In vitro shoot multiplication as influenced by repeated subculturing of shoots of contemporary fruit rootstocks

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Abstract

Vujović T., Ružić Dj., Cerović R., 2012. ***In vitro* shoot multiplication as influenced by repeated subculturing of shoots of contemporary fruit rootstocks**. Hort. Sci. (Prague), *39*: 101–107.

In vitro shoots of vegetative rootstocks for cherry (Gisela 5 and Gisela 6), plum (Fereley Jaspi) and pear (Pyrodwarf) were repeatedly subcultured for 10 subcultures on Murashige and Skoog medium of unchanged hormonal composition. Shoot formation capacity decreased over repeated subculturing in all genotypes. The first significant decrease in multiplication index was observed after first subculture in Gisela 6 and Fereley Jaspi, while in Gisela 5 the decline occurred after second subculture, and remained at that level. As for Gisela 6 and Fereley Jaspi, multiplication index was mainly stable from second to forth subculture, whereupon the second decline in shoot formation was observed. Although Pyrodwarf showed very low multiplication capacity, shoot multiplication slightly increased over the first three subcultures and then declined. This irreversible decline could be due to residual effects of hormones. However, no visible morphological variations or aberrations of shoots were found in successive subcultures in any genotype. Quality of shoots in terms of shoot length varied during subculturing, but the highest quality was observed in later subcultures (from fifth subculture onwards). After subculturing, several media were evaluated for induction of rhizogenesis in order to achieve high rooting rates in tested rootstocks. The highest rooting ability (100%) among genotypes was observed in Fereley Jaspi, followed by Pyrodwarf and Gisela 6 (the best rooting percentage being 90% in both) and Gisela 5 (70%). Rooted shoots were successfully acclimatized under the mist system in greenhouse.

Keywords: cherry; plum; pear; micropropagation; rooting

Fruit rootstocks are traditionally propagated either by relatively slow and labor-intensive vegetative methods (division and cuttings techniques) or from seed, which often results in a non-uniform material. The application of tissue culture methods for vegetative propagation of temperate fruit rootstocks started in the mid-70s, and a considerable number of improved protocols were developed ever since. Generally, the goal of micropropagation is obtaining rapid, large-scale and low-cost production of genetically identical, physiologically uniform and pathogen-free plants (Rathore et al. 2004).

Successful *in vitro* clonal propagation methods are reported in many rootstocks, including plum (Morini et al. 1990; Fortuna et al. 1996; Nacheva et al. 2002; Vujović et al. 2007), cherry (Ružić, Cerović 1987; Muna et al. 1999; Ružić et al. 2003; Sedlák et al. 2008) and pear rootstocks (Yeo, Reed 1995; De Paoli et al. 2002; Ružić et al. 2008). While most of the studies were focused on the influence of nutrient media, including mineral composition, carbohydrates content and type/concentration of plant growth regulators, the influence of repeated subculturing on shoot multiplica-

Supported by the Ministry of Education and Science of Serbia, Projects No. TR-20013A, TR-31064.

tion and growth received less attention in literature (Grant, Hammat 1999).

Generally, subculture effect on multiplication rate of *in vitro* cultures varies from one species to another. A decrease in multiplication potential during long-term growth and repeated subculturing of shoots on medium of constant hormonal composition was reported in six ornamental species and cultivars of Rosaceae (Norton, Norton 1986), two cultivars of *Potentilla fruticosa* (Remphrey et al. 1993), various decorative plants (Varjda, Varjda 2001), pineapple (Hamad, Taha 2008) etc. However, the point of decline is highly dependent on treatments applied (hormonal composition of medium, incubation period etc.). Nevertheless, in dwarf raspberry (*Rubus pubescens* Raf.), Debnath (2004) noticed that shoot multiplication index as well as shoot length and leaf number increased with subculturing up to the third subculture period, and then remained constant. Similarly, the increase in shoot production with extended time of culturing was reported in cherry and apple rootstocks, and was attributed to rejuvenation of mature tissues during *in vitro* culturing (Grant, Hammat 1999).

Given that plant propagation by tissue culture is usually aimed at high multiplication rate and its maintenance during *in vitro* growth, the objective of this study was to assess the effect of repeated subculturing on shoot multiplication of different fruit rootstocks grown on media of constant hormonal composition.

