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A New Method for Rapid In Vitro Propagation of Apple and Pear

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***Abstract*. Improved in vitro clonal propagation methods are valuable tools for nurseries and growers, and are essential for manipulation and improvement of tree fruit germplasm using the tools and techniques of biotechnology. We have developed a rapid shoot multiplication procedure for clonal propagation of apple, *Malus ×domestica* cv. Gale Gala and pear, *Pyrus communis* L. cv. Bartlett. Rapid clonal multiplication was achieved after the following series of steps: pre-conditioning of micropropagated shoots, sectioning pre-treated stems into thin slices, placing slices onto shoot induction medium and incubating directly under cool-white fluorescent lights or after a brief dark incubation. Multiple induction of shoots recovered from stem slice explants within three weeks of culture. A maximum of 37% of cultured apple stem slices, and 97% of pear stem slices, showed induction of shoots. More shoots were recovered on phytagel solidified shoot induction medium than on agar. Cultured stem slices of both apple and pear showed maximum recovery of shoots from shoot induction medium supplemented with thidiazuron (TDZ) compared to medium supplemented with BAP and kinetin. Under ideal conditions, pear stems generated four times the shoots as the same quantity or length of apple shoots. Micropropagated shoots were rooted and transferred to the greenhouse and field nursery for further evaluation. Chemical names used: *N*-phenyl-*N′*-1,2,3-thidiazol-5-ylurea (thidiazuron or TDZ); 6-benzylaminopurine (BAP).**

Conventional clonal propagation methods such as budding and grafting are used successfully by many nursery growers. While successful, these methods are slow, labor intensive and may require large amounts of land. Alternatively, successful in vitro clonal propagation methods are reported in apple and pear for commercial applications in the tree fruit industry. Over the last two decades, several research groups established in vitro conditions that were suitable for micropropagation of apple (Marin et al., 1993; Noiton et al., 1992; Skirvin et al., 1986; Webster and Jones, 1991; Welander, 1985; Yepes and Aldwinckle, 1994; Zimmerman, 1986) and pear (Viseur, 1987; Xiao-Shan and Mullins, 1984) cultivar and rootstock material. These methods hold promise for producing more plant material in a shorter period of time with less labor and at lower costs. These in vitro propagation methods yield an average of 4 to 6 shoots from a single source shoot over a period of 4 weeks (the transfer generation). However, it often requires several transfer generations to recover a sufficient number of clonal plants necessary for this method to be effective in commercial application.

Varietal expansion of apple and to some extent pear expected to meet the demands of changing market and orchard needs throughout the world. Recent trends in the apple industry indicate that older cultivars such as 'Delicious' and 'McIntosh' are gradually becoming obsolete with the introduction of new improved cultivars (Barritt, 1999). Consumer preference drives the continuous production of new cultivars, and most consumer purchases are based on the varietal name (Barritt, 1999). Future projections for the apple industry also indicate that demand for new varieties will continue to grow through the turn of the century [U.S. Dept. of Agriculture (USDA) World Agricultural Outlook Board, 1999]. As new cultivars continue to replace older cultivars, a rapid in vitro vegetative propagation method offers an accelerated method of production of new tree fruit cultivars/rootstocks that may be extremely valuable for commercial and private nursery growers.

We have used in vitro propagated shoots to develop a rapid clonal multiplication procedure in apple (cv. Gale Gala) and pear (cv. Bartlett) by applying microsurgery to axillary buds. Using this method, hundreds of plants were recovered from a single source stem within a short period of time. The study reported here describes results of an enhanced shoot multiplication system in both apple and pear.

Materials and Methods

Micropropagation of apple and pear. Micropropagation of apple (cv. Gale Gala) and pear (cv. Bartlett) was established using the buds of mature trees (Van Well Nursery, Wenatchee, Wash.). Cultures were maintained under cool-white fluorescent lights (38–40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in a growth chamber with 24 ± 2 °C and 16/8h photoperiod. Apple micropropagation medium (GM, Table 1) consisted of phytagel (0.25%) solidified medium with MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968), glycine (26.6 µM), sucrose (87.6 mM), cefotaxime (209.4 to 418.8 µM), 6-benzylaminopurine (BAP, 4.4 µM), kinetin (13.9µM) and pH 5.8. Pear cultures were maintained in a phytagel solidified medium (BM, Table 1) with salts of Quoirin and Lepoivre (1977) (QL) salts, Staba vitamins (Staba, 1969), glycine (26.6 µM), sucrose (87.6 mM), cefotaxime (209.4 µM), BAP (2.2 µM) and kinetin (4.6 µM). Green shoots were separated and transferred to fresh medium every four weeks (transfer generation).

