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In vitro multiplication and rooting of GF677 rootstock

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ABSTRACT

Purpose: Micropropagation of GF677 rootstock, the most widely clonal rootstock used in peach orchards, is an important method for large-scale production of disease-free plants. In this research, effects of media, plant growth regulators and carbohydrates in order to optimize the efficient micropropagation protocol of GF677 rootstock has been investigated. **Research method:** In vitro shoots were multiplicated on MS, WPM and DKW media supplemented with 0, 0.5, 1, 2 mg L^{-1} BA. Proliferated shoots were rooted on MS, WPM and DKW media containing 0, 0.5, 1, 2 mg L^{-1} IBA. In another experiment, the effect of carbohydrate type was investigated. **Findings:** High shoot number and node number as well as shoot fresh weight were achieved with shoot tips when cultured on MS medium supplemented with 1 or 2 mg L^{-1} BA. The highest percentage of rooted shoots was obtained on MS or WPM media supplemented with 1 or 2 mg L^{-1} IBA. Maximum root number was regenerated on WPM medium containing 1 mg L^{-1} IBA. Sorbitol was found to be more effective carbon source on shoot multiplication than sucrose, while the highest average of root number and root length were observed in the medium containing sorbitol and sucrose medium, respectively. Survival rate during the acclimatization in the greenhouse was 67%. **Limitations:** Plant acclimatization needs to be studied for commercial production. **Originality/Value:** This protocol has proven useful for micropropagation of GF677 rootstock.

INTRODUCTION

Prunus rootstocks are an essential component in optimizing productivity of grafted cultivars. Cultivars of these species are normally grafted onto a compatible rootstock to restrict scion vigor, provide better anchorage, improve nutrient uptake, or allow better adaptation to biotic and abiotic stresses that are characteristic of certain soils or climates. Most currently available rootstocks come from interspecific crosses (Gainza et al., 2015; Guajardo et al., 2015). Peach × almond hybrids have shown good performance as rootstocks for several stone fruit species. GF677, discovered as a natural hybrid in France, is used worldwide and at present, it is probably the clonal rootstock for both almond and peach trees mostly planted during the last few years (Felipe et al., 1997; Hartmann et al., 2020). It adapts highly to calcareous soils, and has moderate tolerance to drought and root asphyxia (Byrne et al., 2012; Rubio-Cabetas, 2016). GF677 has a higher productivity with the traditional cultivars of almond, peach and nectarine (Legua et al., 2012; Mestre et al., 2015; Rubio-Cabetas, 2016).

Micropropagation is widely used to propagate numerous Prunus rootstocks (Balla & Mansvelt, 2012; Mayer et al., 2017). It offers a suitable method to provide the growers of sufficient quantities of rootstocks in a very short time and in an extremely small space, as well as of pathogen-free planting materials (Balla & Mansvelt, 2012; Hasanloo et al., 2014). Different factors such as the rootstock genotype, the culture medium, the micro-environmental conditions in the culture vessels and inside the acclimatization greenhouse, can influence the success of this technique (Mayer et al., 2017; Miri & Roughani, 2018a & b).

The micropropagation of GF677 rootstock has been reported previously (Ahmad et al., 2007; Bagheri et al., 2017; Güney et al., 2016; Kamali et al., 2001; Özden et al., 2011; Nazary Moghaddam Aghaye & Yadollahi, 2012; Sepahvand et al., 2012; Tatari & Mousavi, 2014). In these studies, the composition of the nutrient media (water, macro- and micro-elements, sugars, growth regulators) has been mainly studied. The combination of these factors has been less studied, while their interactions seem to have a significant impact on success of in vitro growth and development. According to this, the aim of this study was to investigate the interaction of the media, plant growth regulators and carbohydrates on the in vitro multiplication and rooting of GF677.

MATERIALS AND METHODS

Explant source and in vitro establishment

Semi-hardwood shoots of GF677 were excised from mother plants in early summer. The segments were firstly washed with running tap water for 1 hour to remove any residues. Sterilization comprised the use of 2.6% (v/v) sodium hypochlorite for 15 min and rinses with sterile distilled water three times. Further, they were cut into single node segments (1 cm) and inoculated vertically in glass jars containing 25 ml of hormone-free MS medium (Murashige & Skoog, 1962) supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar for shoot establishment. The pH was adjusted at 5.7-5.8 prior autoclaving at 121 °C for 15 min. All explants were cultured under 25 ± 2 °C, 16 h of photoperiod and light intensity of 2500 lux provided by cool fluorescent lamps. After a period of three weeks, the produced shoots were excised and subcultured for three weeks, in a same, fresh medium in order to increase the number of shoots.