MATERIAL AND METHODS

Plant material. Four contemporary fruit rootstocks, i.e. cherry rootstocks Gisela 5 and Gisela 6 (*Prunus cerasus* × *Prunus canescens*), plum rootstock Fereley Jaspi (*Prunus salicina* Lindley 'Methley' × *Prunus spinosa* L.) and pear rootstock Pyrodwarf (*Pyrus communis* L.), imported from Germany (Lodder-Unterlagen, Ltd., Dülmen) to Serbia within the framework of the project "Introduction of certification into propagation of fruit planting material (obtaining nuclear stock and establishment of mother plantings with fruit cultivars and rootstocks)", were used for establishing *in vitro* cultures.

Establishment of aseptic culture. Initial cultures of all rootstocks were established using actively growing leaf buds selected from screenhouse-grown plants. Surface sterilization procedure involved washing explants under running water for 2 h, sterilization in 70% ethanol (1 min and 20 s), and 12 min-soaking in 10% (v/v) commercial bleach solution (0.4%, w/v, final concentration of sodium hypochlorite), followed by triple rinsing with sterile water. Buds 0.3–0.8 cm large were isolated under the stereomicroscope and placed onto MS nutritive medium (Murashige, Skoog 1962) containing 2 mg/l N6-benzyladenine (BA), 0.5 mg/l indole-3-butyric acid (IBA) and 0.1 mg/l gibberellic acid (GA_3). The following parameters were monitored: percentage of contamination, percentage of necrotic explants and percentage of explants with leaf rosettes initiation.

Shoot multiplication as influenced by repeated subculturing. Upon establishing of aseptic culture, uniform single shoots were multiplied on MS medium of constant hormonal composition. Shoot multiplication of cherry and plum rootstocks (Gisela 5, Gisela 6 and Fereley Jaspi) was monitored on medium supplemented with 1 mg/l BA, 0.1 mg/l IBA and 0.1 mg/l GA_3. For multiplication of Pyrodwarf pear rootstock we used MS medium containing 0.5 mg/l BA, 0.1 mg/l α-naphthyl acetic acid (NAA) and 0.1 mg/l GA_3. All multiplication media contained 30 g/l sucrose and 8 g/l agar. The pH value was adjusted to 5.7 before autoclaving at 121°C, 150 kPa for 20 min. Shoots were repeatedly subcultured for ten times at a constant three-week subculture interval (Marino et al. 1985; Yeo, Reed 1995). Multiplication parameters, i.e. multiplication index and length of axial and lateral shoots were determined upon each subculture. The multiplication index was defined as the number of newly formed shoots (> 0.5 cm) per initial shoot tip recorded after the stated subculture interval.

Shoot cultures were grown in 100 ml culture vessels containing 50 ml of multiplication medium, at 23 ± 1°C and 16 h-photoperiod (light intensity, 8.83 W/m^2).

Rooting and acclimatization. Shoots of all genotypes were rooted on MS medium with mineral salts reduced to ½-strength and organic complex unchanged. Rooting treatments included as follows: (1) medium supplemented with 1 mg/l IBA and 0.1 mg/l GA_3; (2) medium supplemented with 1 mg/l NAA and 0.1 mg/l GA_3; (3) 1-min dip treatment in NAA dissolved in sterile water (500 mg/l) followed by growth on hormone-free (HF) medium. The percentage of rooted plants was determined after 28 days along with the number and length of roots, and height of the rooted plants. Rooted shoots were removed from culture vessels, washed

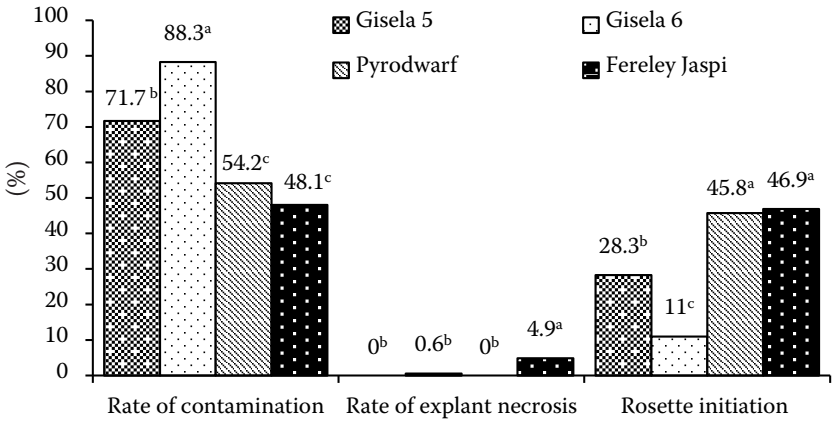

Fig. 1. Establishment of aseptic culture in different rootstocks
Mean values of each parameter in different genotypes followed by the same letter are not significantly different according to the Duncan's multiple range test ($P < 0.05$)

carefully with water to remove adhering medium, transferred to plastic pots containing sterile soil substrate and acclimatized on a 'mist' bench in greenhouse for two weeks.

