Micropropagation of Red Leaf Peach Hybrid (*Prunus persica* L.)

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Abstract

The hybrid Rutgers red leaf peach was obtained as a part of the selection programme of the Fruit Growing Institute, Plovdiv. Its leaves are dark red to violet in colour and it exhibits moderate growth. The present study was established to develop a micropropagation protocol for this hybrid. The influence of modified Murashige and Skoog (MS) and Quoirin and Lepoivre (QL) media on the multiplication rate and growth of the generated shoots was studied. The nutrient media were enriched with 2.5 µM 6-benzylaminopurine (BAP), 0.005 µM indole-3-butyric acid (IBA), 0.3 µM gibberellic acid (GA_3) and varying concentrations (separately and in combination) of sucrose and sorbitol. The nutrient media for root induction contained all the components according to MS, with 25% macroelements and varying concentrations of the auxin IBA (0 µM, 1.5 µM, 2.5 µM and 5.0 µM). The best multiplication rate was achieved on media supplemented with sucrose (15 g L^{-1}) and sorbitol (15 g L^{-1}). The highest rooting percentage was obtained on a medium containing 1.5 µM IBA. After acclimatization a number of plants were selected and used for field tests as peach rootstocks and/or ornamental plants.

INTRODUCTION

The studies of many scientists show that different cultivars of peach and other orchard types have specific nutrient media and cultivation requirements if they are to be successfully micropropagated (Hammerschlag et al., 1987).

Many factors influence the growth and development of in vitro propagated plants during the multiplication stage – light, temperature, nutrient medium composition and speed of gaseous interchange between the culture vessels and the environment. All these have been the subject of intensive studies during recent years (Desjardins, 1995; Nowak and Shulaev, 2003; Pospísilová et al., 2007). However, to what extent in vitro plants need a source of carbohydrates remains insufficiently explored (Leifert et al., 1995; Swedlund and Locy, 1993). Sucrose is the most widely-used carbohydrate in plant tissue cultures, primarily because of its ability to support relatively good culture and microplant growth and it is also comparatively cheap (Swedlund and Locy, 1993). Regardless of the widespread use of sucrose in culture media throughout the years, increasing attention has being directed to other saccharides, which may be more suitable for different plant species (Spiegel-Roy and Saad, 1997; Jain et al., 1997).

Many carbohydrates are synthesized and transported in the plants (Loescher, 1987) and impact the processes of differentiation and dedifferentiation of the plant tissues and their physiological state. These synthesized carbohydrates vary according to botanical families and even species. For this reason it is no surprise that different carbohydrates in the nutrient medium can cause specific in vitro growth behavioural characteristics. Many authors noted specific cellular differentiation patterns in some plant types and categorized these according to carbohydrate source (Lemos and Blake, 1996; Karhu, 1997; Bellettre et al., 1999). It has been suggested that the best results are achieved when the types and proportions of the carbohydrates, characteristic of the phloem sap of the respective plant species are used in vitro (Hammat, 1993).

Researchers working with *Malus* and *Prunus* species described the importance of adding sorbitol to the nutrient medium (Bieleski, 1982). Sorbitol, a sugar alcohol, which,

together with sucrose is an initial photosynthetic product and is a main transport form of carbon translocated in many species of the *Rosaceae* family. (Bieleski, 1982; Gutieres and Gaudillere, 1996).

There are several reports detailing the positive effects of sorbitol on callus initiation, organogenesis, stem multiplication and rooting of apple, pear and apricot (Karhu, 1997; Marino et al., 1991; Kadota et al., 2001). Ružic et al. (2005) reported that sorbitol added at a concentration of 115 mM significantly improved the micropropagation of the cherry 'Lapins' and the pear rootstock Pyrodwarf while fructose promoted the development of the rootstock 'Tabel Edabriz'.

Rooting is a critical step for plant propagation either by conventional methods or by tissue culture. The successful rooting of microshoots in vitro conditions is influenced by the genotype, the auxin type and its concentration. In in vitro rooting of peach cultivars 'Nemaguard' and 'Meet-Ghamre,' the highest rooting percentages were obtained on one half strength MS medium supplemented with either 2.0 mg L^{-1} NAA, 1.5 mg L^{-1} IAA, or 1.5 mg L^{-1} IBA (Fouad et al., 1995). Imani and Abdollaha (2006) reported sufficient rooting of *Prunus persika* × *Prunus amygdalus* Batch. hybrid after four weeks exposure to 1.0-3.0 mg L^{-1} IBA in the dark.

The aim of the present study was to develop an efficient system for in vitro micropropagation of red leaf peach hybrid obtained in the Fruit Growing Institute at Plovdiv, Bulgaria.

MATERIALS AND METHODS

Plant Material

The experiments were made using plants of the peach hybrid Rutgers red leaf – open pollination (o.p) obtained in the Fruit Growing Institute at Plovdiv.

