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Model experiments for establishment of in vitro culture by micrografting in apple

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Summary: Micrografting was used in our experiments for establishment of *in vitro* culture from one rootstock ('JTE-F') and three scion cultivars ('Remo', 'Rewena' and 'Reanda') of apple. Shoot tips of these cultivars were harvested from field and grafted onto *in vitro* rootstock cultivars. Their survival and development were studied. 42–93% of shoot tips survived and developed further depending on cultivar. Impermanent browning of sticking agar-agar could be observed in 21–25% of the micrografts depending on cultivars but discolouration of agar-agar ceased within one week and did not cause any death of shoot tips. We used micrografting successfully for establishment of *in vitro* culture from cultivars, from which earlier with conventional methods the culture establishment was not possible because of hard tissue browning. However, further studies are necessary to ensure the survival and development of shoots after removing them from micrografts.

Key words: culture establishment, micrografts, survival, tissue browning

Abbreviations: BAR: 6-benzylaminopurine riboside, BA: 6-benzylaminopurine, IBA: indole-3-butyric acid, GA_3: gibberellic acid

Introduction

Tissue browning during *in vitro* establishment of explants from woody plants is a problem frequently observed which impede the successful micropropagation of woody plants (*Wang* et al., 1994, *Block & Lankes,* 1996). Browning and eventual death of tissues trigger excessive production of polyphenols as a defence reaction. Browning occurs through the action of polyphenol oxidase and peroxidases induced by wounding. A lot of methods have been developed for preventing or controlling browning of explants in a number of plant species including apple. The use of antioxidants (ascorbic acid, citric acid) and adsorbents (activated charcoal, polyvinyl pyrrolidone) during establishment was suggested by several reports (*Zimmerman & Broome,* 1980, *Wang* et al., 1994, *Pan & Staden,* 1998, *Modgil* et al., 1999). Their addition could suppress the release of phenolic compounds, but browning could be not totally hindered. Another factor in reducing phenol oxidation was reported a short treatment of explants in liquid culture prior to placing them on agar-solidified media (*Yepes & Aldwinckle,* 1994, *Dobránszki* et al., 2000 a,b), as well as treatments of source plants before excision (*Block & Lankes,* 1996) or taking explants at right time of the year (*Wang* et al., 1994, *Modgil* et al., 1999, *Dobránszki* et al., 2000 a). In preliminary experiments in our laboratory, we could reduce tissue browning successful in the case of the majority of rootstocks and scions (*Dobránszki* et al., 2000 a,b) but in some varieties it seemed the establishment of *in vitro* culture impossible due to strong browning caused by polyphenols.

The aim of our present study was to develop a suitable and reliable method for establishment of shoot tip culture that can prevent tissue browning and could make successful culture establishment possible.

Material and method

In the model-experiments first the field grown shoot apices of 'JTE-F' were used as scions and *in vitro* shoots of rootstock 'JTE-H' were used as stock.

As rootstock 3-week-old *in vitro* shoots of 'JTE-H' were used. They were developed on shoot multiplication medium containing MS-salts and –vitamins (*Murashige & Skoog,* 1962) supplemented with 100 mg l^{-1} myo-inositol, 0.7 % agar-agar, 3 % saccharose and 0.5 mg l^{-1} BAR , 0.3 mg l^{-1} IBA and 0.2 mg l^{-1} GA_3. The pH of the medium was adjusted to 5.8 before autoclaving. Cultures were grown at 22±2 °C with 16 h photoperiod provided by warm-white lamps (Tungsram) at a PPF of 105 µMol s^{-1} m^{-2}. After removing of bottom leaves they were placed on rooting medium consisting of MS macro- and microelements at half strength supplemented with 0.5 mg l^{-1} B_1 vitamins, 100 mg l^{-1} myo-inositol, 0.75 % agar-agar, 2 % saccharose, and 3.0 mg l^{-1} IBA. The pH of the medium was adjusted to 5.5 with 1 N

KOH. Shoots were cultured in darkness at 22 ±2 °C for one week and they were used then as rootstocks.

