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Effects of mineral composition and acidity of media, saccharose level, brand and quantity of agar on rooting of fruit rootstocks *in vitro*

T. ORLIKOWSKA

Research Institute of Pomology and Floriculture, 96-100 Skierniewice, Poland

Abstract

The influence of the mineral composition and acidity of media, saccharose level, brand and quantity of agar on *in vitro* shoot rooting of P 60 and P 2 apple and quince S 1 root-stocks were compared. Among the tested salt compositions (full WMP and 1/2 WMP, full MS and 1/2 MS) the most suitable for rooting was composition of WMP. Further modifications in quantity of nitrogen, H_3BO_3, $CaCl_2$, and $MgSO_4$ of WMP medium did not have a positive effect on rooting. 30 g l^{-1} saccharose gave better results than 20 g l^{-1}. Acidity of WMP medium tested in a pH range of 4.0 to 6.5 did not affect rooting of P 60 rootstock but media adjusted to pH 4.5 and 5.0 were better for rooting of recalcitrant P 2 rootstock. Among 4 agar brands (*Difco Bacto, Oxoid* No.1, Japanese commercial fibre and Japanese commercial powder) tested at 6 g l^{-1}, the most effective was the last one, but its quantity had no effect in the range of 3 to 10 g l^{-1} for rooting of P 60 rootstock.

Introduction

Micropropagation of fruit trees - clonal rootstocks and scion cultivars has been done in a number of commercial laboratories for more than 10 years. Although protocols for *in vitro* rooting are known and given in abundant publications, there are still some difficulties in practice, which decrease the efficiency and profitability of micropropagation. Theoretically, shoots of produced in well adapted cultures should be able to root on media with auxin. The criteria for selection of shoots for rooting are based on morphology, but morphology is not always adequate to physiological ability to root. Frequently shoots produce one or a few roots. The quality of rooting and especially the number of roots, however, are crucial for adaptation to *in vitro* conditions, especially for growth rate in the first season and generally for plant quality in the nursery.

In this paper some factors which may improve rooting of fruit rootstocks and may be recommended in practice are presented.

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Material and methods

Cultures of dwarf apple (*Malus domestica* L.) (P 60 and P 2) and quince (*Cydonia oblonga* Mill) S 1 rootstock were established according to the method given in earlier publications (Orlikowska 1988, 1991). Multiplication of quince was carried out on Murashige and Skoog (1962) mineral salts modified with a double dose of $MgSO_4$ and KH_2PO_4 with vitamins B_1 - 1.0, B_6 - 0.5, nicotinic acid - 0.5, glycine - 2.0, inositol - 100.0, BAP - 1.0 [all in mg l^{-1}], sucrose 30 g l^{-1} and *Difco Bacto* agar - 6 g l^{-1}. For multiplication of rootstock shoots apple rootstocks the medium consisted of MS mineral salts with a double dose of $MgSO_4$; vitamins, saccharose and agar the same as for quince and with 1.2 mg l^{-1} BAP, 0.05 mg l^{-1} NAA, 40 mg l^{-1} phloroglucinol, 80 mg l^{-1} adenine sulfate.

The uniform shoots, with no visible physiological disorders, about 1.5 cm long, produced within 4 week passages in at least 1-year-old cultures were taken for rooting experiments. Following modifications of WPM medium (Lloyd and McCown 1981) were tested for quince: diminishing of nitrogen level, replacement of NH_4NO_3 by $Ca(No_2)_3$, omitting or doubling of H_3BO_3, $CaCl_2$ and $MgSO_4$. The following factors were investigated in rooting of apple rootstocks: comparison of different mineral salt compositions - WPM, MS and 1/2 MS, WPM and 1/2 WPM, two doses of saccharose - 20 and 30 g l^{-1}, four agar brands at level of 6 g l^{-1} - *Difco Bacto, Oxoid* No. 1, Japanese commercial fibre and Japanese commercial powder, quantities of Japanese commercial powder agar ranging from 3 to 10 g l^{-1}, and acidity of WPM medium ranging from pH 4.0 to 6.5. To all the rooting media the same vitamine as listed for proliferation were added.

