whilst, for the other cultivar (Rabi), to obtain the highest rooting percentage, increasing IBA concentration

to more than 2 mg/L was necessary and it caused a dramatic increase (by almost 65%) on rooting percentage (Tables 4 and 5). Interestingly for both genotypes, the highest rooting percentage and mean number of roots per shoot (60%, 1.85 ± 0.47 and 75.0%, 2.4 ± 0.46, respectively) were observed when adventitious shoots were cultured in the root induction medium supplemented with 1 mg/L NAA (Table 5 and Figure 1).

Table 4. Analysis of variance for rooting in GF677 and Rabi

S. O. V.	d. f.	Mean Squares		
		Rooting Percentage	Root Number	Rooting Length
Genotype	1	$0.2304^{n.s.}$	$0.1121^{n.s.}$	131.73**
PGRs	1	2.3592^{**}	0.8066^{**}	23.8778n.s.
Genotype × PGRs	1	$0.3317^{n.s.}$	$0.0375^{n.s.}$	87.0582**
Concentration	4	2.8995^{**}	0.6927^{**}	101.3016**
Genotype × Concentration	4	$0.3513^{n.s.}$	0.1250^{*}	44.5714**
PGRs × Concentration	4	0.9826^{**}	0.3254^{**}	28.5872**
Genotype ×PGRs × Concentration	4	$0.0840^{n.s.}$	$0.0223^{n.s.}$	24.0541*
Error	380	0.1629	0.0401	7.8329
Total	399			

*Genotype: GF677 and Rabi; PGRs: IBA and BAP; Concentrations: 0.0, 0.5, 1.0, 2.0, 3.0 mg/l

Table 5. *In vitro* rooting response for GF677 and Rabi adventitious shoots

		GF677 Hybrid rootstock			Rabi cultivar		
Plant growth regulators	Concentration (mg/L)	Rooting (%)	Roots/shoot ± SEM	Root length (cm) ± SEM	Rooting (%)	Roots / shoot ± SEM	Root length (cm) ± SEM
	0	0.0	0.0	0.0	0.0	0.0	0.0
IBA	0.5	35	0.35 ± 0.11^{b}*	2.62 ± 0.82	25b	0.45 ± 0.22^{b}	1.32 ± 0.59^{b}
	1	20	0.25 ± 0.12^{b}	1.6 ± 0.82	15^{b}	0.4 ± 0.30^{b}	1.12 ± 0.64^{b}
	2	40	1.05 ± 0.36^{a}	2.06 ± 0.73	20^{b}	0.4 ± 0.25^{b}	0.79 ± 0.41^{b}
	3	35	0.65 ± 0.23^{ab}	1.35 ± 0.54	65^{a}	1.75 ± 0.36^{a}	5.47 ± 0.96^{a}
NAA	0.5	25	0.30 ± 0.12c	0.62 ± 0.29c	40b	0.85 ± 0.28b	2.96 ± 0.87
	1	60	1.85 ± 0.47a	2.17 ± 0.50a	75a	2.4 ± 0.46a	3.83 ± 0.65
	2	60	1.80 ± 0.43ab	1.77 ± 0.47ab	60ab	1.60 ± 0.46ab	4.77 ± 0.98
	3	35	0.80 ± 0.31bc	0.85 ± 0.29bc	60ab	1.6 ± 0.49ab	4.24 ± 0.97

*Values with the same letters in the same column are not significantly different at α=0.05 using Duncan's Multiple Range Test, respectively.

Deflasking and acclimatization

In vitro plantlets grew actively during acclimatization process and no stress symptoms were observed after transferring to the greenhouse. After 2 months, 90% of the potted plantlets survived and plantlet sizes ranged from 15 to 25 cm in height (Figure 1).

Discussion

The results indicated that four factors were critical for shoot induction from somatic tissues: (1) genotype, (2) type of explant tissues (petiole), (3) medium composition and (4) growth conditions. Under identical conditions, regeneration efficiency was considerably affected by genotype,