Establishment of In Vitro Culture

Explants (shoot tips and axillary buds) were excised from field grown motherstock at the end of May, 2007. They were disinfested according to standard procedures: washed with running water for 15 minutes, dipped in 95% ethanol for 30 seconds, immersed in 5% calcium hypochlorite $Ca(OCl)_2$ solution for 5, 7 or 9 min followed by three rinses/washes with sterile distilled water for 1, 5 and 10 minutes respectively. After disinfection, the explants were implanted on a modified MS (Murashige and Skoog, 1962) nutrient media with and without the addition of growth regulators. The source plant material was cultured on MS nutrient medium, supplemented with 2.5 μM BAP, 0.005 μM IBA, 0.3 μM GA_3, 30.0 g L^{-1} sucrose, 8.5 g L^{-1} agar and a pH value of 5.6 before autoclaving.

Multiplication

Multiplication media were based on MS and QL formulations and enriched with 2.5 μM BAP, 0.005 μM IBA, 0.3 μM GA3 and 6.5 g L^{-1} agar with a pH value of 5.6 before autoclaving. Different concentrations of sucrose, sorbitol (alone and in a combination) were used in the experiments (Table 1).

Microshoots measuring 5-7 mm in height were implanted in 22 mm (diameter) test-tubes containing 5.0 ml of nutrient medium. The following indices were reported following two three week subcultures on the respective nutrient media:

- The number of newly formed shoots up to 5 mm and greater than 5 mm in height;
- The mean length of the newly formed shoots (mm);
- The increase in biomass accumulation per plantlet on fresh weight (FW) and dry weight (DW) basis (30 plantlets were measured per nutrient media).

The cultures were incubated in a growth chamber maintained at a temperature of 22±2°C, a 16 hour photoperiod under fluorescent tubes (OSRAM 40 W) emitting 40 μmol m^{-2} s^{-1} PPFD.

Rooting

The shootlets obtained in the process of multiplication were elongated on hormone-free MS nutrient medium for 10 days. Apical cuttings (approximately 15-20 mm in length) were inserted on the rooting medium. The nutrient media for root induction contained all the components according to MS, with 25% macroelements, sucrose at 20.0 gL^{-1} and varying concentrations of the IBA (0, 1.5, 2.5 and 5.0 μM). The cultures were incubated as described above. The percentage of rooted plants was evaluated after 30 days.

Acclimatization

Plant acclimatization was carried out in pots containing a 50:50 (peat:perlite) mix in growth chambers maintained at 22±2°C temperature and a 16-hour photoperiod maintained under fluorescent lamps (OSRAM 40 W) emitting 60 μmol m^{-2} s^{-1} PPFD.

Data Analysis

For each nutrient medium variant, 20 shoots were used. There were three repetitions. Data was analyzed by analysis of variance ANOVA and the means were separated using Duncan's multiple range test (DMRT) ($P < 0.05$).

RESULTS AND DISCUSSION

Establishment of Explants in In Vitro Conditions

The sterilization procedures used were standard ones for fruit species and resulted in a high percentage (85-100%) of sterile explants. It was observed that treatment with calcium hypochlorite for five minutes for the shoot tip explants and seven minutes for the stem segments resulted in high explant survival levels. It was also observed that the explants were fresh, green in colour and readily adapted to the in vitro conditions.

Multiplication

The results from the present study showed that there were no significant differences in the development of the plants on media based on MS or QL salts when supplied with identical carbohydrate concentrations, with the exception of treatments MP1/QP1 and MP3/QP (Fig. 1); MP3/QP3 and MP5/QP5 for shoot length (Fig. 2) and biomass increase MP1/QP1 and MP2/QP2 (Fig. 3). Battistini and De Paoli (2002) reported that salts based on QL formulation were more suitable for some peach rootstocks. In this work both MS and QL salt formulations proved to be suitable for the micropropagation of the red leaf peach hybrid.

The type and the concentration of carbohydrates in the culture media significantly influenced development of the hybrid in in vitro conditions. From the media variants, (based on the MS salt formulation), the highest multiplication rate was achieved when sucrose and sorbitol were combined - variants MP2, MP4 and MP5 (Fig. 1). However, these combinations did not produce propagules with the greatest length (Fig. 2). This occurred in treatments MP1 and MP5 respectively. Maximum fresh weight occurred when both sorbitol and sucrose were added at 15 g L^{-1} each (MP2) whilst there was no difference between dry weight in treatments MP2, MP4 and MP5 (Fig. 3). The plants cultured on the medium containing 30 g L^{-1} sucrose as a single carbohydrate source (MP1) was not significantly different from treatment MP5 where the ratio of sucrose to sorbitol was two to one. In both these treatments maximum shoot length occurred. Despite this, these two treatments did not maximize biomass production either on fresh or dry weight basis.