During first 4 d (quince) or 5 d (apple) the investigated cultures were incubated in the dark, then under 4 W m^{-2} provided by white fluorescent radiation at 16/8 h photoperiod. The incubation temperature during dark and light treatments was 25 °C.

The experiments were conducted in 100 ml Erlenmeyer flasks filled with 25 ml of medium. Four or five shoots were put into one flask. Five flasks constituted one treatment. All experiments were repeated twice and randomized design was used. Analysis of variance for root number, root and shoot length was conducted on the means from one flask. Percentage of rooted shoots had to be transformed prior to analysis according to Bliss, and rate of rooted shoots according to Freeman-Tukey. The results in the tables are presented as back-transformed values. Means which are followed by the same letter do not differ at 5 % level of significance.

Results

The relationship between mineral composition of media and rooting: The highest rate of rooted shoots and significantly higher number of roots per rooted shoot of P 60 rootstock were obtained on WPM salts in comparison to full MS and 1/2 MS (Table 1). On full MS salt medium callusing of the bases of shoots and retarding the growth of shoots were observed. In another experiment, where full WPM and 1/2 WPM salts were compared, the higher number of roots was observed on 1/2 WPM - 12.4 compared to 9.3 on full WPM. There were no differences in percentage of rooting, as

well as in length of shoots and roots. However, shoots rooted on 1/2 WPM had miniaturized leaves, smaller diameter and more red pigment.

Table 1. Rooting of shoots of P 60 apple rootstock on media of different mineral composition and saccharose quantity. IBA - 1.0 mg l^{-1} phloroglucinol - 40 mg l^{-1} riboflavin - 1.0 mg l^{-1}, the first 5 d in the dark, 4 shoots per flask.

Media	Saccharose [g l^{-1}]	Number of rooted shoots	Number of roots per rooted shoot	Length of roots [mm]
WPM	20	3.30 cd	6.50 e	1.40 b
WPM	30	3.60 d	7.50 f	1.50 b
1/2 MS	20	2.70 c	4.50 c	1.20 a
1/2 MS	30	3.10 cd	5.50 d	1.80 c
MS	20	0.40 a	1.70 a	1.10 a
MS	30	1.50 b	2.50 b	1.40 b

Means followed by the same letter do not differ at 5 % level of significance.

Diminishing the total amount of nitrogen in WPM medium to one half and replacement of NH_4NO_3 by $Ca(NO_3)_2$ significantly reduced the length of roots and shoots of quince S 1 (Table 2). Elimination or doubling of H_3BO_3 and $CaCl_2$ did not affect rooting significantly, but there was a tendency towards that the omission stimulated formation of more and longer roots in comparison to control, and doubling. With a double dose of $MgSO_4$ similar rooting was observed as in the control but the lack of this salt reduced rooting significantly. On the medium without $MgSO_4$ leaves were green-yellow. Abundant secondary roots were observed on media where the amount of nitrogen was reduced and on the medium without $CaCl_2$. On media with a double dose of $CaCl_2$ and where ammonium nitrate was replaced by calcium nitrate secondary roots were missing.

Influence of sucrose level on rooting: Increase of saccharose quantity from 20 g l^{-1} as in the original formula of WPM medium to 30 g l^{-1} resulted in enhancement of percentage of rooted shoots, as well as number and length of roots of P 60 apple rootstock (Table 1).

Effect of medium acidity on rooting: Acidity of WPM medium ranging from pH 4.0 to 6.5 had no effect on rooting of P 60 apple rootstock. The only difference observed was earlier appearance of roots on medium adjusted to pH 5.0. Earlier rooting was observed also for recalcitrant apple rootstock P 2. On medium of pH 5.0 the percentage of rooting was 60, whereas at other pH it ranged from 41 to 45 %. The number of roots was the highest at pH 4.5 and 5.0 (5.0 and 5.2 respectively) in comparison with 2.5 to 3.6 at other pH (Table 3).

Table 2. Rooting of shoots of quince S 1 on media of different amount of mineral components. IBA - 1.0 mg l^{-1}, L-proline - 200 mg l^{-1}, riboflavin - 1.0 mg l^{-1}, the first 4 d in the dark.

