Efficient Axillary Shoot Proliferation and \textit{in Vitro} Rooting of Apple cv. ‘Topaz’

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Abstract

‘Topaz’ is a modern Czech apple cultivar well accepted by consumers and scab-resistant, providing reasons for the significant spread of cv. ‘Topaz’ in European orchards, especially in the organic fruit production industry. Growing the apple trees on their own roots provides some advantages in comparison with grafted trees. Micropropagation is the method of choice for plantlet production for this purpose as well as for the establishment of healthy mother stock trees as a source of scions. The efficiency of axillary shoot proliferation was examined on four media differing in plant growth regulators and their concentrations, and from three explant types: intact or decapitated and defoliated microshoots placed vertically and one-nodal segments placed horizontally. All media consisted of Quoirin and Lepoivre (QL) macroelements and Murashige and Skoog (MS) microelements. Furthermore, rooting efficiency on six different media/treatments was analyzed. Media with 1 mg/L 6-benzylaminopurine (BA) or BA (0.5 mg/L) + 1.5 mg/L kinetin (Kin) produced similar number of microshoots per inoculated one (2.5 and 2.4, respectively). Medium with 1 mg/L thidiazuron (TDZ) produced significantly higher number of shoots (3.6) but they were fasciated. Three different explant types also produced similar numbers of microshoots. High rooting efficiency (68.7%), a high number of roots per shoot (6.6) and the best quality of shoots were obtained in rooting medium containing 2 mg/L of indole-3-butyric acid (IBA). An efficient method of shoot proliferation was established, and, since rooting was the most critical step, an efficient procedure for rooting apple cv. ‘Topaz’ was established.

Keywords: culture establishment, explant type, medium, micropropagation, rooting, woody species

Introduction

Demand for apple varieties with high sugar content and high acidity is increasing worldwide (Godec, 2004). ‘Topaz’, a new Czech cultivar, belongs to this group. ‘Topaz’ is a hybrid between ‘Vanda’ and ‘Rubin’, cultivars developed in the Czech Republic. The skin color is yellow, almost completely overlaid with red and crimson flush (Kellerhals and Eigenmann, 2006). One of the major problems in apple cultivation is apple scab disease caused by the fungus \textit{Venturia inaequalis} (MacHardy, 1996). ‘Topaz’, besides its fruit quality preferred by consumers, is also scab-resistant (Godec, 2004; Szklarz, 2008); these features are the reason for its significant spread throughout European orchards in the last 15 years. Moreover, Sansavini (2004) reported that cv. ‘Topaz’ is the most widespread scab-resistant apple cultivar in the European organic fruit production industry, which is currently undergoing a boom.

Micropropagated rooted apple plants can be used for the establishment of healthy mother stock trees and be a source of scions for grafting on rootstocks, but the real potential lies in the establishment of orchards with own root apple trees. Ermen (2008) reported that own root apple trees have some advantages in comparison with grafted ones. These advantages are: better tree health due to the uptake of nutrients according to genotype requirements (and not limited by rootstocks), better fruit set, better fruit quality and storage life, and better resistance to pests and diseases. More land is needed for own root apple trees than apples grafted on dwarfing rootstocks, which could be offset by doubling the lifespan of own root trees (Ermen, 2008). Micropropagated ‘Topaz’ trees grown on their own roots in organic orchards could therefore be a good choice.

Production of the first apple plantlets \textit{in vitro} was reported by Elliott (1972) and Walkey (1972). Since then, many scion and rootstock genotypes have been micropropagated \textit{in vitro}, as reviewed by Dobrănszky and Teixeira da Silva (2010). The success of shoot multiplication depends not only on the genotype (Lane and McDougald, 1982), but also on plant growth regulators (PGRs) and the interactions between these two factors. Shoot multiplication of apple is based on media containing cytokinins as the major PGR, and with lower concentrations of auxin and sometimes gibberellin (Dobrănszky and Teixeira da Silva, 2010).
Considering the fact that many woody species are difficult to root through cuttings (Tereso et al., 2008), adventitious root formation is a key step in micropropagation (De Klerk et al., 1997). Sharma et al. (2007) reported that consistent high frequency rooting of apple has been more difficult to achieve than shoot multiplication. Moreover, for successful acclimatization of plants, critical traits are the number of roots per shoot and the length of the roots. Like the multiplication rate, rooting ability is also genotype dependent (Lane and McDougald, 1982; Sharma et al., 2007; Yepes and Aldwinckle, 1994), and rootstocks usually root with greater ability than scions (Dobránszky and Teixeira da Silva, 2010).