Assessing the QL media variations, maximum shoot induction occurred when 30 g L^{-1} sucrose (QP1) or sucrose and sorbitol in combination of 15 g L^{-1} each (QP2) were used (Fig. 1). The plants inoculated on these media demonstrated a significant difference in shoot length and fresh weight biomass with the former producing significantly longer and heavier propagules. When QP1 was compared with MS1, there was no difference in

propagule dry weight with both treatments producing significantly lower values. In our experiments, the separate use of sorbitol in both the MS and QL salts based nutrient media (MP3 and QP3) resulted in low multiplication rate, significantly shorter shoot length and a poor increase in both dry and fresh weight biomass (Figs. 1, 2, 3 and 4). This contrasts with the work of Karhu (1997); Marino et al. (1991); Kadota et al. (2001) experimenting with rosaceous plants such as apricots and Japanese pear. On the contrary, our results track the data of Imani and Abdollahi (2006). However, they reported that combining sucrose (2.5%) and sorbitol (0.5%) improved the proliferation of *Prunus Persica* L.× *Prunus amygdalus* Batsch hybrid compared to the independent application of sucrose (3%) while this work shows that the effect of combining the two sugars (irrespective of ratio) improved propagule dry weights.

Based on the results from our experiments and experimental observations, two media variants (MP2 and QP2) can be recommended for multiplication of the red leaf peach hybrid. Both these media were supplemented with 15 g L^{-1} sucrose and 15 g L^{-1} sorbitol. They produced propagules of the best quality.

Rooting

The application of IBA in the nutrient medium significantly affected rooting of the hybrid where the optimum concentration of the auxin was 1.5 µM (Fig. 5). The auxin IBA is applied widely in rooting orchard types of peach. Battistini and De Paoli (2002) reported successful rooting of many peach rootstocks in higher concentrations of IBA. This work showed the opposite result. In fact, it was found that higher concentrations lead to callus formation at the base of some plantlets and to a lower rooting percentage. The genotype and the size of the treated microplants probably react to different levels of auxin application.

CONCLUSIONS

Both MS and QL salt formulations proved to be suitable for micropropagation of the red leaf peach hybrid.

The best multiplication rate with good quality of plantlets was achieved on media supplemented with combination of sucrose (15 g L^{-1}) and sorbitol (15 g L^{-1}).

The highest percentage of rooting was obtained on media with 1.5 µM IBA.

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Tables

Table 1. Nutrient media for multiplication.

Variants	Macroelements	Sucrose (Su) ($g\ L^{-1}$)	Sorbitol (Sb) ($g\ L^{-1}$)
MP1	MS	30	
QP1	QL	30	
MP2	MS	15	15
QP2	QL	15	15
MP3	MS		30
QP3	QL		30
MP4	MS	10	20
QP4	QL	10	20
MP5	MS	20	10
QP5	QL	20	10

Figures

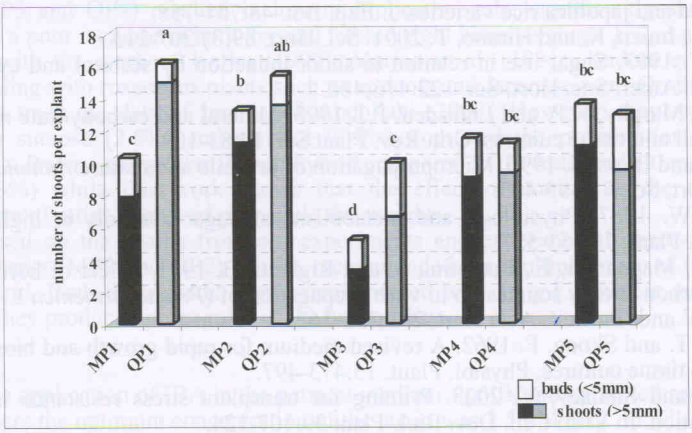

Fig. 1. Effect of different Sucrose (Su) and Sorbitol (Sb) concentrations in MS and QL based nutrient media on the multiplication rate of red leaf peach hybrid for the number of shoots longer than 5 mm. Bar graphs with different letters are significantly different according to DMRT (P<0.05).

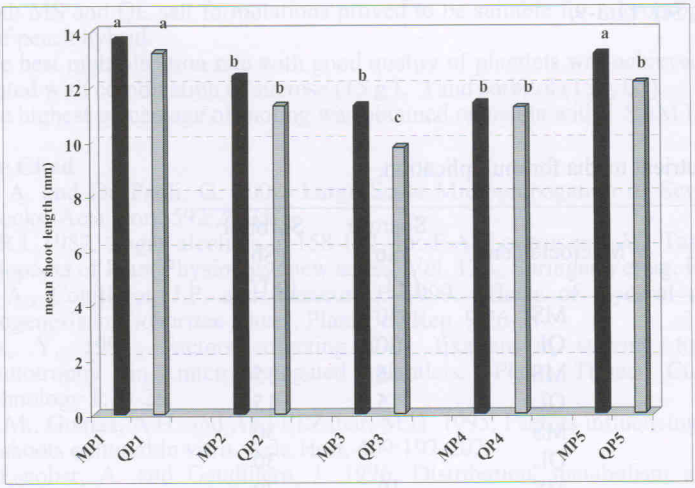

Fig. 2. Effect of the Sucrose (Su) and Sorbitol (Sb) in MS and QL based nutrient media on mean propagule length of red leaf peach hybrid. Bar graphs with different letters are significantly different according to DMRT (P<0.05).