

Increased root and shoot production during micropropagation of cherry and apple rootstocks: effect of subculture frequency

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Summary Shoot cultures of apple rootstock cv. M9 and cherry rootstock cv. F12/1 were established and then divided into several sublines that were subcultured at 28–42-day intervals. Consequently, similarly aged cultures received various numbers of subcultures. Cultures kept at 24 °C showed an increase in shoot and root production over time. There were differences in shoot and root production between apple lines, but there were no differences among sublines. In cherry, altering the subculture interval affected rooting competence, which increased with time. Cherry cultures maintained at 4 °C gave rise to cultures that were as easy to root as cultures kept at 24 °C with more frequent subculturing. We conclude that total time in culture is the most important factor bringing about physiological changes in these genotypes of micropropagated apple and cherry.

Keywords: *Prunus avium*, *Malus pumila*, rooting competence, tissue culture, subculture.

Introduction

Tree improvement programs, whether by conventional breeding (Nicoll 1993), or recombinant DNA technology (Tzfira 1998), often rely on vegetative propagation to preserve superior genotypes because of high inherent heterozygosity and self-incompatibility mechanisms. Mature woody plants are typically more difficult to propagate vegetatively than their juvenile counterparts (Hackett 1985, Greenwood and Hutchison 1993).

In some tree species, micropropagation may circumvent the effects of aging or maturation, or both, by restoring shoot vigor and adventitious rooting competence (Webster and Jones 1989, Noiton et al. 1992, Hammatt 1994, Hammatt and Grant 1993, 1997). Improved rooting is also sustained *ex vitro*, in stool beds or conventional shoot cuttings derived from micropropagated plants (Howard et al. 1989, Jones and Webster 1989, Kristiansen 1991, Webster and Jones 1992). Hedged micropropagated plum trees produced cuttings with improved rooting 9 years after establishment (Howard et al. 1989). With apple, results suggest a positive correlation between the total time that shoot lines have spent in culture and subsequent ease of conventional vegetative propagation (Webster and Jones 1992).

It has been suggested that such phenotypic changes following long-term micropropagation result from rejuvenation of mature tissues (Webster and Jones 1989, Jones and Webster 1992, Noiton et al. 1992, Hammatt and Grant 1993, 1997). However, little is known about the mechanism that underlies this rejuvenation process. In apple, Webster and Jones (1989) and Noiton et al. (1992) concluded that such changes were a result of subculturing. However, both studies failed to distinguish between subculture number and total time in culture. The current experiments were initiated to test the hypothesis that rates at which shoot and root production increase during micropropagation are a function of subculturing rather than total time in culture.

Materials and methods

Culture initiation

Rooted shoots from an apple (*Malus pumila* Mill.) rootstock cv. M9 stoolbed, earthed up in 1992, were lifted in February 1993, and stored at 4 °C in darkness. In October 1993, rooted shoots were transferred to a peat-based growing medium and maintained in an illuminated (16-h photoperiod) greenhouse at 20 °C. On November 19, 1993, shoots emerging from buds were removed and used to initiate cultures. On June 18, 1996, shoot tips were excised from mature cherry (*Prunus avium* L.) cv. F12/1 scions that had been grafted on *P. avium* × *P. pseudocerasus* cv. Colt rootstock during 1980 and were maintained as a field-grown hedge with hard annual pruning each spring. Shoots from both species were surface-sterilized (10 min in 10% (v/v) aqueous solution of commercial bleach solution) and rinsed with sterile water six times. Following washing, shoots were reduced in size to produce an inoculum approximately 5 mm in length, with leaf primordia > 10 mm in length before transfer singly to shoot culture medium.

Culture media

Cultures of both species were initiated on MS medium (Murashige and Skoog, 1962), modified as described by Hammatt and Grant (1997), and supplemented with 4.4 µM benzyladenine (BA), 0.29 µM gibberellic acid (GA₃), and 0.49 µM indolebutyric acid (IBA). In addition, 1 mM 1,3,5-trihydroxybenzene and 87.7 mM sucrose were added. Cultures were

grown in 30-cm³ polystyrene scintillation vials (PSVs; Greiner Labortechnik, U.K.) containing 8 ml of the modified MS medium.

After 42 days, apple shoot cultures were transferred to 60-cm³ glass jars with white polypropylene lids containing 20 cm³ of modified MS medium, whereas cherry shoot cultures were transferred to PSVs containing modified MS medium supplemented with 2.2 μ M BA. Cultures of both species were subcultured every 28 days by separating individual shoots from proliferating shoot cultures before transfer to fresh medium. Shoots from both species were rooted in modified MS medium supplemented with 87.7 mM sucrose and 14.7 mM IBA, contained in PSVs.