Shoot proliferation

Microshoots (~1 cm) were cut off and transferred into fresh MS, DKW (Driver & Kuniyuki, 1984) and WPM (Lloyd & McCown, 1980) media supplemented with different concentrations

of BA (0, 0.5, 1 and 2 mg L^{-1}), 30 g L^{-1} sucrose and 7 g L^{-1} agar. The iron source of all culture media was replaced with 96 mg L^{-1} Fe-NaEDDHA. After six weeks of incubation, the multiplication rate (%), shoot number, shoot length, node number as well as fresh and dry weight of shoots were recorded.

In order to assess the effect of carbon source on in vitro multiplication of GF677 explants, microshoots were transplanted to the best treatment of culture medium and BA concentration containing 30 g L^{-1} sucrose or sorbitol as carbon source. The explants remained at these media for six weeks, after which the optimal medium was selected.

Rooting

For the rooting experiments, individual shoots (approximately 1.5 cm in length), were transferred to the three modified culture media described above supplemented with different concentrations of IBA (0, 0.5, 1 and 2 mg L^{-1}), 20 g L^{-1} sucrose and 6 g L^{-1} agar. The percentage of root induction, root number, root length as well as fresh and dry weight of plantlets were recorded after six weeks.

To optimize the best root induction medium, the selected treatment of culture medium and IBA concentration was chosen as the basal rooting medium and was supplemented with 20 g L^{-1} sucrose or sorbitol. Rooting parameters were measured as above after six weeks.

Acclimatization

Plantlets with well-developed roots were gently removed from the culture vessels and washed under running tap water to remove the adhering medium. Subsequently, they were transplanted to plastic trays containing a mixture of peat moss, cocopeat and perlite in a ratio of 2:2:1 (v/v), and covered with clear plastic to provide a high relative humidity. The plants were kept in a shaded greenhouse at 23°C. Plantlets were gradually acclimatized by progressively opening the cover over a period of 3 weeks. After 40 days, plants were maintained in the small pots and sprayed every 10 days with 20N-20P-20K commercial fertilizer. The survival rate was calculated after two months.

Statistical analysis

The experiment was arranged according to the completely randomized design (CRD) with four replications (each replication consisted of 4 glass jars with 2-3 explants). To assess the culture media and BA or IBA, the experiments were carried out based on factorial. Analysis of variance (ANOVA) and Pearson's correlation coefficient were performed using SPSS software version 21.0. Statistically significant differences among means were detected using Duncan's multiple range test at $P \leq 0.05$ and $P \leq 0.01$.

RESULTS AND DISCUSSION

Effect of media and BA on shoot multiplication

Analysis of variance revealed that interaction of media and BAP concentration had a significant effect on all shoot multiplication traits (Table 1).

Increasing BA levels brought an increase in shoot multiplication rate (%) in all three culture media. The highest shoot multiplication rate (%) was obtained in MS medium supplemented with 0.5-2 mg L^{-1} BA or WPM containing 2 mg L^{-1} BA. In MS medium, with increasing concentrations of BA, shoot number was significantly increased so that the maximum average of shoot number was observed in MS medium supplemented with 1 or 2 mg L^{-1} BA, in which the average number of new shoots was 4.7 and 5.8, respectively. No vitrification was observed on media with higher BA concentrations. The highest node number

and shoot fresh weight were also related to these two treatments. MS medium containing 1 or 2 mg L^{-1} BA or WPM containing 1 mg L^{-1} BA resulted in the production of the highest shoot length (2.3-2.4 cm) in comparison with other treatments. The highest shoot dry weight was due to MS medium containing 0.5-2 mg L^{-1} BA or WPM supplemented with 2 mg L^{-1} BA (Table 1).

