The effect of PGRs on *in vitro* shoot multiplication of GF677 hybrid (*Prunus persica x P. amygdalus*) rootstock on GNH medium

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Abstract

GF677 rootstock (peach ×almond) is an important rootstock for peach. It was produced by Bernhard in French at Grand Farad Research Centre in 1965. The present study focused on the effect of some PGRs on propagation and root induction on GNH medium as part of providing an applicable protocol for micro-propagation of mentioned rootstock. Results indicated that BAP at concentration of 0.5 mg/l plus 0.1 mg/l IBA produced the highest normal auxiliary shoots. Media supplemented with ZR produced only single shoots. The greatest rate of rooting (up to 40%) was obtained from induction medium containing 2 mg/l of IBA.

Keywords: GF677, GNH, BAP, ZR, propagation, rooting.

Introduction

GF677 rootstock propagation is strongly demanded to provide almond orchards in Iran. It is a good rootstock for peach orchards in moderate or poor fertility soils. Vegetative propagation of peach can be achieved by hardwoods, semi-hardwoods and soft wood cuttings as well as by suckers, root cuttings and also tissue culture approaches (Loreti *et al.*, 1985). However, its vegetative propagation is quite difficult (Styliaides *et al.*, 1988). *In vitro* culture of GF677 is usually based on using MS, WPM, TK and other media which result in low rates of shoot proliferation and/or some problems such as vitrification (during tissue culture) and also low rooting rates. Such situations lead to a high cost of *in vitro* propagation of this rootstock. Our previous efforts on the modification of MS medium led to introduce a novel medium termed as GNH (Garoosi, Nezami and Haddad), which significantly solved the propagation problems (unpublished results).

It is well known that cytokinins promote cell division and cell expansion in plant tissue culture. BAP as a synthetic cytokinin in combination with appropriate auxins has been used in micropropagation of nut fruits (Ruzic and Vujovic, 2008), PR 204/84 hybrid (*Prunus persica* × *P. amygdalus*) (Fotopoulos and Sotiropoulos, 2005) and cherry (Durkovic, 2006) under *in vitro* conditions with good results. The effect of alone or in combination with synthetic and natural cytokinins such as BAP and auxins on quantity and quality vegetative growth of *Prunus* genera, specially GF677, is not well known. However Ansar *et al.* (2009) have reported noticeable results using a combination of BAP and zeatin (Z) in olive propagation. They could obtain a high regeneration rate, by using only low concentrations of BAP in the presence of high amounts of zeatin.

It is well known that ethylene acts as one of the important inhibitors of rooting in many species e.g. rice (Chen *et al.*, 1992),

pea cuttings (Nordstorm and Elisson 1993), tomato leaf discs (Coleman *et al*., 1980), *prunus avium* shoot cultures (Biondi *et al.,* 1990) and apple (Ma *et al* 1998). AgNO3 (as an inhibitor of ethylene activity) and $CoCl_2$ (as an ethylene biosynthesis inhibitor) (Yang 1985), play the main role in improving rooting by decreasing the ethylene contents in woody plants (Ma *et al.,* 1998). Concerning the role of exogenous auxin on rooting, there are many reports which indicate its necessity for apple root induction (Zimmerman and Fordham, 1985), almond (Caboni, *et al.,* 1997), PR 204/84 (Fotopoulos and Sotiropoulos, 2005) and also its interaction with ethylene in Pea rooting (Nordstrom and Eliasson, 1993).

In this study the effect of two kinds of cytokinins in GNH basal salt medium and various levels of IBA on shoot multiplication and root induction was investigated.

Materials and Method

Meristem-tip and lateral breaking buds were used as explants. Young shoots were collected from 14 year old rootstock GF677 trees in Jun /Few. The sectioned shoots with 1-2 buds were rinsed in tap water for 2 hours, then sterilized by 96% ethanol for 3-4 sec; 10% sodium hypochloride (v/v) (including 5.25% (m/v) NaOCl) for 10 min., and ultimately rinsed 3 times with sterilized distilled water. Then the explants were transferred to TK (Tabachnik and Kester 1977) medium supplemented with 1 mg/l BAP. Shootlets on TK were cut and multiplied on MS medium supplemented with 0.5 percent pectin and 1 mg/l BAP for further experiments on a novel medium (GNH, Table1).