Media	Rooted shoots [%]	Number of roots per rooted shoot	Length of a root [mm]	Length of rooted shoots [mm]
WPM salts (control)	99.20 bc	5.10 bcd	8.10 de	3.50 b
1/2 nitrogen salts	97.40 bc [x]	6.00 d	6.40 bc	2.30 a
NH_4NO_3 omitted replaced by 280 mg of $Ca(NO_3)_2$	99.20 bc [y]	4.20 ab	5.90 b	2.50 a
H_3BO_3 doubled	92.10 ab	4.60 bc	7.90 de	3.20 b
$CaCl_2$ doubled	96.80 bc [y]	5.00 bc	7.20 cd	3.10 b
$MgSO_4$ doubled	99.50 c	5.00 bc	8.30 e	3.10 b
H_3BO_3 omitted	98.80 bc	5.50 cd	8.70 e	3.20 b
$CaCl_2$ omitted	96.30 bc	5.40 cd	9.00 e	3.10 b
$MgSO_4$ omitted	80.30 a	3.70 a	4.70 a	2.60 a[z]

Means followed by the same letter do not differ at 5% level of significance.
[x] Abundant secondary roots; [y] Secondary roots absent; [z] Yellow leaves

Table 3. Rooting of shoots of P 2 apple rootstock on media of different acidity. WPM medium with 30 g l^{-1} saccharose, IBA - 1.0 mg l^{-1}, phloroglucinol - 80 mg l^{-1}, L-arginine - 200 mg l^{-1}, the first 5d in the dark, 40 shoots per treatment.

pH medium (adjusted before autoclaving)	Rooted shoots [%]	Number of roots per rooted shoot
4.00	41.70	2.70
4.50	46.30	5.00
5.00	60.00	5.20
5.50	45.00	3.60
6.00	41.70	2.50
6.50	45.80	3.30

Influence of brand and amount of agar on rooting: From four agar brands added to media in amount of 6 g l^{-1} the best rooting of shoots of P 60 apple rootstock was obtained with Japanese commercial powder (Table 4). Significantly worse rooting

was observed on media solidified by Japanese commercial fibre, *Oxoid* No. 1 and *Difco Bacto* agars.

The amount of Japanese commercial powder ranging from 3 to 10 g l^{-1} did not affect the percentage of rooted shoots (100 % at each amount) and length of shoots, but number and length of roots were diminished with higher quantity of agar (Table 5). The diminishing was not important for practical purposes. Only the basal parts of shoots submerged in the medium of the lowest quantity of agar were vitrified.

Table 4. Rooting of shoots of P 60 apple roostock on media of different agar brands. WPM medium with 30 g l^{-1} saccharose, IBA - 1.0 mg l^{-1}, phloroglucinol - 80 mg l^{-1}, L-arginine - 200 mg l^{-1}, the first 5 d in the dark, 4 shoots per flask.

Agar brands [6 g l^{-1}]	Number of rooted shoots	Number of roots per rooted shoot	Length of roots [mm]	Length of rooted shoots [mm]
Japanese commercial powder	4.00 c	11.50 c	3.60 c	1.60 c
Japanese commercial fibre	3.80 b	9.30 b	2.70 b	1.50 c
Oxoid No. 1	3.80 b	9.30 b	2.10 a	1.40 b
Difco Bacto	3.10 a	8.10 a	2.20 a	1.30 a

Means followed by the same letter do not differ at 5% level significance.

Table 5. Rooting of shoots of P 60 roostock on media of different quantity of Japanese commercial agar. WPM medium with 30 g l^{-1} saccharose, IBA - 1.5 mg l^{-1}, phloroglucinol - 80 mg l^{-1},L-arginine - 200 mg l-1, the first 5 d in the dark, 40 shoots per treatment. There were 100% rooted shoots in each treatment.

