

## Efficient Axillary Shoot Proliferation and *in Vitro* Rooting of Apple cv. 'Topaz'

Snježana KEREŠA<sup>1</sup>, Anita MIHOVILOVIĆ BOŠNJAK<sup>1</sup>, Marijana BARIĆ<sup>1</sup>,  
Ivanka HABUŠ JERČIĆ<sup>1</sup>, Hrvoje ŠARČEVIĆ<sup>1</sup>, Ante BIŠKO<sup>2</sup>

<sup>1</sup>University of Zagreb, Faculty of Agriculture, Department of Plant Breeding, Genetics and Biometrics, Svetošimunska 25, 10000 Zagreb, Croatia; [skeresa@agr.hr](mailto:skeresa@agr.hr)

<sup>2</sup>Croatian Centre for Agriculture, Food and Rural Affairs, Hondlova 2/11, 10000 Zagreb, Croatia

### 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 *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 *in vitro* was reported by Elliott (1972) and Walkey (1972). Since then, many scion and rootstock genotypes have been micropropagated *in vitro*, as reviewed by Dobránszky 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 (Dobránszky 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 (Dobrąnszky 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 flushing 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 GA<sub>3</sub>, 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<sup>-2</sup>s<sup>-1</sup>).

### *Axillary shoot proliferation*

After establishment, cultures were grown on B<sub>1</sub> 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 Lepoivre, 1977) and MS microelements and vitamins with addition of 3% sucrose, 0.1 g/L myo-inositol, 0.5 mg/L GA<sub>3</sub>, 0.1 mg/L IBA,

and 0.8% agar (Difco Bacto); pH 5.8. Four proliferation media: B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, and B<sub>4</sub> had the following constitution: B<sub>1</sub> = BPM + 1 mg/L BA, B<sub>2</sub> = BPM + 1 mg/L TDZ, B<sub>3</sub> = BPM + 0.5 mg/L BA, B<sub>4</sub> = 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 B<sub>1</sub> 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 B<sub>1</sub> 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<sup>-2</sup>s<sup>-1</sup>) 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<sup>-2</sup>s<sup>-1</sup>). 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 contamina-

tion (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.