Effect of BAP, ZR and IBA on shoot multiplication

Following proliferation of sufficient number of shootlets, they were placed in the GNH medium containing BAP and ZR at the concentration of 0.0, 0.5, 0.75, 1 and 2 mg/l, in combination with IBA at 0.0, 0.01 and 0.1 mg/l concentrations (Tables 2 and 3). In another study, the effect of BAP and ZR at 0.0, 0.25, 0.5, 0.75 and 1 mg/l in GNH medium were investigated (Table 4). Each treatment was considered with seven replications and data, including shoot multiplications and size of multiple healthy shoots were recorded 25 days later.

Effect of different concentrations of auxin on rooting

Rooting was induced for 10 d with IBA at 0.0, 0.5, 1, 2 and 3 mg/l concentrations (Table 5) on the half strength GNH medium, except iron and vitamins, including: active charcoal (25 mg/l), phloroglucinol (150 mg/l), 0.42 mg/l $AgNO_3$, 0.95 mg/l $CoCl_2.6H_2O$, 20 g/l sucrose and 0.6% plant agar. Cultures were kept in dark. Each treatment was replicated four times with five shootlets per replication. After induction, shoots were transferred to a fresh medium without growth regulators. After three weeks, rooting efficiency, the number of roots per shootlet and root length were recorded. All media contained plant agar (7 g/l) and 20 g/l of sucrose. Before adding agar, medium pH was adjusted to 5.7, autoclaved and maintained in 25 ± 2ºC under cool white florescent tubular lamps (65 µmol/ sm^2) with a 16/8 h photoperiod. Proliferated shoots were subcultured in fresh medium every three weeks.

Acclimatization of rooted plantlets from nodal explants

After removing the remaining agar from the rooted plantlets, they were placed into 10 × 9-cm plastic pots containing pitmoss/perlite mixture (1:3), covered with a transparent poly ethylene cap (for 1 week), and placed in a growth chamber in 96% humidity under a 16/8h photoperiod (60 µmol/m^2s^1) and 25 ± 2 °C condition.

Table 1. GNH macronutrients, micronutrients and vitamins

Compound	Concentration (mg/l)
Macronutrients	
NH_4NO_3	1650
$Ca(NO_3)_2.4H_2O$	800
KNO_3	25
KH_2PO_4	300
$NaH_2PO_4.H_2O$	50
$MgSO_4.7H_2O$	540
Micronutrients	
H_3BO_3	6.2
$CuSO_4.5H_2O$	0.025
$MnSO_4.H_2O$	22.3
$Na_2\ MoO_4.H_2O$	0. 25
$ZnSO_4.7H_2O$	8.6
$FeSO_4.7H_2O$	27.8
Na_2-EDTA.$2H_2O$	37.3
$CoCl_2$.6H2O	0.025
Organics and Vitamins	
Glycine	2
Myo-inositol	100
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Thiamine-HCl	0.1

Humidity was gradually decreased down to 60% during acclimatization. Plantlets were watered weekly. Acclimatization was carried out over a period of 4 to 5 weeks to adapt the plantlets to room temperature and humidity conditions before transferring to the greenhouse.

Statistical analysis

ANOVA method was used to determine the effect of growth regulators on shoot proliferation and rooting of explants. A Duncan's new multiple range test using SPSS 16.0 software was used to discriminate differences between treatments.

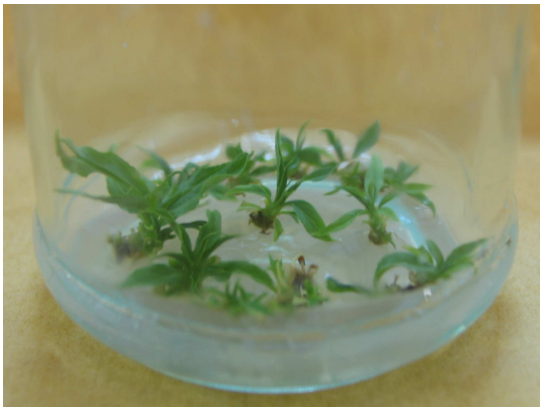

Fig. 1. Proliferation medium, GNH supplemented with 0.5 mg/l BAP plus 0.1mg/l IBA.

Table 2. Effect of various concentrations of IBA and BAP on total and healthy numbers of shoots.