All media were supplemented with 0.6 % (w/v) agar and adjusted to pH 5.65 before autoclaving (5 min at 121 °C). Cultures were maintained at 24.6 \pm 2 °C with a 16-h photoperiod of 70 μ mol m⁻² s⁻¹ from Phillips 70-W, Type 84 fluorescent tubes, positioned 25 cm above the shelf on which cultures were located.

Subculture treatments

On Day 73 after establishment of the initial apple culture, two randomly selected shoot cultures (designated Lines 8 and 10) were both divided into three shoots, each of which was used to establish a subline (Figure 1). The apple shoot sublines were each assigned to a 28-, 35- or 42-day subculture treatment. On Day 105 after establishment of the initial cherry culture, three randomly selected shoot cultures were each subdivided into two sublines. One subline continued to be subcultured every 28 days, and the other subline was subcultured every 42 days.

For both species, to eliminate effects of varying times since subculture on shoot and root production, numbers of shoots per culture were recorded 28 days after each subculture (Figure 1). Twenty-eight days after each subculture, shoots (up to 20 per subline) 10–15 mm in length were excised below a node, their basal two-thirds stripped of leaves and then inserted (two per PSV) in rooting medium. The proportions of shoots that rooted and the number of roots per rooted shoot were recorded after 28 days in rooting medium.

To extend the subculture interval beyond 42 days, on Day 133, after establishment of the initial cherry culture, some

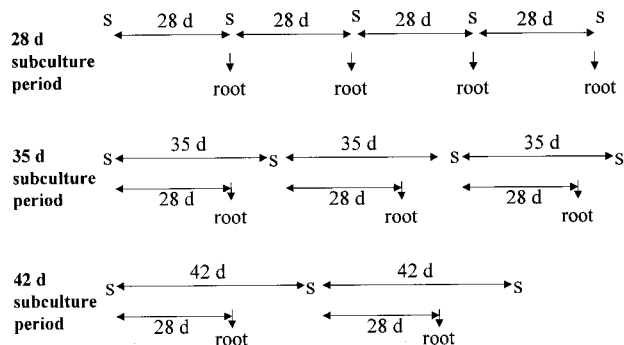


Figure 1. Schematic representation (not to scale) of the shoot subculture protocol used for apple and cherry cultures. Rooting of shoots was assessed 28 days after subculture. Abbreviation: S = subculture.

cultures were transferred to a cooled incubator (4 \pm 2 °C, 16-h photoperiod of 10–20 μ mol m⁻² s⁻¹ irradiance from 20-W, Type 33 fluorescent tubes) and subsequently subcultured in May and November 1997. On November 9, 1997, after 509 days in culture, three randomly selected shoot culture lines were returned to favorable growth conditions (24.6 \pm 2 °C) and subcultured every 28 days. After a further 28 and 56 days, 20 shoots from each shoot line were transferred to rooting medium.

Statistical analyses

All data were analyzed with Genstat 5 software (Genstat 5 Committee, 1993). Mean numbers of shoots and roots per rooted shoots were analyzed by linear regression, including a quadratic term for apple Line 8. Data for shoot and root numbers were weighted by the number of shoot cultures used to determine the mean value. Proportions of shoots that rooted were analyzed by a generalized linear model (McCullagh and Nelder 1989) with binomial error, using the logit link function. A quadratic term was added to the model for apple Line 8. Goodness of fit of the binomial models was tested by comparing residual deviances with the appropriate χ^2 value. For Line 8, there was no significant lack of fit; consequently, changes in deviance associated with the introduction of terms into the models were compared with χ^2 values to derive approximate tests of significance. For Line 10, because there was significant overdispersion, approximate tests of significance were carried out by comparison of deviance ratios for fitted terms with *F* values. Differences in the effects of subculture frequency on shoot and root formation between sublines was determined by comparing regression coefficients with values from the *t*-distribution appropriate for the residual degrees of freedom.

Results

Shoot production by apple cultures

Shoot production increased in both lines in response to increasing time in culture ($P < 0.001$); however, the response curves of the two lines differed. A simple linear model adequately explained shoot production by Line 10 in response to increasing culture time ($P < 0.01$; Figure 2). By contrast, a significant quadratic term ($P < 0.001$) contributed to variance in shoot production by Line 8, with a progressive increase in shoot production up to Day 430, and then a decline thereafter (Figure 2). At the outset of the experiment, Lines 8 and 10 produced significantly fewer shoots when subcultured at 35-day intervals compared with either 28- ($P = 0.001$ for Line 8 and $P < 0.05$ for Line 10) or 42-day ($P < 0.05$ for both lines) intervals. There was no difference in shoot production between the 28- and 42-day subculture intervals. There was no interaction between time in culture and frequency of subculture.