Healthy green shoots of 'JTE-F' grown on the field were harvested in May and surface sterilized. Surface sterilization was made in two steps after removing all of the leaves from shoot pieces. First step of sterilization was a washing in tap water contained 0.05% Tween-20 for one hour in a shaker at 150 rpm and at 26 °C. After washing shoot apices were sterilized in 70% ethanol for 0.5 minute, then in 0.1% $HgCl_2$ solution for 5 minutes. After surface sterilization shoot tip explants were washed three-times in sterilized distilled water and flooded in the solution of citric acid (1 g l^{-1}) and after cutting of basal part of shoot pieces they were placed on S medium (*Jámbor-Benczúr & Márta-Riffer*, 1990). Next day the second step of sterilization was made by 25 % Clorox for 5 minutes and then explants were washed three-times with sterilized distilled water. Explants were placed into the solution of citric acid (0.15 mg l^{-1}), ascorbic acid (0.1 mg l^{-1}) and gibberellic acid (0.1 mg l^{-1}) till the grafting.

Directly before grafting the rootstock was decapitated and all leaves were removed. A vertical slit was made on the stump and the scion base cut in a v-shape was fitted in this slit. Before fitting scion was dipped into fluid 1% agar-agar-solution supplemented with 5 mg l^{-1} indole acetic acid (IAA) and so they were stuck to the vertical slit of rootstocks as described earlier (*Dobránszki* et al., 2000 c). The grafts were placed on rooting medium, which contained MS macro- and microelements at half strength supplemented with 50 mg l^{-1} myo-inositol, 2 ml l^{-1} Wuxal, 0.7 % agar-agar, 3 % saccharose and 0.1 % activated charcoal. Micrografts were cultured at 22±2 °C with 16 h photoperiod at a PPF of 105 µMol s^{-1} m^{-2}.

After 30 days the scion were removed and placed on shoot multiplication medium containing MS-salts and –vitamins supplemented with 100 mg l^{-1} myo-inositol, 0.7 % agar-agar, 3 % saccharose and 1.0 mg l^{-1} BAR, 0.1 mg l^{-1} IBA and 0.2 mg l^{-1} GA_3.

The method was applied for establishment of *in vitro* culture from some scion cultivars: 'Reanda', 'Remo' and 'Rewena'. The field grown shoots of these scions were harvested and pre-treated in the same way as in the model-experiment but during August. However, only first step of surface sterilization occurred because of the browning of the explants.

Experiments were repeated three-times.

Results and Discussion

Survival and development of shoot-tips of different scions after grafting are presented in *Table 1*.

When 'JTE-F' rootstock cultivar was used as scion, 93% of grafted shoot-tips developed further and on the average 6.4 new leaves developed on them by the end of the 4th week *(Figure 1. A)*. Directly after grafting, on the 1st day, browning of sticking agar-agar was observable in 21% of the micrografts. However, after 1 week no any browning was detectable in it. It is presumable, that the rootstock of micrografts was able to suppress the further release of polyphenolic compounds and/or could help in their degradation. These shoot-tips were cultured on rootstocks for 30 days. After that they were cut from rootstocks and placed on MS-based shoot-multiplication medium for propagation. The multiplication rate after 4 weeks reached on average 3.1 and no any damage of shoots was detectable during the further subcultures *(Figure 1. B)*.