The basal medium and cytokinins display as important factors on shoot proliferation. The composition of the medium required by various Prunus cultivars and rootstocks are not the same. Therefore, choosing the optimal medium is crucial for the success of in vitro shoot morphogenesis (Arab et al., 2014). The results revealed that MS medium was superior for shoot multiplication of GF677 rootstock than WPM and DKW, although there was no significant difference in the multiplication rate (%) and shoot length between MS and WPM media. The differences among these media may be explained on the basis of total ionic strength, a high level of which can be inhibitory to in vitro growth of woody plant species, since DKW has 8% higher total ion concentration than MS (Bell et *al*., 2009). In addition, components of the culture medium (macro- and micro-elements, vitamins) can also contribute to the morphogenesis response of species (Fallahpour et al., 2015). Our results are in agreement with those of Bagheri et al. (2017), and Tatari and Mousavi (2014) that MS medium containing BAP was the best medium for proliferation of GF677 rootstock, although the number of shoots was 1.2 to 2.6 times more than those obtained by Kamali et al. (2001), Nazary Moghaddam Aghaye and Yadollahi (2012), and Tatari and Mousavi (2014). MS was the most commonly used basal medium for micropropagation of GF677 (Ahmad et al., 2007; Özden et al., 2011; Nazary Moghaddam Aghaye & Yadollahi, 2012; Sepahvand et al., 2012), however, other media such as DKW, WPM based media was shown to be superior to MS for some woody species (Aftabi et al., 2013; Fallahpour et al., 2015 & 2019). Sometimes in some species, there is no significant difference between the culture medium, which indicates a species-specific response (Davoudabadi Farahanie et al., 2020; Mousavi et al., 2017).

Use of cytokinins in the media is generally considered necessary for in vitro shoot proliferation (Arab et al., 2014). Cytokinins promote cell division, overcome the apical dominance and stimulate axillary shoot growth (Miri et al., 2003b; Miri & Roughani, 2018a). BA has been the most commonly used cytokinin for proliferation of peach rootstocks (Balla & Mansvelt, 2012). In our study, BA at concentrations of 1 or 2 mg L^{-1} was more effective on shoot multiplication (Fig. 1a). These results agree with results obtained by Özden et al. (2011), and Nazary Moghaddam Aghaye and Yadollahi (2012) with GF677 rootstock that recorded the highest shoot proliferation at BA concentration of 1 mg L^{-1}.

Effect of media and IBA on in vitro rooting

Results of analysis variance showed that interaction of media and IBA concentrations had significant effects on in vitro rooting parameters (Table 2). The highest percentage of rooted shoots was obtained on MS or WPM media supplemented with 1 or 2 mg L^{-1} IBA. In all three media, increasing the concentration of IBA to 1 mg L^{-1} enhanced the root number but in 2 mg L^{-1} IBA it was decreased. Maximum root number (6.1 roots/shoot) was regenerated on WPM containing 1 mg L^{-1} IBA. The longest roots were obtained on WPM, MS containing 0.5-2 mg L^{-1} IBA and DKW supplemented with 1 mg L^{-1} IBA. WPM medium containing 1 or 2 mg L^{-1} IBA or MS medium containing 1 mg L^{-1} IBA resulted in the highest shoot fresh and dry weight (Table 2).

In micropropagation of Prunus sp., rooting is considered a critical stage, since it determines the plant survival during the acclimatization (Rogalski et al., 2003). The nutrient concentrations in the media would affect the rooting so that, reducing the media nutrients by half would improve the rooting (Arab et al., 2018). According to this, for woody species,

usually the more diluted media are used such as WPM with lower concentrations of nitrogen and potassium salts (Lloyd & McCown 1980). Most studies on in vitro rooting of GF677 rootstock have used ½MS medium (Bagheri et al., 2017; Özden et al., 2011; Nazary Moghaddam Aghaye & Yadollahi, 2012). In our study, although WPM and MS media were superior in terms of most rooting traits, but the highest number of root was obtained with WPM. The number of roots is a significant factor for increased plant survival percentage during the acclimatization phase and it is considered as a qualitative trait of rooting response (Tsafouros & Roussos, 2018).

Exogenously auxins are able to induce adventitious roots (Arab et al., 2018). We found that the concentrations of 1 or 2 mg L^{-1} IBA caused the maximum rooting (Fig. 1b). IBA has been suggested as a suitable hormone for in vitro rooting of Prunus rootstocks (Arab et al., 2018; Balla & Mansvelt, 2012). This is because that IBA is more stable and less sensitive to auxin degrading enzymes, and would slowly be metabolized by the peroxidase enzyme (Arab et al., 2018; Miri, 2017).