Root production by apple cultures

In both Lines 8 and 10, the proportion of shoots that rooted increased with time in culture ($P < 0.001$; Figure 3), and for each subline, the slopes of the response curves did not differ

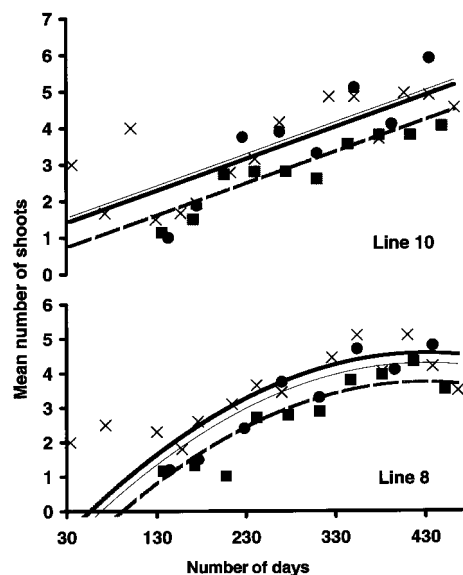


Figure 2. Time course of mean number of apple cv. M9 shoots produced per culture. Cultures were subcultured at 28- (X; fitted model = thick, solid line), 35- (■; fitted model = thick, broken line) and 42-day (●; fitted model = thin, solid line) intervals.

significantly from one another. A significant ($P < 0.01$; Figure 3) quadratic term indicated that rooting competence of shoots from Line 8 started to decline toward the end of the sampling period. For Line 10, subculture frequency had no effect on the proportion of shoots that rooted. Therefore, data from the three sublimes were pooled to produce a response curve (Figure 3). For Line 8, by contrast, there was a significant effect of subculture frequency on the overall rooting abilities of shoots (Figure 3). Comparison of regression coefficients suggested that shoots subcultured at 35-day intervals rooted less frequently than shoots subcultured at 28-day intervals ($P < 0.05$).

In Line 8, root number per shoot increased with increasing time in culture ($P < 0.001$; Figure 4) until the end of the study when there was a significant decline in root production ($P < 0.00$). Shoots subcultured every 42 days produced more roots ($P < 0.001$) than shoots subcultured at either 28- or 35-day intervals; however, rates of increase in mean root numbers did not differ among sublimes. Mean numbers of roots in Line 10 were unaffected by increasing time.

Root production by cherry cultures

Overall, the proportion of shoots that produced adventitious roots increased with time in culture ($P < 0.001$; Figure 5). Although there were overall differences in the proportion of shoots from each line that rooted ($P < 0.001$), there was no significant interaction between culture line and subculture interval; therefore, data for the three replicate lines were pooled for further analysis. A significant interaction ($P < 0.001$) between subculture interval and time in culture suggests that the rate at which the proportion of shoots that rooted increased differed between shoots subcultured at 28- and 42-day intervals. The proportion of shoots maintained at

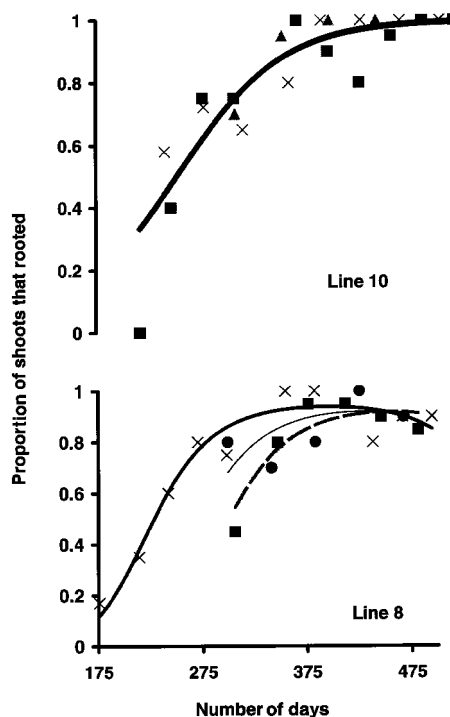


Figure 3. Proportions of apple cv. M9 shoots producing adventitious roots during micropropagation. Shoots were excised from shoot cultures and subcultured at 28- (X), 35- (■) or 42-day (●) intervals. For Line 8, models are fitted for the 28- (thick, solid line), 35- (thick, broken line) and 42-day (thin, solid line) subculture intervals.