The aim of this study was (1) to determine the effect of media constitution and explant type on axillary shoot proliferation of apple cv. ‘Topaz’ and (2) to investigate in vitro rooting efficiency of microshoots on different media/treatment.

Materials and methods

Culture establishment

Post-dormant shoot tips of annual scionwood of apple cv. ‘Topaz’, approximately 10 cm long, were collected in the experimental orchard at the end of the winter period and buds were forced to flush in a growth chamber at 23°C with a 16 h photoperiod of cool white light. Shoot segments with flushing buds were washed in tap water and surface-sterilized by immersion in 70% ethanol for 1 min, followed by immersion in 5% sodium hypochlorite (5% active chlorine) with the addition of 0.1% Tween 20 and 150 mg/L of ascorbic acid for 10 min. Segments were rinsed four times in sterile distilled water supplemented with 150 mg/L of ascorbic acid.

Shoot apices approximately 1 mm in size were aseptically isolated under stereomicroscope from flushng terminal and lateral buds and each placed separately on the surface of 10 ml establishment medium in 12 cm high tubes. Establishment medium consisted of MS (Murashige and Skoog, 1962) salts, 3% sucrose, 0.1 g/L myo-inositol, 0.8% agar (Difco Bacto), 1.5 mg/L BAP, 0.2 mg/L IBA, 0.5 mg/L GA₃, 150 mg/L ascorbic acid and 1 g/L activated charcoal. Explants were cultured on establishment medium for 30 days at 22°C, with a 16 h photoperiod of cool white light (40 μE m⁻² s⁻¹).

Axillary shoot proliferation

After establishment, cultures were grown on B₃ medium until a sufficient number of microshoots was produced for further experiments. To test the effect of medium constitution on axillary shoot proliferation, four different media were used. Basal proliferation medium (BPM) consisted of QL macroelements (Quoirin and Lepoitre, 1977) and MS microelements and vitamins with addition of 3% sucrose, 0.1 g/L myo-inositol, 0.5 mg/L GA₃, 0.1 mg/L IBA, and 0.8% agar (Difco Bacto); pH 5.8. Four proliferation media: B₁, B₂, B₃, and B₄ had the following constitution: B₁ = BPM + 1 mg/L BA, B₂ = BPM + 1 mg/L TDZ, B₃ = BPM + 0.5 mg/L BA, B₄ = BPM + 0.5 mg/L BA + 1.5 mg/L Kin. In this experiment, shoots 0.8-1 cm long were placed vertically in medium. Shoots were multiplied and subcultured in Magenta vessels at four-week intervals. Culture conditions were the same as during the establishment phase.

An additional experiment was set up in order to investigate the influence of explant type on axillary shoot proliferation. Three explant types were placed on B₃ medium, namely, intact microshoots 0.8-1 cm high placed vertically (microshoots 1), microshoots 0.8-1 cm high with an excised apex and removed leaves and petioles placed vertically (microshoots 2), and one nodal segments without petioles placed horizontally. The efficiency of axillary shoot proliferation in both experiments was expressed as the mean number of shoots (at least 0.5 cm high) per one inoculated shoot/explant. Shoot proliferation rate was expressed as the ratio between proliferated and total number of placed explants.

Rooting

For rooting, 4 week old axillary shoots, 1-1.5 cm long, developed on B₃ proliferation medium, were cultured on six media/treatments. Basal rooting medium (BRM) consisted of QL macroelements and MS microelements and vitamins, 2% sucrose, 0.1 g/L myo-inositol, 0.8% agar (Difco Bacto), pH 5.8. Four rooting media had the following constitution: R₁ = BRM + 1 mg/L IBA, R₂ = BRM + 2 mg/L IBA, R₃ = BRM + 1 mg/L IAA, R₄ = BRM + 2 mg/L IAA. For the first four days, shoots were cultivated on these four media in the dark, at 22°C. Later, rooting was encouraged under a photoperiod of 16 h of cool white fluorescent light (40 μE m⁻² s⁻¹) at 22°C. In treatments R₅ and R₆, microshoots were pre-treated for 20 hours in solutions (pH 5.8) of MS vitamins and 80 mg/L IBA (R₅) or 70 mg/L IAA (R₆). Shoots were kept at 22°C, with a photoperiod of 16 h of cool white fluorescent light (40 μE m⁻² s⁻¹). After 20 hours, shoots were transferred to BRM and kept under the same culture conditions. Experiments were carried out in Magenta vessels.