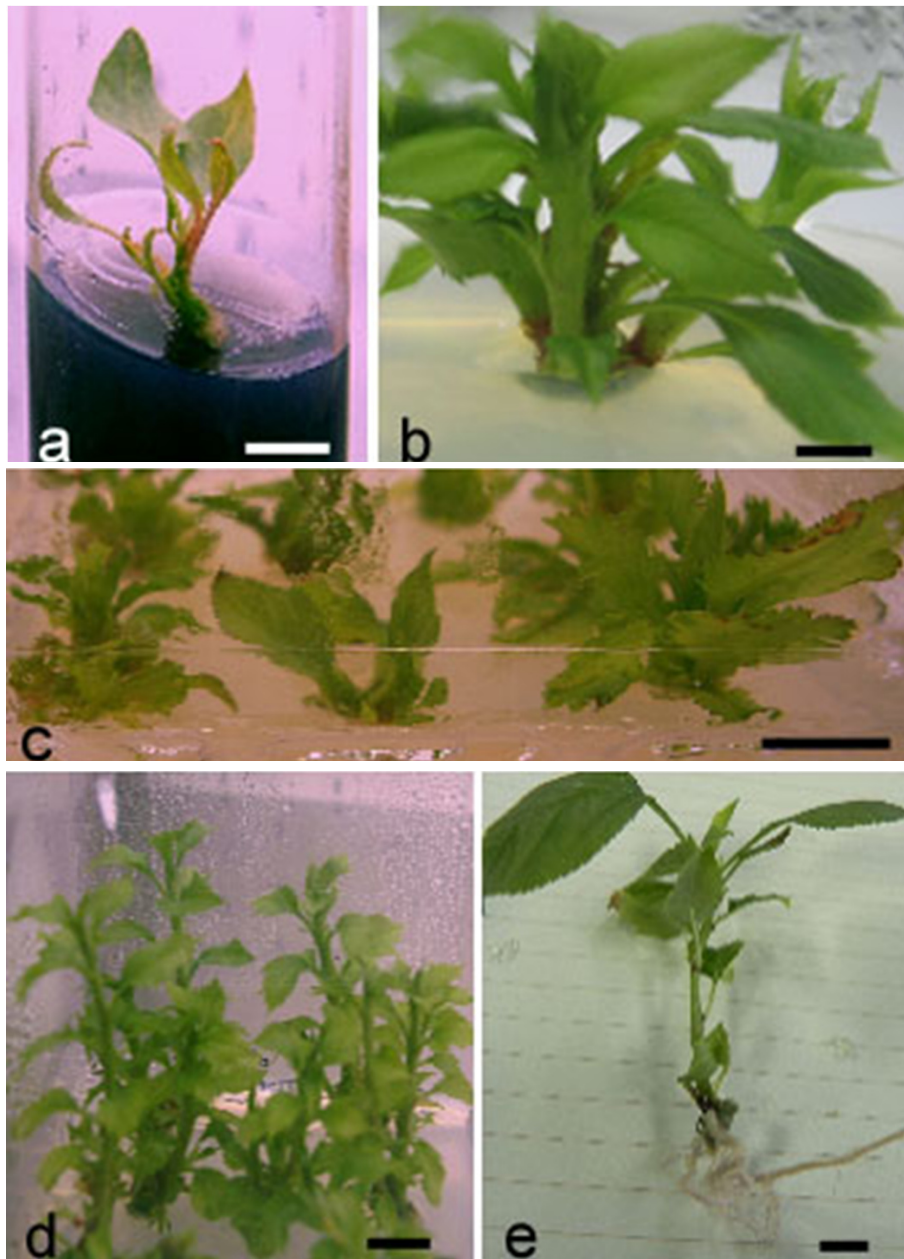


Fig. 1. Micropropagation and *in vitro* rooting of apple cv. 'Topaz' (bar = 1 cm). (a) leaf rosette on the establishment medium; (b) axillary shoot proliferation; (c) fasciated shoots on medium with 1 mg/L TDZ; (d) normal appearance of shoots developed on medium with BA; (e) rooted plantlet

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 B<sub>1</sub> medium. When enough microshoots were produced, the experiment in which shoot proliferation was examined was set up with four different media (B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub> and B<sub>4</sub>). In addition, three different explant types were examined on B<sub>1</sub> 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 B<sub>1</sub>-B<sub>4</sub> media on axillary shoot proliferation (Fig. 1b) was monitored in three successive experiments. Media supplemented with 1 mg/L BA (B<sub>1</sub>) or 0.5 mg/L BA and 1.5 mg/L Kin (B<sub>4</sub>) 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 (B<sub>3</sub>) lower number of shoots was obtained. BA alone or in combination with other PGRs is the most commonly used cytokinin for apple micropropagation (Dobrąnszky and Teixeira da Silva, 2010; Lane and McDougald, 1982; Modgil *et al.*, 1999; Magyar-Tábori *et al.*, 2002). Using a similar PGR constitution as in B<sub>1</sub> and B<sub>3</sub> 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. 'Tydeman's' early 'Worcaster', which was not the case in present study. A signifi-

cantly higher number of shoots (3.6) was obtained on B<sub>2</sub> 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 (B<sub>1</sub>, B<sub>3</sub> and B<sub>4</sub> 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. 'Tydeman's' early 'Worcaster' 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 Rodríguez 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 (Kereša 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 (Sedlák *et al.*, 2001) has reported on successful *in vitro* establishment and shoot proliferation of particular pear and apple cultivars, including apple cv. 'Topaz'.

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

Medium	Mean number of shoots per explant	No. of cultures examined and shoot proliferation rate (%)
B1	2.5 b	132 (76)
B2	3.6 a	102 (77)
B3	1.9 b	90 (58)
B4	2.4 b	96 (67)

Note: Values followed by the same letter are not significantly different at  $p < 0.05$  according to LSD test

Tab. 2. Efficiency of axillary shoot proliferation as affected by explant type

Explant type	Mean number of shoots per explant	No. of cultures examined and shoot proliferation rate (%)
Microshoots 1-vertical	2.5 a	132 (76)
Microshoots 2-vertical	2.7 a	120 (96)
One-nodal segments-horizontal	2.6 a	128 (85)

Note: Values followed by the same letter are not significantly different at  $p < 0.05$  according to LSD test

Tab. 3. Percentage of rooting, number of roots per shoot and the length of the longest root as affected by rooting medium/treatment