Quantity of agar [g l^{-1}]	Number of roots per rooted shoot	Length of roots [mm]
3.00	11.00	6.90
4.00	10.60	6.90
5.00	10.00	6.10
6.00	10.40	5.50
7.00	11.20	4.20
8.00	11.20	3.80
9.00	9.40	3.70
10.00	9.40	3.50

Discussion

The abundance of nutritional elements in substrates used for rooting of hard and soft cuttings is not important because the endogenous components are basipetally transported to the rooting zone (Eliasson 1978). The movement of minerals is stimulated by IBA treatment (Blazich *et al.* 1983). Microshoots produced *in vitro* probably do not have a sufficient quantity of endogenous minerals to support the growth of roots. Induction of roots *in vitro* can be initiated without minerals (Zimmerman and Fordham 1985) but development of roots should be continued in peat substrate. In the scheme where the whole process takes place on the same medium, the mineral composition plays an important role (Dunstan 1981, Hasegawa 1980, Machnik and Orlikowska 1981, Welander 1983) and the lowering the concentration of MS salts is beneficial to rooting. Hammerschlag (1982) stated that concentration of MS salts was not important for rooting of *Prunus cerasifera.* In the results presented here full WPM salts were superior to full and diluted 1/2 MS salts and to WPM salts diluted to 1/2.

WPM medium has been recommended for proliferation of woody plants by virtue of possessing a low total ion strength, apropriate proportion Ca to Mg, minimal concentration of Cl^- and not containing ballast elements (McCown and Lloyd 1981). However, this medium used for proliferation of fruit rootstocks always caused vitrification of most explants (Orlikowska, data not published). WPM medium had the optimal level of nitrogen, including the ammonium form for rooting, and further reducing or omitting of ammonium caused disturbances in growth of shoots and roots. Elimination of $CaCl_2$ did not affect rooting, probably because a sufficient quantity of calcium was provided by $Ca(NO_3)_2$. It has been suggested that boron plays multifunctional role in plant metabolism (Lewis 1980), but control of enzymes reducing auxin activity should be the most important one for rooting (Jarvis *et al.* 1983). In the experiment presented here a similar role was probably played by riboflavin, which destroyed the excess of auxin in the medium during the light phase. A complete lack of $MgSO_4$ reduced rooting significantly but did not make it impossible. This shows that if a complex of factors securing rooting is at the optimal level, some individual mineral components are not obligatory in the medium.

Saccharose as a main source of energy and osmotic agent should be included into the rooting media. There were no differences in rooting on media with saccharose ranging from 7 to 35 g l^{-1} (Dunstan 1981) and from 15 to 60 g l^{-1} (Zimmerman 1983), although Pua and Chong (1985) reported that for apple rootstock, 30 g l^{-1} gave better results than 10, 50 and 70 g l^{-1}. In the experiment presented here 30 g l^{-1} sucrose increased the number of roots, rate of rooted shoots and length of roots in comparison with 20 g l^{-1}.

Acidity of the rooting medium did not affect rooting in P 60 rootstock, but for recalcitrant P 2 rootstock, the medium of pH 5.0 increased the percentage and number of roots. Independence of apple rooting from acidity, ranging from pH 4.5 to 8.0 was stated by Welander (1983). Geneve *et al.*(1982) reported that pH ranging from 3.0 to 7.0 did not affect rooting of *Vigna radiata* seedlings, although the highest quantity of ethylene was detected at pH 7.0. Zatyko and Molnar (1990) suggested

that low pH (3 and 4) increases the number of roots and length of *Aronia melanocarpa* shoots.

The kind and quantity of agar affect physical and chemical properties of the medium (Debergh 1983). Influence of agar type on proliferation was investigated by Singha (1982). In the present study, rooting of P 60 apple rootstock depended on agar brand. The best for rooting - Japanese commercial powder - is however completely inadvisable for proliferation of rootstock because it causes total vitrification (Orlikowska, unpublished data). The beneficial effect of this agar on rooting is better demonstrated on recalcitrant rootstocks, which did not form roots on other agars at all (Orlikowska and Machnik, unpublished data). However, concentration of this agar in the medium does not affect rooting, in practice. This was also found by Zimmerman (1983). Therefore reducing the amount of agar is even to 3 g l^{-1} is possible. Nevertheless with this quantity shoots take a horizontal position and twist what resulting in difficulties during planting in the greenhouse.

According to the obtained results the suggested medium for rooting of rootstocks consists of WPM salts and vitamins with 30 g l^{-1} saccharose, adjusted to pH 5.0 and solidified by 4 g l^{-1} of agar.

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