Experimental design and statistical analysis

All experiments were set up as a completely randomized design. The effect of media constitution on shoot proliferation was monitored for three subcultures, each time with at least 30 microshoots inoculated per each medium. To estimate the effect of explant type on shoot proliferation, the experiment was repeated three times, each time with at least 40 inoculated explants of each type. Data from rooting experiments were collected from three successive experiments, each containing 10 plantlets per medium/treatment. ANOVA and Fisher’s least significant differ-
ence (LSD) test or Duncan’s multiple range test at \( p < 0.05 \) were used for statistical analyses. Analyses were performed using SAS version 9.1 (SAS Institute, 2008).

**Results and discussion**

**Culture establishment**

Contamination of explants was not detected as the flushing buds at the end of the winter period (early spring) were used, which along with usual disinfection procedures, gave rise to the establishment of a completely aseptic culture. Spring is known to be the ideal season for the establishment of *in vitro* cultures with minimum contamination (Hammerschlag, 1986). The rate of uncontaminated/contaminated explants depends very strongly also on the different phytosanitary stage of the donor plant (Laimer da Câmara Machado *et al.*, 1991); in the present study young (four year old), healthy donor plants appropriately treated with fungicide were used.

Explants survived the establishment period and formed leaf rosettes with 100% efficiency (Fig. 1a). Explant browning due to the oxidation of phenols is the usual problem associated with field-grown trees (Modgil *et al.*, 1999), but from preliminary experiments with several cultivars (Kereša and Mihovilović, unpublished results), it was noticed that browning depends on apple genotype as well.

![Fig. 1. Micropropagation and *in vitro* rooting of apple cv. ‘Topaz’ (bar = 1 cm). (a) leaf rosette on the establishment medium; (b) axillary shoot proliferation; (c) fasciated shoots on medium with 1 mg/L TDZ; (d) normal appearance of shoots developed on medium with BA; (e) rooted plantlet](image-url)
Wang et al. (1994) suggested the use of antioxidants and adsorbents for the prevention of explant browning during establishment. Ascorbic acid (150 mg/L) used during the sterilization procedure and in combination with activated charcoal (1 g/L) in the establishment medium fully prevented explant browning due to phenol oxidation in the current study.

Formation of axillary shoots from established microshoots started two months later on B1 medium. When enough microshoots were produced, the experiment in which shoot proliferation was examined was set up with four different media (B1, B2, B3, and B4). In addition, three different explant types were examined on B1 medium.

**Influence of medium constitution on axillary shoot proliferation**

MS macroelements in the media for shoot proliferation were replaced by QL macroelements due to hyperhydricity problems. In preliminary experiments where the MS macroelements were used, shoots had frequently enrolled, thickened leaves. QL medium has a lower ammonium ion concentration, a higher calcium concentration and chlorine ions are almost eliminated. This formulation avoided hyperhydricity problems.

The effect of the type and concentration of different plant growth regulators (PGRs) in B1-B4 media on axillary shoot proliferation (Fig. 1b) was monitored in three successive experiments. Media supplemented with 1 mg/L BA (B1) or 0.5 mg/L BA and 1.5 mg/L Kin (B2) had a similar effect on shoot proliferation producing 2.5 and 2.4 shoots per inoculated shoot, respectively (Tab. 1). On medium with 0.5 mg/L BA alone (B3) lower number of shoots was obtained. BA alone or in combination with other PGRs is the most commonly used cytokinin for apple micropropagation (Dobrzensky and Teixeira da Silva, 2010; Lane and McDougald, 1982; Modgil et al., 1999; Magyar-Tabori et al., 2002). Using a similar PGR constitution as in B1 and B2 medium, Mahna and Motallebi Azar (2007) found the same results for cv. ‘Golden Delicious’. The optimal BA concentration for maximal shoot proliferation depends on the cultivar (Lane and McDougald, 1982). Using BA in combination with Kin, Modgil et al. (1999) increased the multiplication ratio of apple cv. ‘Tydemans’ early ‘Worcester’ which was not the case in present study. A significantly higher number of shoots (3.6) was obtained on B3 medium supplemented with TDZ. However, shoots and leaves were smaller and abnormal in appearance (fasciated) (Fig. 1c) on medium with TDZ, and the formation of large shoot clumps with adventitious shoots was observed that is consistent with findings of Van Nieuwkerk et al. (1986). These shoots were not suitable for further rooting. In contrast, shoots developed on media containing BA or BA + Kin (B1, B3 and B4 medium) had a normal appearance (Fig. 1d).