in which Rabi cultivar was apparently more recalcitrant to regeneration than GF677 (Figure 2). This difference may be originated from the genotypes because Rabi is an almond cultivar but GF677 is an almond × peach hybrid rootstock. Ainsley *et al.* (2000) reported direct shoot regeneration of 16.6% and 19.4% in two almond cultivars of Ne Plus Ultra and Nonpariel, respectively. The type of explants was the next effective factor for obtaining successful morphogenesis in both genotypes. In the primary experiments, the effect of different parts of leaf (petiole, leaf midrib and lamina) on shoot regeneration was surveyed (the results are not shown). However, regeneration was only observed from petioles. Despite the production of mass calli in different parts of leaf, there were only a few competent cells for shoot regeneration at petiole area and, in other parts, the ability of morphogenesis seemed to be weak. Gentile *et al.* (2002) could obtain shoot regeneration from only preconditioned apices of some peach genotypes. In addition, according to Rugini and Muganu (1998), one of the important factors for such a limitation might be related to the presence of a few competent cells in the mass produced callus from fully differentiated tissues for only shoot regeneration with the majority of cells being non-regenerative. In this context, some exegenic factors such as BA might still induce morphogenesis (Welander 1988; Yepes and Aldwinckle 1994). Moreover, importance of the presence of petioles (as another important factor) for obtaining regeneration from *Prunus spp.* leaves has been previously reported by other authors (Antonelli and Druart 1990; Escalettes and Dosba 1993; Miguel *et al*. 1996; Gentile *et al*. 2002). For GF677, the percentage of shoot regeneration (54%) was considerable. A part of this result may be relevant to the formulated medium for GF677 micro-propagation [(Table 1) (Nezami *et al.* 2010]. The produced leaves from this medium were wide, healthy and dark green. However, there was an attempt in this work to prove this by designing more related experiments. According to Escalettes and Dosba (1993), the macronutrient of QL medium (Quoirin and Lepoivre 1977) enabled regeneration from different genotypes of apricot whereas regeneration was not obtained with full- or half-strength MS (Murashige and Skoog 1962). In contrast to the results obtained with other *Prunus spp*. (Escalettes and Dosba 1993; Miguel *et al*. 1996; Gentile 2002), nitrogen sources of QL (Table 1) were modified in the primary experiments, which led to the significant difference in the increase of regeneration efficiency compared with the QL medium (the results are not shown). The results also approved that the presence of BAP was very effective for obtaining succession in direct shoot regeneration. This was supported by the results reported by Gentile *et al*. (2002), Hammerschlag *et al*. (1985) and Tang *et al*. (2002). In spite of all this, NAA had no positive effect on direct shoot regeneration of both genotypes (Figure 4), which agreed with the results reported by Perez-Tornero *et al*. (2000) on *P. armenica*, and Espinisa *et al*. (2006) on *P. Serotina*. However, this result was in contrast with the results reported by Bhagwat and Lane (2004) and Hasan *et al.* (2010) on direct shoot regeneration of GF677 using leaf segment as

explants in which their treatments did not apply free auxin medium. In this study, interaction of the studied factors played decisive role on the characters under study, so that, an increase in NAA level changed the required BAP for shoot regeneration, which is in coincidence with previous reports by others on *Prunus* genus (Perez-Tornero *et al.* 2000; Hasan *et al.* 2010). Over all, the regeneration efficiency increased with the increase in BAP concentrations up to 3 mg/L; but, then it dropped when BAP increased to 4 mg/L.

The current study indicated that the presence of either IBA or NAA was necessary for root induction, in which NAA was more effective (Table 5); this was in agreement with the results reported by Perez-Tornero *et al.* (2000). Moreover, the previously obtained results indicated that using some ethylene inhibitors such as silver nitrate and cobalt chloride significantly increased the rooting rate of GF677 (Nezami *et al*. 2010). Therefore, in this study, both inhibitors were used at a constant concentration. The leaves of plantlets in such conditions showed altered anatomy such as poor cuticle development and low amount of epicuticular waxes (Preece 2010). Reasonable success (90%) was obtained during acclimatization of the plantlets to the *ex vitro* environment using high humidity and then by gradual lowering of relative humidity. Also, another important problem of this phase was fungal contamination. Murai *et al.* (1997) could obtain only 20% acclimatized apricot shoots and their important problem was fungal infection whereas, in the current study, this problem was solved by removing the gelling agent covering around the roots and taking particular environmental care. It can be concluded that: a) GNH and MQL were suitable media for shoot proliferation and direct regeneration, respectively, in both plant sources, b) BAP solely induced adventitious shoot regeneration up to 54%, c) in comparison with IBA, NAA significantly increased rooting rate and d) 90% success was obtained during acclimatization process. In at all, in this research it was developed a successfull and repeatable protocol for proliferating direct shoot regeneration for GF677 hybrid rootstock and also Rabi, a new Iranian late flowering cultivar on new GNH medium, along with presentation of primary basic conditions for further genetic engineering studies in these rootstocks.

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