Statistical analysis. All data were analyzed by ANOVA, followed by the Duncan's multiple range test. Data presented in the form of percentage were subjected to arcsine transformation.

RESULTS AND DISCUSSION

The establishment of aseptic culture is the first critical step in *in vitro* propagation process. There are many reports on successful application of sodium hypochlorite for surface sterilization of initial explants (Norton, Norton 1986; Grant, Hammatt 1999; Debnath 2004). In our material, bleach, as the source of sodium hypochlorite, proved ineffective in disinfecting explants derived from screenhouse-grown plants, probably due to the high level of surface contamination of mother plants. Contamination rates of explants after surface sterilization ranged between 48.1% (Fereley Jaspi) and 88.3% (Gisela 6) (Fig. 1). Contamination rate is influenced by growth conditions, and according to Hartman and Kester (1983), mother plants should be grown in greenhouse under disease- and insect-free conditions, at low humidity and watered so as to avoid overhead irrigation. Also, Muna et al. (1999) believed that sodium hypochlorite is more effective in greenhouse-grown mother plants due to their weak and susceptible cuticles. However, the toxicity to tissues caused by sodium hypochlorite in our experiment was either absent (Gisela 5 and Pyrodwarf) or very low (Gisela 6 and Fereley Jaspi), which enabled us to obtain sufficient number of aseptic cultures, along with those

Table 1. Effect of successive subcultures on multiplication parameters of cherry rootstocks Gisela 5 and Gisela 6 on MS medium supplemented with BA 1, IBA 0.1 and GA_3 0.1 mg/l

Subculture	Gisela 5			Gisela 6		
	multiplication index	length of axial shoot (cm)	length of lateral shoots (cm)	multiplication index	length of axial shoot (cm)	length of lateral shoots (cm)
1.	2.18^{a*}	1.05^{bc}	0.59^{ab}	3.00^{a}	0.80^{d}	0.53^{d}
2.	2.10^{a}	0.95^{bc}	0.57^{b}	2.13^{bc}	1.03^{abc}	0.59^{c}
3.	1.57^{b}	0.92^{bc}	0.60^{ab}	1.82^{cd}	0.82^{d}	0.63^{bc}
4.	1.60^{b}	1.00^{bc}	0.60^{ab}	2.48^{b}	0.93^{bcd}	0.64^{bc}
5.	1.56^{b}	1.32^{a}	0.61^{ab}	1.48^{de}	0.89^{cd}	0.62^{bc}
6.	1.59^{b}	1.07^{b}	0.59^{ab}	1.74^{de}	1.00^{abc}	0.67^{b}
7.	1.59^{b}	1.06^{bc}	0.58^{ab}	1.39^{e}	1.08^{ab}	0.83^{a}
8.	1.24^{b}	1.00^{bc}	0.65^{a}	1.73^{de}	1.11^{a}	0.61^{bc}
9.	1.29^{b}	0.82^{c}	0.54^{b}	1.65^{de}	1.02^{abc}	0.78^{a}
10.	1.50^{b}	1.03^{bc}	0.58^{ab}	1.70^{de}	0.91^{bcd}	0.65^{bc}

*mean values of multiplication parameters within each column followed by the same letter are not significantly different according to the Duncan's multiple range test ($P < 0.05$)

Table 2. Effect of successive subcultures on multiplication parameters of plum rootstock Fereley Jaspi on MS medium supplemented with BA 1, IBA 0.1 and GA_3 0.1 mg/l

Subculture	Multiplication index	Length of axial shoot (cm)	Length of lateral shoots (cm)
1.	3.24^{a*}	0.89^{f}	0.59^{bc}
2.	1.95^{bcd}	0.90^{f}	0.57^{c}
3.	1.83^{bcd}	0.91^{def}	0.59^{bc}
4.	2.05^{bc}	1.02^{bcde}	0.66^{ab}
5.	1.26^{e}	1.15^{a}	0.70^{a}
6.	2.17^{bc}	0.97^{cdef}	0.60^{bc}
7.	1.46^{de}	1.05^{abc}	0.71^{a}
8.	2.33^{b}	1.13^{ab}	0.57^{c}
9.	1.67^{cde}	1.03^{abcd}	0.56^{c}
10.	1.95^{bcd}	0.94^{cdef}	0.60^{bc}

*mean values of multiplication parameters within each column followed by the same letter are not significantly different according to the Duncan's multiple range test ($P < 0.05$)

which initiated leaf rosettes to establish further experiments (Fig. 1).