Influence of explant type on axillary shoot proliferation

In order to examine the influence of explant type on axillary shoot proliferation, three explant types were used, including intact microshoots placed vertically (microshoots 1), microshoots with an excised apex and removed petioles placed vertically (microshoots 2), and one-nodal segments without petioles placed horizontally. The mean number of shoots per explant varied between 2.5-2.7 and did not differ significantly with different explant type (Tab. 2) that is in agreement with Modgil et al. (1999) who found in apple cv. ‘Tydemans’ early ‘Worcester’ that decapitated shoots placed vertically grew further by showing apical dominance. However, Modgil et al. (1999) also reported that more axillary shoots were produced by placing nodal segments horizontally, but it was not specified if those segments were one-nodal segments or longer. Mackay and Kitto (1988) assumed that increased axillary shoot proliferation by placing the explant in horizontal position could be attributed to greater uptake of the medium constituents (including cytokinins) due to increased contact with the medium. Alekhno and Vysotskii (1986), investigating shoot proliferation in roses, reported that growing shoots with more than one node in a horizontal position almost doubled axillary branching as compared with growing shoots in a vertical position. Mederos and Rodriguez Enríquez (1987) reported that petiole fragments in nodal explants had an inhibitory effect on rose axillary proliferation. In spite of the horizontal placement of one-nodal segments and removal of petioles, it could not produce a greater number of shoots from this kind of explant. The reasons for this could be the fact that strictly one-nodal segments were used. When placing nodal segments of cherry rootstock ‘Gisela 5’ with more than one node horizontally, it was also produced more shoots than placing microshoots vertically (Kerėsė and Mihovilović, unpublished results).

To the best of our knowledge, this study is the first in which axillary shoot proliferation along with successful adventitious rooting of apple cv. ‘Topaz’ was systematically investigated. Only one previous study (Sedláková et al., 2001) has reported on successful in vitro establishment and shoot proliferation of particular pear and apple cultivars, including apple cv. ‘Topaz’.

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**Tab. 1. Efficiency of axillary shoot proliferation as affected by medium constitution**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Mean number of shoots per explant</th>
<th>No. of cultures examined</th>
<th>Shoot proliferation rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>2.5 b</td>
<td>132 (76)</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>3.6 a</td>
<td>102 (77)</td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>1.9 b</td>
<td>90 (58)</td>
<td></td>
</tr>
<tr>
<td>B4</td>
<td>2.4 b</td>
<td>96 (67)</td>
<td></td>
</tr>
</tbody>
</table>

Note: Values followed by the same letter are not significantly different at p<0.05 according to LSD test.
approximately 2 mg/L (10 μM) of IBA was optimal for adventitious rooting of apple cv. ‘Jork 9’ as well (De Klerk et al., 1997), although this experiment was conducted in continuous dark for 21 days. Darkness in the first few days of rooting in apple encourages root initiation (Welander, 1985; Zimmerman and Fordham, 1985). Although it was incubated shoots in the dark for only four days (for media R1 to R4), the percentage of rooting in media R2 was comparable with the results of Modgil et al. (1999) who incubated shoots of apple cv. ‘Tydeman’s Early Worcester’ in the dark for 9 days.

Media with a low (1 mg/L) auxin concentration, either IBA or IAA (R1 and R3 medium, respectively), showed the lowest root induction efficiency.

Conclusions

Efficient axillary shoot proliferation and adventitious rooting was established for apple cv. ‘Topaz’. BA at a concentration of 1 mg/L (B1) or 0.5 mg/L in combination with 1.5 mg/L Kin (B4 medium) showed the same efficiency and could be recommended for axillary shoot proliferation of cv. ‘Topaz’. Three different explant types: vertically placed intact microshoots, vertically placed decapitated and defoliated microshoots and horizontally placed one-nodal segments showed the same shoot multiplication efficiency. High rooting efficiency and good quality of shoots was recorded when shoots were rooted in media with 2 mg/L IBA (R2) or IAA (R4). Due to the significantly higher number of roots per shoot in R2 compared to R4 medium, R2 medium could be recommended for apple cv. ‘Topaz’ rooting.

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References