Medium/treatment	Percentage of rooting	No. of roots/shoot	Root length (cm)
R1	37.5 b	3.8 b	4.7 a
R2	68.7 ab	6.6 a	3.2 ab
R3	25.0 b	2.8 b	3.5 ab
R4	62.5 ab	3.7 b	2.3 b
R5	81.2 a	6.8 a	3.8 ab
R6	56.2 ab	2.6 b	3.7 ab

Note: Values followed by the same letter within column are not significantly different at  $p < 0.05$  according to Duncan's multiple range test

### Rooting

Emergence of the root primordia was visible from the 12<sup>th</sup> day. The highest percentage of rooted shoots (81.2%) was obtained in the R5 treatment when explants were exposed to a high (80 mg/L) IBA concentration for 20 hours and then transferred to hormone-free medium (BRM). The same treatment induced a high number of roots/shoot, and sufficiently long roots (Tab. 3). These results were comparable to the results of Tereso *et al.* (2008), who achieved very high root induction of almond with a similar procedure of shoot pre-treatment in 0.4 or 0.9 mM IBA solution. Applying shorter pre-treatment (3 hours) with 90 mg/L followed by 80 mg/L of IBA and then transferring the shoots to auxin-free medium, Sharma *et al.* (2007) obtained 86% rooting of apple rootstock M7 microshoots. Nothing was reported, however, on the quality of shoots rooted in this way.

In this study, however, the quality of the shoots rooted in R5 treatment was poor due to shoot apex and leaf decay (browning). It cannot be, therefore, recommended for 'Topaz' rooting, despite the highest root induction and root number per shoot. It is obvious that a very high IBA concentration in pre-treatment had a certain toxic effect. At the same time, pre-treatment with high IAA concentration (70 mg/L, R6) did not show such an adverse effect. The reason for this could be the rapid photo-oxidation of IAA in tissue culture media (50% in 24 hours) in comparison with only 10% photo-oxidation of IBA as reported by Nissen and Sutter (1990).

A high percentage of rooting (68.7 and 62.5%, respectively) and excellent quality of shoots without any sign of decay (Fig. 1e) was obtained on R2 and R4 media that were supplemented with 2 mg/L of IBA or IAA, respectively. As the R2 medium induced higher root number per shoots (6.6) than the R4 medium (3.7), and roots were sufficiently long (3.2 cm), it could be recommended for rooting microshoots of cv. 'Topaz'. A concentration of

approximately 2 mg/L (10  $\mu$ M) 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 (Welandar, 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 ( $B_1$ ) or 0.5 mg/L in combination with 1.5 mg/L Kin ( $B_4$  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.

### Acknowledgements

This work was done under scientific project No. 178-1191193-2045 funded by the Ministry of Science, Education and Sport of the Republic of Croatia.