During multiplication stage, the decline in shoot formation capacity over repeated subcultures was observed in all genotypes. The first significant decrease in multiplication index was observed after the first subculture in Gisela 6 and Fereley Jaspi, while in Gisela 5 decline in shoot number formed occurred after the second subculture and remained constant afterwards (Tables 1 and 2). As for Gisela 6 and Fereley Jaspi, multiplication index was mainly stable from second to fourth subculture, whereupon the second decline in shoot formation was observed. During further subculturing, shoot formation capacity of Gisela 6 remained at the same level, while in Fereley Jaspi multiplication index significantly varied in successive subcultures towards the end of the experiment, but never reached the value obtained in first subculture. Likewise, the analysis of *in vitro* branching pattern of two ornamental cultivars of *Potentilla fruticosa* L. showed that shoot multiplication was at its maximum at the beginning of the experiment and then declined (Remphrey et al. 1993). Morphogenetic potential of the tissue gradually decreased (at a more or less steady level) in both cultivars, which indicated that apical control increased during successive subculturing (Remphrey et al. 1993). However, in our experiment, multiplication index decreased sharply in the second/third subculture and once more in the fifth subculture, which might be due to the residual effects of hormones (inhibitor accumulation). According to Hussain et al. (2007), a sharp decline in shoot formation of *Sterculia urens* Roxb. (second subculture onwards) may be overcome by the reduction of cytokinin (thidiazuron) concentration, which favor continuous production of shoots. Also, Vardja and Varjda (2001) reported that a decline in shoot multiplication of *Gerbera* could be delayed to the ninth subculture by reducing the hormone concentration at the fourth subculture and employing the HF medium by the seventh subculture. However, Hamad and Taha (2008) reported that extending the incubation period from 30 to 75 days reversed the decline in shoot formation rate over the first three subcultures. They also examined the patterns of shoot formation during 75 days of incubation period. High percentage of shoot formation during the first 30 and the last 15 days of the 75 day-incubation period in each of the four subcultures indicated that it was the lack of promoter rather than accumulation of inhibitors that caused a sharp decline by the fourth subculture (Hamad, Taha 2008).

Although in our experiment pear rootstock Pyrodwarf showed very low multiplication capacity on MS medium containing BA 0.5, NAA 0.1 and GA_3 0.1 mg/l, shoot multiplication slightly increased over the first three subcultures and then declined (Table 3). Norton and Norton (1986) reported similar patterns in shoot multiplication of 6 ornamental species and cultivars of Rosaceae.

Table 3. Effect of successive subcultures on multiplication parameters of pear rootstock Pyrodwarf on MS medium supplemented with BA 0.5, NAA 0.1 and GA_3 0.1 mg/l

Subculture	Multiplication index	Length of axial shoot (cm)	Length of lateral shoots (cm)
1.	1.23^{c*}	1.51^{abc}	0.88^{a}
2.	1.40^{ab}	1.13^{b}	0.68^{bc}
3.	1.50^{a}	1.31^{bcd}	0.77^{ab}
4.	1.21^{cd}	1.55^{abc}	0.72^{b}
5.	1.27^{bc}	1.63^{ab}	0.85^{a}
6.	1.12^{cde}	1.32^{bcd}	0.57^{cd}
7.	1.00^{e}	1.81^{a}	–
8.	1.00^{e}	1.22^{cd}	–
9.	1.05^{de}	1.41^{bcd}	0.50^{d}
10.	1.14^{cde}	1.40^{bcd}	0.57^{cd}

*mean values of multiplication parameters within each column followed by the same letter are not significantly different according to the Duncan's multiple range test ($P < 0.05$)