Concentration (mg/l)		Number of healthy shoots per explant within specified length (cm)[a]			
IBA	**BAP**	**Total shoots produced per explants**	**1>**	**1<x<2**	**2<**
0	0	$1 \pm 0^{c*}$	1.0 ± 0.0^{b}	-	-
0	0.5	2.16 ± 0.3^{bc}	1.16 ± 0.6^{b}	-	-
0	0.75	2.6 ± 0.67^{abc}	2.0 ± 0.94^{ab}	-	-
0	1	3.8 ± 0.73^{ab}	3.2 ± 0.91^{ab}	-	-
0	2	3.75 ± 1.1^{ab}	2.0 ± 1.14^{ab}	-	-
0.01	0.5	2.16 ± 0.3^{bc}	2.16 ± 0.3^{ab}		-
0.01	0.75	3.0 ± 0.85^{abc}	1.33 ± 0.81^{ab}	0.8 ± 0.4^{a}	-
0.01	1	4.6 ± 0.57^{ab}	2.57 ± 0.75^{ab}	-	-
0.01	2	2.83 ± 0.47^{abc}	2.83 ± 0.47^{ab}	-	-
0.1	0.5	3.5 ± 0.22^{ab}	3.5 ± 0.22^{a}	-	-
0.1	0.75	3.3 ± 0.42^{abc}	2.67 ± 0.55^{ab}	-	-
0.1	1	4.8 ± 1.1^{a}	3.14 ± 1.28^{ab}	-	-
0.1	2	3.6 ± 1.4^{ab}	3.2 ± 1.58^{ab}	-	-

*Means ± standard error. The same letters indicate no significance at α=0.05.

Results

Effect of BAP and IBA on shoot multiplication

The highest shooting was obtained with 1mg/l BAP and 0.1 mg/l IBA, whereas the combination of 0.1 mg/l IBA and 0.5 mg/l BAP resulted in significantly greater numbers of length healthy shoots with 1 cm in length, compared to the other treatments. No shootlets were observed with 2 cm in length. Therefore, the combination of 0.5 mg/l BAP and 0.1 mg/l IBA was applied to further experiments (Fig. 1 and Table 2).

Table 3. Effect of various concentrations of IBA and ZR on total and healthy number shoots at different size for rootstock GF677**.**

Concentration (mg/l)		Number of healthy shoots per explant within specified length (cm)			
IBA	**ZR**	**Total shoots produced per**	**1>**	**1<x<2**	**2<**
0	0	$1.0 \pm 0.0^{c*}$	1.0 ± 0.0^{a}	-	-
0	0.5	1.5 ± 0.22^{bc}	1.4 ± 0.24^{a}	-	-
0	0.75	1.0 ± 0.0^{c}	1.0 ± 0.46^{a}	-	-
0	1	2.2 ± 0.58^{abc}	2.0 ± 0.44^{a}	-	-
0	2	2.1 ± 0.46^{abc}	0.85 ± 0.46^{a}	-	0.66 ± 0.37^{a}
0.01	0.5	2.66 ± 0.49^{ab}	2.0 ± 0.54^{a}	0.4 ± 0.24^{a}	-
0.01	0.75	1.66 ± 0.33^{bc}	1.0 ± 0.31^{a}	0.67 ± 0.31^{a}	0.5 ± 0.45^{a}
0.01	1	1.33 ± 0.33^{c}	1.17 ± 0.16^{a}	-	1.0 ± 0.64^{a}
0.01	2	3.16 ± 0.6^{a}	1.33 ± 0.84^{a}	-	-
0.1	0.5	1.5 ± 0.34^{bc}	1.25 ± 0.25^{a}	-	1.0 ± 0.64^{a}
0.1	0.75	1.5 ± 0.5^{bc}	1.2 ± 0.73^{a}	0.25 ± 0.27^{a}	-
0.1	1	1.2 ± 0.18^{c}	0.71 ± 0.28^{a}	0.33 ± 0.3^{a}	0.5 ± 0.45^{a}
0.1	2	3.0 ± 0.57^{a}	1.0 ± 0.36^{a}	-	-

*Means ± standard error. The same letters indicate no significance at α=0.05.

Effect of ZR and IBA on shoot multiplication

In the medium containing IBA and ZR, the highest shoot multiplication was observed in the combination of 2 mg/l ZR and either 0.01 or 0.1 mg/l IBA. There were no significant differences between treatments in producing healthy shootlets. In some treatments the length of obtained shootlets reached 2 cm. However, a low rate of shootlet multiplication with thin shootlets having very large and wide leaves were obtained in media containing ZR (Table 3).