## References

- Alekhno GD, Vysotskii VA (1986). Clonal micropropagation of roses. *Fiziol Biokhim Kul' Ras* 18:489-493.
- De Klerk G-J, Ter Brugge J, Marinova S (1997). Effectiveness of indoleacetic acid, indolebutyric acid and naphthaleneacetic acid during adventitious root formation *in vitro* in *Malus 'Jork 9'*. *Plant Cell Tiss Org* 49:39-44.
- Dobránszky J, Teixeira da Silva JA (2010). Micropropagation of apple—a review. *Biotechnol Adv* 28:462-488.
- Elliott RF (1972). Axenic culture of shoot apices of apple. *New Zea J Bot* 10:254-258.
- Ermen HF (2008). Growing Apple Trees on their Own Roots. Orange Pippin. Available at [<http://www.orangepippin.com/articles/own-roots>].
- Godec B (2004). New scab resistant apple cultivars recommended in Slovenia. *J Fruit Ornament Plant Res (special ed)* 12:225-231.
- Hammerschlag FA (1986). Temperate fruits and nuts, 221-236 p. In: Zimmerman RH, Griesbach RJ, Hammerschlag FA, Lawson RH (Eds.). *Tissue culture as a plant production system for Horticultural crops*. Martinus Nijhoff Publishers, Dordrecht.
- Kellerhals M, Eigenmann C (2006). Evaluation of apple fruit quality within the EU project Hidras. Available at [[http://www.ecofruit.net/2006/ecofruit\\_12th\\_30.pdf](http://www.ecofruit.net/2006/ecofruit_12th_30.pdf)].
- Laimer da Câmara Machado M, Hanzer V, Kalthoff B, Weiss H, Mattanovich D, Regner F, Katinger FWD (1991). A new, efficient method using 8-hydroxy-quinolinol-sulfate for the initiation and establishment of tissue cultures of apple from adult material. *Plant Cell Tiss Org* 27:155-160.
- Lane WD, McDougald JM (1982). Shoot tissue culture of apple: Comparative response of five cultivars to cytokinin and auxin. *Can J Plant Sci* 62:689-694.
- MacHardy WE (1996). *Apple scab: biology, epidemiology and management*. APS Press, St. Paul, Minnesota.
- Mackay WA, Kitto SL (1988). Factors affecting *in vitro* shoot proliferation of French tarragon. *J Am Soc Hortic Sci* 113:282-287.
- Magyar-Tábori K, Dobránszky J, Jámor-Benczúr (2002). High *in vitro* shoot proliferation in the apple cultivar 'Jonagold' induced by benzyladenine analogues. *Acta Agron Hung* 50:191-195.
- Mahna N, Motallebi Azar A (2007). *In vitro* micropropagation of apple (*Malus x domestica* Borkh.) cv. 'Golden Delicious'. *Comm Appl Biol Sci Ghent University* 72:235-238.
- Mederos S, Rodríguez Enríquez MJ (1987). *In vitro* propagation of 'Golden Times' roses. Factors affecting shoot tips and axillary buds growth and morphogenesis. *Acta Hortic* 212:619-624.
- Modgil M, Sharma DR, Bhardwaj SV (1999). Micropropagation of apple cv. 'Tydeman's' 'Early Worcester'. *Sci Hortic* 81:179-188.
- Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plantarum* 15:473-497.
- Nissen SJ, Sutter EG (1990). Stability of IAA and IBA in nutrient medium of several tissue culture procedures. *Hort Sci* 25:800-802.
- Quoirin M, Lepoivre P (1977). Improved media for *in vitro* culture of *Prunus* sp. *Acta Hortic* 78:437-442.
- Sansavini S (2004). Europe's Organic Fruit Industry. *Chronica Hortic* 44:6-11.
- SAS Institute (2008). SAS/STAT software: User's Guide. Version 9.1. SAS Inst., Cary, NC.
- Sedlák J, Paprstein F, Bilavčík A, Zámecník C (2001). Adaptation of apple and pear plants to *in vitro* conditions and to low temperature. *Acta Hortic* 560:457-460.
- Sharma T, Modgil M, Thakur M (2007). Factors affecting induction and development of *in vitro* rooting in apple rootstocks. *Indian J Exp Bio* 45:824-829.
- Szklarz M (2008). Productive value of seven apple cultivars with different susceptibility to apple scab (*Venturia inaequalis* Che.). *J Fruit Ornament Plant Res* 16:325-331.
- Tereso S, Miguel CM, Mascarenhas M, Roque A, Trindade H, Maroco J (2008). Improved *in vitro* rooting of *Prunus dulcis* Mill. cultivars. *Biol Plantarum* 52:437-444.
- Van Nieuwkerk JP, Zimmerman RH, Fordham I (1986). Thidiazuron stimulation of apple shoot proliferation *in vitro*. *Hortic Sci* 21:516-518.
- Walkey DG (1972). Production of apple plantlets from axillary-bud meristem. *Can J Plant Sci* 52:1085-1087.
- Wang Q, Tang H, Quan Y, Zhou G (1994). Phenol induced browning and establishment of shoot-tip explants of 'Fuji' apple and 'Jinhua' pear cultured *in vitro*. *J Hortic Sci* 69:833-839.
- Welander M (1985). *In vitro* shoot and root formation in the apple cultivars Åkerö. *Ann Bot* 55:249-261.
- Yepes LM, Aldwinckle HS (1994). Micropropagation of thirteen *Malus* cultivars and rootstocks, and effect of antibiotics on proliferation. *Plant Growth Regul* 15:55-67.
- Zimmerman RH, Fordham I (1985). Simplified method for rooting apple cultivars *in vitro*. *J Am Soc Hortic Sci* 110:34-38.