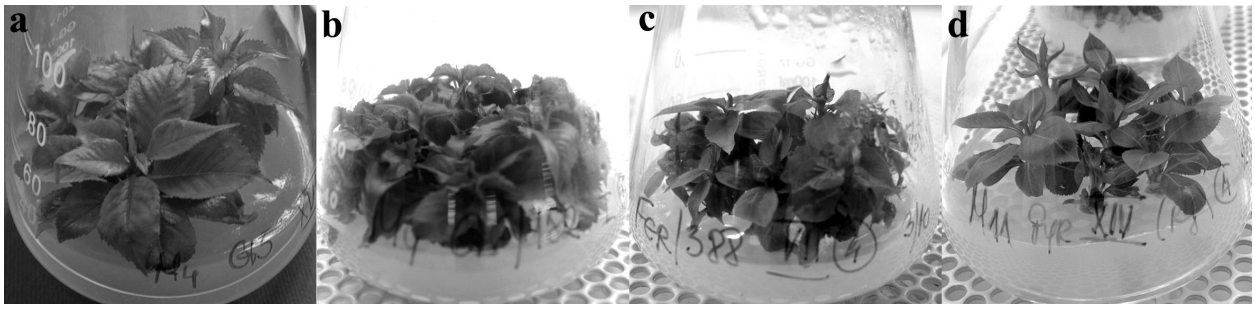

Fig. 2. *In vitro* shoots of different rootstocks in the multiplication stage: (a) Gisela 5; (b) Gisela 6; (c) Fereley Jaspi; (d) Pyrodwarf

According to the authors, irreversible decline could be either due to genetic or epigenetic change resulting from repeated fluxes in cytokinin, nutrient status or sucrose, or to elimination of seasonal environmental fluctuation (Norton, Norton 1986).

No visible morphological variations or aberrations of shoots were found in successive subcultures in any genotype (Fig. 2). Although quality of both axial and lateral shoots in terms of shoot length varied during subculturing, the highest values of these parameters were observed in later subcultures (Tables 1–3). Hamad and Taha (2008) also reported that the subcultures improved shoot elongation at short-lasting incubation (30 or 45 days). In contrast, Norton and Norton (1986) recorded a decrease in shoot length and leaf size after several subcultures, which indicate that besides being affected by external factors, growth is highly influenced by genotype.

After subculturing, the highest rooting ability among genotypes was observed in Fereley Jaspi grown on MS medium containing mineral salts reduced to ½-strength, IBA 1 and GA_3 0.1 mg/l (Table 4, Fig. 3c). Gisela 5 showed similar rooting rates on media containing 1 mg/l IBA or NAA (65% and 70% respectively), although number of roots and plant height were significantly higher when IBA was supplemented (Table 4, Fig. 3a). Concerning the number of roots/shoot, IBA was also found to be more effective than NAA in other *Prunus* representatives (Koubouris, Vasilakakis 2006). In contrast to results obtained for Gisela 5, shoots of Gisela 6 did not root on medium containing IBA (Table 4). The best rooting rate (90%) in this genotype was achieved with 1-min dip treatment in NAA followed by growing on HF medium (Fig. 3b). Pyrodwarf exhibited higher rooting rate (90%, Fig. 3d) along with other rooting parameters on medium supplemented with 1 mg/l IBA than on the one containing NAA (40%, Table 4). Screening of *Pyrus* germplasm for *in vitro* rooting response performed by Reed (1995) also showed that IBA or IBA dip procedures were especially suitable for different *Pyrus communis* representatives.

Acclimatization of rooted shoots was highly successful (above 90%) for most rootstocks, with exception of Gisela 5 where shoots exhibited significantly lower potential (61.8%) to acclimatize (Fig. 4). According to Yepes and Adwinckle (1994), lack of vascular connections between roots and shoots was implicated in low survival of *in vitro* rooted apple plantlets after transfer to the soil. Similarly, indirect *in vitro* rhizogenesis through callus formation can be one of the reasons for low percentage of acclimatization in Gisela 5, as was shown in pear cultivar Bartlett (Bommineni et al. 2001).