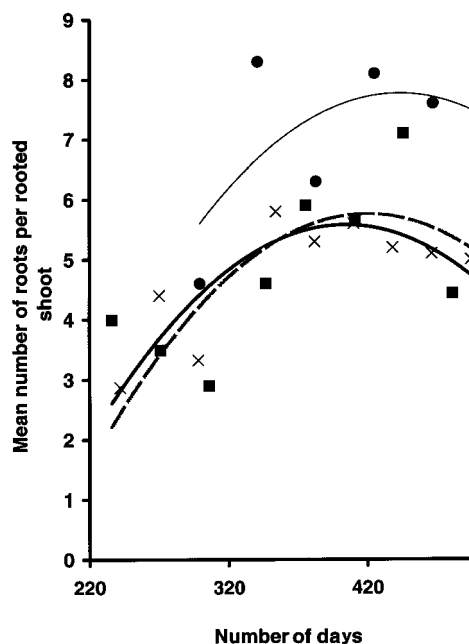


Figure 4. Mean number of roots produced per rooted apple cv. M9 shoot after extended periods of micropropagation. Shoots were excised from shoot cultures maintained with 28- (X; fitted model = thick, solid line), 35- (■; fitted model = thick, broken line) and 42-day (●; fitted model = thin, solid line) subculture intervals.

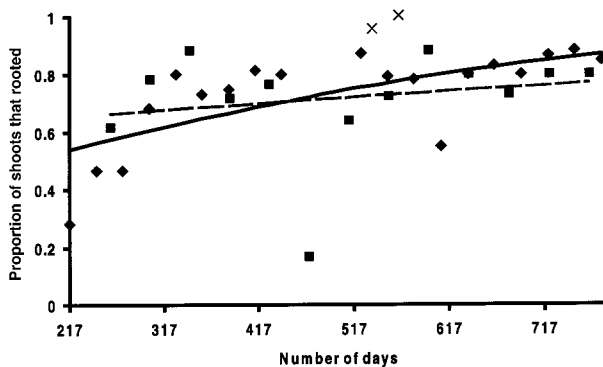


Figure 5. Proportions of cherry cv. F12/1 shoots producing adventitious roots during micropropagation. Shoots were excised from shoot cultures and subcultured at 28- (◆; predicted values = solid line) or 42-day (■; predicted values = broken line) intervals, or an extended subculture interval at 4 °C (X).

4 °C that rooted was similar to that of shoots kept at 24 °C (Figure 5).

Root numbers increased with time in culture ($P < 0.05$; data not shown) and differed overall between shoot lines ($P < 0.001$) and subculture intervals ($P < 0.001$). However, there was no interaction between subculture interval and time in culture, suggesting that the increasing rate of root production with increasing time in culture was not affected by subculture interval.

Discussion

Both shoot and root production increased with increasing time in culture for cultures of the same age but differing in number of subcultures. We conclude that, for the two genotypes studied, the ability to produce roots and shoots was dependent on the total time spent in culture, but was not affected by the frequency of subculture as has been suggested previously (Webster and Jones 1989, Noiton et al. 1992). However, apple and cherry differed in their responses to subculture frequency.

In apple, different competencies among the sublines for either shoot or root formation were established early in the life of each culture line and were unaffected by subculture interval. This difference cannot be ascribed to differences between stock plants because there were also differences among sublines established from the two shoot tips. Similar differences were also observed among the three cherry culture lines; however, the cherry culture lines did not differ from one another in their response to increasing time in culture. Similarly, apple sublines did not differ from one another in response to increasing time in culture.

In cherry, subculture interval affected rates at which rooting competence of shoot cultures increased with time; however, the effect of subculture frequency on rooting competence was not as great as overall time spent in culture. When cold-treatment was used to extend the subculture interval, the cultures were able to root at least as well as those cultured at 24 °C with

more frequent subculturing. Maintaining cultures at 1–4 °C with illumination has previously been used to reduce the need for subculturing in other woody species (Lundergan and Janick 1979, Chun and Hall 1986).

Micropropagation of apple is unlikely to be a useful technique for producing trees commercially for several reasons. Self-rooted, micropropagated scion cultivars tend to be too vigorous (Webster et al. 1986, Jones and Hadlow 1989, Zimmerman and Miller 1991), and micropropagated apple rootstocks produce excessive numbers of suckers and burr knots (Webster and Jones 1992). The most likely commercial opportunity for micropropagation of apple will be the use of micropropagated stockplants for conventional propagation either by cuttings or stoolbeds. Webster and Jones (1992) showed that apples were easier to propagate from stockplants of a micropropagated origin than from stockplants of a conventional origin. Furthermore, rootstocks from micropropagated stockplants performed as well as rootstocks from conventional stockplants (Jones and Webster 1993). We conclude that the amount of subculturing required to produce easy-to-root stockplants by micropropagation can be reduced, which, in turn, should reduce their cost.