Phenotypic Pearson correlation coefficients

A positive significant correlation was observed among all traits in both multiplication and rooting stages (Table 3 and 4). These results are contrary to the findings of Erfani et al. (2017) who stated that there is a negative significant correlation between the length and number of shoots due to limited capacity of food-making of the explants. From the correlation analysis, it seems that the composition of most culture media are suitable and sufficient for shoot proliferation and rooting of GF677, so that it does not limit the growth and development of explants.

Effect of carbohydrates on shoot multiplication and in vitro rooting

The type of carbon sources in the culture medium showed the significant effects on shoot number, shoot length and node number (Table 5). Sorbitol was found to be more effective carbon source on shoot multiplication than sucrose, so that the shoot number, shoot length and node number in the culture medium containing sorbitol increased by 13.6%, 24.7% and 51.9%, respectively, compared to the culture medium containing sucrose.

Root number and root length varied significantly with the type of two carbon sources (Table 6). The highest number of roots was observed in the medium containing 20 g L^{-1} sorbitol, while the maximum average of root length was found on the sucrose medium.

The exogenous supply of carbon sources has an important role on the in vitro morphogenesis of several woody species as an energy and carbon source, as well as an osmotic agent (Ahmad et al., 2007; De Paiva Neto & Otoni, 2003; George et al., 2008). Sucrose is almost universally used for micropropagation purposes as it is so generally utilizable by tissue cultures (George et al., 2008). However, our results showed that sorbitol is a better carbon source for shoot proliferation and root induction as compared to sucrose. This result agrees with results reported elsewhere for other species of Rosaceae family such as apple (Miri et al., 2003a) and Garnem rootstock (Erfani et al., 2017). The positive influence of sorbitol on proliferation of Rosaceous shoots could be ascribed to the fact that these plants produce sorbitol as a major photosynthetic product, translocate it within the phloem and metabolize it in the sinks. This preeminent response with sorbitol may be associated with the availability of sorbitol dehydrogenase, sorbitol-6-phosphate dehydrogenase and sorbitol oxidase responsible for the metabolism and assimilation of sorbitol in the sink tissues (Yaseen et al., 2013). The enzymatic conversion of sorbitol into fructose and glucose can make sorbitol readily available for the tissues and thus used as carbon structure for new growth.

Poor response of sucrose in shoot proliferation of peach rootstock might be due to the slow break up of sucrose into glucose and fructose (Ahmad et al., 2007).

The results on rooting showed that the average of root length in sucrose-containing medium was higher than sorbitol. This might be because sucrose reduces the osmotic potential of the culture medium less than sorbitol, so it seems that hydration of root cells was more and the roots became longer. This agrees with the data of in vitro rooting of apricot by Marino et al. (1993), which showed that the roots were shorter and thicker with sorbitol. They suggested that the reduction in root length may be due to the higher osmolality effect of sorbitol.

Plantlet acclimatization

Rooted plantlets were actively growing during the acclimatization process, so that after two months, the height of the plants was about 20-25 cm and 67% survived (Fig. 1c and d).

Table 1. Effect of media and BA concentration on shoot multiplication of GF677 rootstock

Medium	BA (mg L^{-1})	Multiplication rate (%)	Shoot no.	Shoot length (cm)	Node no.	Shoot FW (g)	Shoot DW (g)
WPM	0	12.5 de	1.1 c	1.6 d	12.3 fg	1.0 ef	0.12 fg
	0.5	31.2 cd	2.1 c	2.2 b	13.2 efg	1.9 cde	0.20 cdef
	1	75.0 b	2.3 c	2.3 ab	14.3 def	2.1 bcd	0.22 bcde
	2	87.5 ab	3.6 b	2.2 b	14.7 de	2.7 bc	0.27 abcd
MS	0	38.9 c	1.4 c	1.6 d	15.8 bcd	1.5 def	0.15 efg
	0.5	81.2 ab	4.2 b	2.2 b	16.1 cd	2.8 b	0.29 abc
	1	87.5 ab	4.7 ab	2.3 ab	21.2 a	3.4 a	0.34 a
	2	100 a	5.8 a	2.4 a	19.4 ab	3.7 a	0.31 ab
DKW	0	1.4 e	1.0 c	1.5 d	10.6 g	0.9 f	0.10 g
	0.5	12.5 de	1.2 c	1.5 d	13.0 efg	1.7 def	0.18 defg
	1	25.0 cd	1.5 c	1.8 c	15.0 cd	1.6 def	0.16 efg
	2	36.7 c	1.3 c	1.8 c	17.4 bc	1.6 def	0.16 efg
Sig.		*	**	*	*	*	*