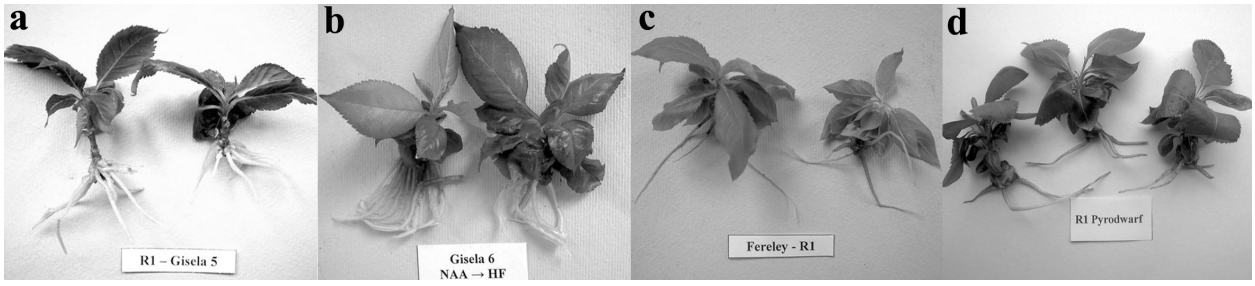

Fig. 3. Rooted shoots of: (a) Gisela 5 on medium supplemented with IBA 1 and GA_3 0.1 mg/l; (b) Gisela 6 on HF medium after 1-min dip treatment in NAA (500 mg/l); (c) Fereley Jaspi on medium supplemented with IBA 1 and GA_3 0.1 mg/l; (d) Pyrodwarf on medium supplemented with IBA 1 and GA_3 0.1 mg/l

Table 4. Parameters of shoots rooting in different rootstocks

Genotype/rooting treatment	% of rooting	No. of roots	Root length (cm)	Height of shoots (cm)
Gisela 5/1	65^{c*}	3.9^{b}	1.82^{bc}	1.54^{b}
Gisela 5/2	70^{c}	2.8^{c}	3.08^{a}	1.31^{cde}
Gisela 5/3	50^{d}	2.2^{cd}	2.13^{b}	1.15^{e}
Gisela 6/1	0^{f}	–	–	–
Gisela 6/2	35^{e}	2.4^{c}	1.76^{bc}	1.47^{bc}
Gisela 6/3	90^{b}	8.1^{a}	3.02^{a}	1.59^{b}
Pyrodwarf/1	90^{b}	4.4^{b}	2.08^{b}	1.89^{a}
Pyrodwarf/2	40de	1.1^{d}	1.48^{c}	1.26^{de}
Fereley Jaspi/1	100^{a}	4.7^{b}	3.00^{a}	1.45^{bcd}

*mean values of rooting parameters within each column followed by the same letter are not significantly different according to the Duncan's multiple range test ($P < 0.05$); 1 – medium supplemented with IBA 1 and GA_3 0.1 mg/l; 2 – medium supplemented with NAA 1 and GA_3 0.1 mg/l; 3 – 1-min dip treatment in NAA (500 mg/l) followed by growth on HF medium

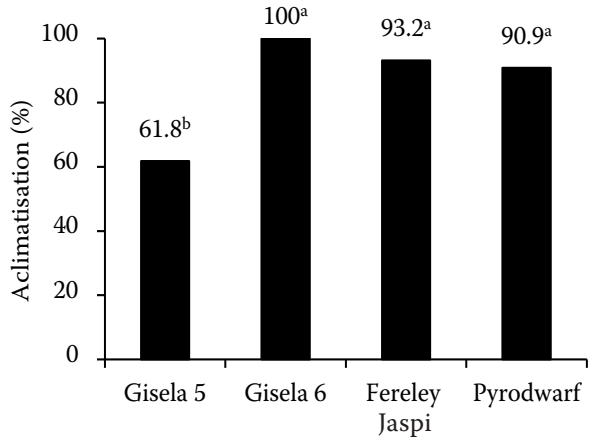

Fig. 4. The comparison of acclimatization rates in rooted shoots of different rootstocks. Values followed by the same letter are not significantly different according to the Duncan's multiple range test ($P < 0.05$)

CONCLUSION

A shoot multiplication protocol could have a large impact on our ability to rapidly multiply *in vitro* desirable fruit rootstocks and ensure plant availability throughout the year accordingly. In this study, the decline in shoot multiplication rate was observed over the repeated subcultures, which implies necessity for further investigation in order to find the proper method of restoring proliferation capacity of *in vitro* shoots and/or delay the decline in rate for several subcultures. Given that the decline in multiplication index was already observed after the second subculture, it is neccesary to determine if and when cytokinin concentration should be reduced, or if hormon-free medium can be empoyed to delay the decrease.

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Received for publication October 14, 2011
Accepted after corrections December 9, 2011

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