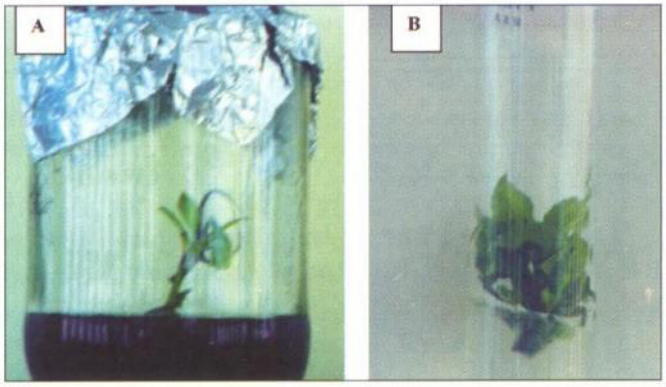

Figure 1. **A:** 4-week-old micrograft with 'JTE-F'as scion and **B:** 3-week-old plantlets of 'JTE-F' on multiplication medium after removing 'JTE-F' from micrograft.

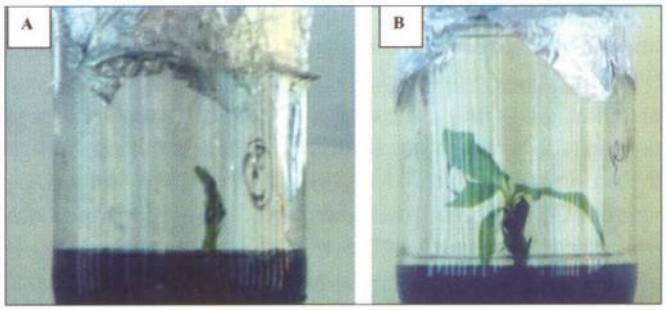

Figure 2. **A:** 2-week-old and **B:** 4-week-old micrografts with 'Remo' as scion.

Table 1. Survival and development of shoot–tips after using them as scions in grafting experiments and the development of new leaves on the different scions after grafting*

Scions	Number of grafts	Number of grafts displaced	Undeveloped grafts	Scions developed further *in vitro*	Average number of new leaves on the scion per graft			
					1st week	2nd week	3rd week	4th week
JTE-F	14	0	1	13	2.1	3.9	4.7	6.4
Remo	7	0	3	4	0	0	0.2	2.0
Reanda	11	0	6	5	0	0	0	0.2
Rewena	7	0	4	3	0	0	0.1	0.4

*: data from three independent experiments

It was tried previously several times to establish *in vitro* culture from scion cultivars 'Reanda', 'Remo' and 'Rewena with different methods described earlier both on solid and on liquid medium (*Zimmerman & Broome*, 1980, *Wang* et al., 1994, *Yepes & Aldwinckle*, 1994, *Pan & Staden*, 1998, *Modgil* et al., 1999, *Dobránszki* et al., 2000 a, b) but because of intensive tissue browning it was impossible: all of shoot-tips died within 1 week (majority of shoot-tips within 24 hours). After the success of *in vitro* culture establishment by micrografting of JTE-F we tried to apply it in the case of these scion cultivars. The main aim of this work was to try if tissue browning during establishment could be hindered or limited by micrografting in the case of these scions. Our work was hardly hampered because the number of shoot-tips harvestable for culture establishment was limited.

The browning of sticking agar-agar was 25–25% in 'Reanda' and 'Rewena' but no any browning was detected in the agar-agar in the case of 'Remo'. Discolouration of agar-agar ceased within one week likewise as in the case of 'JTE-F'. The rate of further developed shoot-tips was lower in all the scion cultivars than in 'JTE-F': it was 57% in 'Remo', 45% in 'Reanda' and 42% in 'Rewena'. The development of these scions was slower on rootstock compared to the development of 'JTE-F' as it can be seen in Table 1. Figure 2 presents 2- and 4-week-old micrografts with 'Remo' as scion. After 4 weeks scions were removed from rootstocks and placed on multiplication medium. However, the shoots died after 2–3 subcultures presumably because the composition of multiplication medium was not suitable for these genotypes.

In this paper we have demonstrated, that micrografting could be successfully used for establishment of *in vitro* culture from cultivars, from which earlier the culture establishment was not successful with conventional methods because of hard tissue browning. Further examinations are necessary considering the composition of multiplication medium in order to ensure the survival and development of shoots after removing them from micrografts.

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