*, P ≤0.05, and **, P ≤0.01

Table 2. Effect of media and IBA concentration on in vitro rooting of GF677 rootstock

Medium	IBA (mg L^{-1})	Rooting (%)	Root no.	Root length (cm)	Plantlet FW (g)	Plantlet DW (g)
WPM	0	18.7 cd	0.9 gh	2.5 ab	1.0 ef	0.13 ef
	0.5	68.7 b	2.9 de	2.9 ab	1.6 de	0.18 de
	1	100.0 a	6.1 a	3.9 a	3.7 a	0.38 a
	2	93.7 a	4.4 b	3.0 ab	3.3 a	0.35 a
MS	0	12.5 cd	0.7 gh	2.2 cd	0.7 f	0.10 f
	0.5	31.2 cd	1.2 fg	2.9 ab	2.1 cd	0.20 cde
	1	81.2 ab	4.1 bc	3.6 ab	3.0 ab	0.32 ab
	2	100.0 a	3.0 d	3.0 ab	2.4 bc	0.26 bc
DKW	0	6.2 d	0.2 h	0.8 d	0.7 f	0.09 f
	0.5	12.5 cd	1.0 gh	1.3 c	1.0 ef	0.11 f
	1	31.2 cd	3.7 cd	3.3 ab	1.9 cd	0.20 cd
	2	37.5 c	2.0 ef	2.6 b	2.1 cd	0.22 cd
Sig.		**	*	*	**	**

*, P ≤0.05, and **, P ≤0.01

Table 3. Pearson correlation coefficients between pairs of multiplication traits

Trait	Multiplication rate (%)	Shoot no.	Shoot length	Node no.	Shoot FW
Shoot no.	0.79**				
Shoot length	0.74**	0.70**			
Node no.	0.62**	0.59**	0.51**		
Shoot FW	0.77**	0.89**	0.69**	0.59**	
Shoot DW	0.76**	0.82**	0.66**	0.52**	0.94**

**, P ≤0.01

Table 4. Pearson correlation coefficients between pairs of rooting traits

Trait	Rooting (%)	Root no.	Root length	Plantlet FW
Root no.	0.81**			
Root length	0.59**	0.68**		
Plantlet FW	0.76**	0.84**	0.64**	
Plantlet DW	0.77**	0.85**	0.62**	0.99**

**, P ≤0.01

Table 5. Effect of carbohydrate on shoot multiplication of GF677 rootstock

Carbohydrate	Multiplication rate (%)	Shoot no.	Shoot length (cm)	Node no.	Shoot FW (g)	Shoot DW (g)
Sucrose	100 a	5.8 b	2.5 b	19.4 b	3.7 a	0.31 a
Sorbitol	100 a	6.6 a	3.1 a	29.4 a	3.8 a	0.32 a
Sig.	ns	*	*	**	ns	ns

ns, non-significant; *, P ≤0.05 and **, P ≤0.01

Table 6. Effect of carbohydrate on in vitro rooting of GF677 rootstock

Carbohydrate	Rooting (%)	Root no.	Root length (cm)	Plantlet FW (g)	Plantlet DW (g)
Sucrose	100 a	6.1 b	4.0 a	3.7 a	0.38 a
Sorbitol	100 a	7.2 a	3.4 b	4.0 a	0.43 a
Sig.	ns	*	*	ns	ns

ns, non-significant and *, P ≤0.05

Fig. 1. Micropropagation of GF677 rootstock. a. Shoot multiplication on MS medium supplemented with 2 mg L^{-1} BA, 6 weeks after initiation; b. Rooting of shoots on WPM medium containing 2 mg L^{-1} IBA after 6 weeks; c. Acclimatization of plantlet in plastic trays; d. Hardened plants in peat moss, cocopeat and perlite (2:2:1), 8 weeks after successful establishment under greenhouse conditions.

CONCLUSION

An efficient in vitro propagation protocol has been successfully developed using nodal explants from semi-hardwood shoots of GF677 rootstock. MS medium supplemented with 1 mg L^{-1} BA and 30 g L^{-1} sorbitol as well as WPM medium containing 1 mg L^{-1} IBA and 20 g L^{-1} sorbitol are suggested for in vitro proliferation and rooting of GF677 rootstock, respectively. This protocol has the potential for large-scale clonal production of GF677 plantlets.

Conflict of interest

The authors have no conflict of interest to report.

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