Acknowledgments

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References

- Chun, Y.W. and R.B. Hall 1986. Low temperature storage of *in vitro* cultured hybrid poplar (*Populus alba* × *P. grandidentata*) plantlets. In VIth. Intl. Congress Plant Tissue and Cell Culture. Abstracts. Minneapolis, MN, p 13.
- Greenwood, M.S. and K.W. Hutchison. 1993. Maturation as a developmental process. In Clonal Forestry I, Genetics and Biotechnology. Eds. M.R. Ahuja and W.J. Libby. Springer-Verlag, Berlin, pp 14–33.
- Hackett, W.P. 1985. Juvenility, maturation and rejuvenation in woody plants. *Hortic. Rev.* 7:109–155.
- Hammatt, N. 1994. Shoot initiation in the leaflet axils of compound leaves from micropropagated shoots of juvenile and mature common ash (*Fraxinus excelsior* L.). *J. Exp. Bot.* 45: 871–875.
- Hammatt, N. and N.J. Grant. 1993. Apparent rejuvenation of mature wild cherry (*Prunus avium* L.) during micropropagation. *J. Plant Physiol.* 141:341–346.
- Hammatt, N. and N.J. Grant. 1997. Micropropagation of mature British wild cherry. *Plant Cell, Tissue Organ Cult.* 47:103–110.
- Howard, B.H., O.P. Jones and J. Vasek. 1989. Long-term improvement in the rooting of plum cuttings following apparent rejuvenation. *J. Hort. Sci.* 64:147–156.
- Jones, O.P. and W.C.C. Hadlow. 1989. Juvenile-like character of apple trees produced by grafting scions and rootstocks produced by micropropagation. *J. Hort. Sci.* 64:395–401.
- Jones, O.P. and C.A. Webster. 1989. Improved rooting from conventional cuttings taken from micropropagated plants of *Pyrus communis* rootstocks. *J. Hort. Sci.* 64:429–434.

- Jones, O.P. and C.A. Webster. 1993. Nursery performance of 'Cox' apple trees with rootstocks of M9 from either micropropagation or improved conventional propagation from micropropagated plants. *J. Hort. Sci.* 68:763–766.
- Kristiansen, K. 1991. Post-propagation growth of cuttings from *in vitro* and *in vivo* propagated stock plants of *Ficus benjamina*. *Sci. Hortic.* 46:315–322.
- Lundergan, C. and J. Janick. 1979. Low temperature storage of *in vitro* apple shoots. *HortScience* 14:514–515.
- McCullagh, P. and J.A. Nelder. 1989. Generalised linear models. Chapman and Hall, London, pp 511.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol. Plant.* 15:473–497.
- Nicoll, F.J. 1993. Genetic improvement of cherry for farm woodlands. *Qld. J. For.* 87:187–194.
- Noiton, D., J.H. Vine and M.G. Mullins. 1992. Effects of serial subculture *in vitro* on the endogenous levels of indole-3-acetic acid and abscisic acid and rootability in micro-cuttings of 'Jonathan' apple. *Plant Growth Reg.* 11:331–383.
- Tzfira, T., A. Zuker and A. Altman. 1998. Forest-tree biotechnology: Genetic transformation and its application to future forests. *Trends Biotechnol.* 16:439–446.
- Webster, C.A. and O.P. Jones. 1989. Micropropagation of apple rootstock M9: Effect of sustained subculture on apparent rejuvenation *in vitro*. *J. Hort. Sci.* 64:421–428.
- Webster, C.A. and O.P. Jones. 1992. Performance of field hedge and stoolbed plants of micropropagated dwarfing apple rootstock clones with different degrees of apparent rejuvenation. *J. Hort. Sci.* 67:521–528.
- Webster, A.D., T.R. Sparks and A. Blecher. 1986. The influence of micropropagation and chemical mutagens on the growth and precocity of Cox's Orange Pippin and Bramley's seedling apple. *Acta Hort.* 180:25–34.
- Zimmerman, R.H. and S.S. Miller. 1991. Orchard growth and fruiting of micropropagated apple trees. *J. Am. Soc. Hort. Sci.* 116:780–785.