Effect of ZR and BAP on shoot multiplication

The combination of ZR and BAP indicated that the most significant total shoot multiplication was obtained with the combination of 0.75 mg/l ZR and 0.75 mg/l BAP. However, the concentration of 0.75 mg/l BAP and 0.75 mg/l ZR was selected to get greater numbers of healthy shoots up to 1 cm in length. Also, results approved strongly that presence of both ZR and BAP is necessary to obtain growth in length; so that, the highest number of long shootlets was observed in the medium supplemented with 0.75 mg/l BAP plus 0.5 mg/l ZR (Table 4).

Effect of different concentrations of auxin on rooting

The highest rate of rooting (up to 40%) and the number of roots per shootlet (2.62 ± 0.56) was obtained on the induction medium supplemented with 2 mg/l IBA (Table 5). Roots emerged 3-4 days after transferring to hormone free medium (Fig. 2). However, the longest roots, up to 8 cm were observed, in induction medium containing 1 mg/l IBA. *In vitro* plantlets were actively grown during acclimatization process and no stress symptoms were observed after their transfer to the greenhouse and in larger pots (Fig. 3). After 2 months, 90% of the potted plantlets

survived, in which plantlet sizes ranged from 20-30 cm in height.

Table 4. Effect of various concentrations of BAP and ZR on the total number of healthy shoots.

Concentration(mg/l)		Number of healthy shoots per explant within specified length (cm)			
BAP	**ZR**	**Total produced shoot per explant**	**1>**	**1<x<2**	**2<**
0	0	1 ± 0^{c}*	1 ± 0^{cde}	-	-
0	0.25	1.42 ± 0.2^{bc}	0.86 ± 0.26^{de}	0.8 ± 0.36^{b}	-
0	0.5	1.5 ± 0.22^{bc}	1.16 ± 0.30^{bcde}	-	-
0	0.75	1.0 ± 0.0^{c}	0.6 ± 0.24^{e}	-	0.66 ± 0.4^{a}
0	1	2.2 ± 0.58^{abc}	2.0 ± 0.44^{abcde}	-	1 ± 0.8^{a}
0.25	0	2.16 ± 0.40^{abc}	2.0 ± 0.51^{abcde}	-	-
0.25	0.25	2.4 ± 0.87^{abc}	1.6 ± 0.87^{abcde}	-	-
0.25	0.5	2.66 ± 0.61^{abc}	1.5 ± 0.76^{abcde}	0.5 ± 0.52^{b}	1 ± 0.8^{a}
0.25	0.75	3.0 ± 0.57^{ab}	0.83 ± 0.40^{de}	3.0 ± 0.88^{a}	-
0.25	1	3.16 ± 0.40^{ab}	3.16 ± 0.4^{abc}	-	-
0.5	0	2.16 ± 0.3^{abc}	1.16 ± 0.60^{bcde}	-	-
0.5	0.25	3.6 ± 0.50^{a}	2.0 ± 0.70^{abcde}	0.75 ± 0.4^{b}	-
0.5	0.5	3.66 ± 0.21^{a}	3.0 ± 0.63^{abcd}	-	-
0.5	0.75	3.0 ± 0.7^{ab}	1.0 ± 0.44^{cde}	1.0 ± 0.88^{b}	0.5 ± 0.4^{a}
0.5	1	2.8 ± 0.48^{abc}	1.8 ± 0.48^{abcde}	0.25 ± 0.44^{b}	-
0.75	0	2.6 ± 0.67^{abc}	2.0 ± 0.94^{abcde}	-	-
0.75	0.25	2.83 ± 0.47^{abc}	1.83 ± 0.70^{abcde}	-	-
0.75	0.5	3.0 ± 0.57^{ab}	0.83 ± 0.54^{de}	3.5 ± 0.62^{a}	0.5 ± 0.5^{a}
0.75	0.75	4.16 ± 0.87^{a}	3.5 ± 1.02^{a}	-	-
0.75	1	3.0 ± 0.57^{ab}	1.83 ± 0.6^{abcde}	1.0 ± 0.88^{b}	-
1	0	3.85 ± 0.73^{a}	3.2 ± 0.91^{a}	-	-
1	0.25	3.14 ± 0.59^{ab}	2.14 ± 0.55^{abcde}	-	-
1	0.5	3.0 ± 0.93^{ab}	2.16 ± 0.87^{abcde}	-	-
1	0.75	3.4 ± 0.74^{ab}	2.4 ± 0.812^{abcde}	-	-
1	1	3.42 ± 0.75^{ab}	3.4 ± 0.74^{a}	-	-

*Means ± standard error. The same letters indicate no significance at α=0.05.

Fig. 2. Rooted shootlets on GNH medium, supplemented with 2 mg/l IBA.

Table 5. Effect of various levels of IBA on rooting of hybrid rootstock GF677 shoots on GNH medium.

Auxin	mg/l	Rooting efficiency (%)	Number of roots per shoot	Root length (cm)
Control	0	0.0^{c*}	0.0^{c}	0.0^{c}
IBA	0.5	20 ± 9^{ab}	0.33 ± 0.16^{bc}	2.5 ± 1.27^{ab}
	1	20 ± 9.1^{ab}	1.25 ± 0.25^{abc}	8.0 ± 2.16^{a}
	2	40 ± 11^{a}	2.62 ± 0.56^{a}	5.2 ± 1.19^{ab}
	3	35 ± 10^{ab}	2.0 ± 0.3^{ab}	4.14 ± 0.81^{ab}

*Means ± standard error. The same letters indicate no significance at α=0.01.

Fig. 3. Acclimatized plantlets in the greenhouse (8 weeks after potting).

Discussion

Successful proliferation of rootstock GF677 has been reported by Dimassi-Theriou (1989), Zimmerman (1991), Molassiotis *et al.,* (2003). However, there are many reports on problems during micropropagation of this rootstock such as vitrification and chloresis of shoots (Murai *et al.,* 1997; George, 1993; Pearoz-tornero *et al.,* 2000). Rugini and Verma (1982) tried to remove vitrification during proliferation, using MS supplemented with pectin. However, our previous results indicated that during shoot subculture on MS supplemented with pectin such problems were temporarily solved in short term. However, in long-term, the vitrification significantly increased (results not shown).

On the basis of the obtained results, it may be concluded that there are differences in the uptake of cytokinins, recognition by the cells and or mechanisms of function of the cytokinin compounds (Ruzic and Vujovic, 2008). The present study indicated that the applied cytokinins can be divided into two groups, the active group (BAP), which was more effective on GF677 shootlets formation, whereas in the second group, ZR exhibited rather weak effects on multiplication. These results agree with the results obtained by Kadata and Numi (2003) in pear (*Pyrus pyroliferia*.) proliferation. They suggested that BAP is more suitable for shoot multiplication of pear than phenylurea derivates. It is well known that high concentrations of cytokinins of adenine type and cytokinins are often necessary for growth and differentiation. Although, the combination of equal amounts of BAP with ZR had a good result on shoot multiplication, which seems to have a synergistic effect on GF677 shoot multiplication (Table 4); but application of BAP plus IBA at 1.0 and 0.1 mg/l concentrations in media resulted in the best shoot multiplication (Table 2).

The current study indicated that using IBA is necessary for root induction in GNH medium (Table 5). The general role of ethylene in plant tissue culture is not clear, however, it can change the organogenic capacity of explants under *in vitro* conditions (Biddington 1992). Also, our previous results (unpublished) indicated that in spite of the presence of IBA including EIs significantly increased root induction. These observations are in agreement with Ma *et al* (1998) results in which they increased rooting in apple using a number of ethylene inhibitors such as $AgNO_3$, $CoCl_2$ and AVG (Amino ethoxy vinyl glycine). Our results also indicated that auxin, in addition to root induction, affects root numbers per shootlet and root length.

The leaves of shootlets obtained under *in vitro* conditions shows characteristics; such as poor cuticle development, and low amounts of epicuticular waxes. In such conditions, many plantlets may die or became damaged following transplanting into the greenhouse or field conditions. This occurs because of moving from high humidity and a low light environment to inverse conditions (Preece, 2010). To minimize plantlet death, acclimatization of the plantlets to the *ex vitro* environment was carried out using fog to provide sufficient humidity, and gradually lowering relative humidity.

Also to avoid fungal contamination (Murai *et al*., 1997) agar was carefully removed from the roots (Fig 3).

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