Preconditioning. Preconditioning of shoots (i.e., source stems) from micropropagation cultures is a prerequisite step essential to increase the density of leaves (i.e. increase the number of axillary buds) on source stems. High leaf density was achieved by increasing the cytokinin composition in the micropropagation medium. Apple preconditioning medium was the same as apple propagation medium with BAP (4.4 or 8.8 µM) and kinetin (13.9 or 23.2 µM) and pear preconditioning medium was the same as pear micropropagation medium with BAP (13.2 µM) and kinetin (23.2 µM). Preconditioning treatment was carried out for two transfer generations under the growth conditions described earlier.

Making stem slices and recovery of multiple shoots. Preconditioned shoots (1 to 2 cm long) with a high leaf density were selected for the stem slice procedure. Leaves were removed from preconditioned shoots and shoots were then sliced with a sterile double-edged platinum razor blade. Multiple cross-sectional slices <1.5 mm were made along the length of each source shoot. More than one slice must go through each leaf axis (or axillary meristem) to achieve multiple shoots from each slice. Fifteen to thirty stem slices from a 1.0- to 2.0-cm-long preconditioned source stem were placed on shoot induction (or recovery) medium (see below) in a Petri plate (100 × 25 mm) and incubated under cool-white fluorescent lights as described above. If the total number of slices from a single source stem exceeded 30 then the slices were explanted onto two Petri plates. Two to three source stems were used in each treat-

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ment and the procedure was repeated once with selected treatments, and subsequently, several times in the transformation experiments (unpublished data). Some cultures were incubated first in the dark for 2 weeks and then transferred to light conditions.

Effects of plant growth regulators and gelling agents (phytagel or agar) were examined for rapid recovery of multiple shoots. Plant growth regulators and their concentrations were selected based on the earlier experiments (Table 1). Multiple shoots from each stem slice were separated and transferred to micropropagation medium, first to petri plates (15 to 20 shoots per petri plate) and then phytatrays (Sigma) (six to nine shoots per phytatray) and maintained as described earlier. The stem slice procedure was successfully used in transformation of apple and pear (unpublished data).

Rooting of propagated shoots. Single shoots from established cultures were separated and placed onto rooting medium. Although several media were evaluated for induction of roots in both apple and pear, only results from media that showed maximum root induction are presented in this report. Apple rooting medium consisted of MS salts, B5 vitamins, glycine (26.6 μM), casein hydrolysate (25 mg·L^{-1}), sucrose (87.6 mM), indole-3-acetic acid (IAA, 17.1 μM), and pH adjusted to 5.8 prior to autoclaving. Pear rooting medium consisted of QL salts, Staba vitamins, glycine (26.6 μM), sucrose (87.6 mM), casein hydrolysate (25 mg·L^{-1}), indole butyric acid (IBA, 24.6 μM) and pH adjusted to 5.8 prior to autoclaving. Both media contained either phytagel (0.25%) or agar (0.8%). Five to six shoots were placed in each phytatray containing rooting medium. Cultures were incubated under light as described earlier for 6 to 8 weeks. Randomly selected plants were transferred to peat pots filled with greenhouse soil mix and incubated under light at room temperature for 2 weeks. Established plants were then transferred to the greenhouse and later transplanted to large size pots (7.6 L pot). Data on plant survival was collected after establishment in the greenhouse pots. Some of these plants (22 'Gale Gala' and 23 'Bartlett') were also transferred to the field (Van Well Nursery) in Summer 1999 for future evaluation.

Collection and analysis of data. Shoots from each slice and shoots from each source stem were counted four to 6 weeks after stem slice culture. A randomized block experimental design was applied for single variance of analysis (treatments vs. shoots per slice) because of unequal numbers of stem slices in each treatment. Mean values for each treatment were calculated using Statgraphics software (Statgraphics Plus, v. 4.0; Manugistics, Rockville, Md.). One-way analysis of variance was carried out with Statgraphics software to identify differences among treatments using an F test at a 95% level of confidence ($P \leq 0.05$) and results of multiple range test was used to separate homogeneous groups among treatments.

Results

Multiple shoot induction in apple. Induction of apple shoots from stem slices was evident within two weeks of culture (Fig. 1a). A majority of slices showed shoot induction. Some slices turned green without shoots and only a few slices showed multiple induction of shoots (Fig. 1a). These shoots grew and finally established in propagation medium (Fig. 1 b and c). More than 17% of stem slices produced shoots with all treatments in apple (Table 2). Almost all treatments enhanced the frequency of shoot induction (17% to 38%) in the slices exposed to dark conditions prior to their incubation in light (Table 2). There were significant differences in the mean number of shoots among all treatments. Slices cultured on GM1 (light) and GM3 (dark) media showed high mean number of shoots per cultured slice. With the exception of GM1 (light), the mean values of shoots from each cultured slice in GM3 (dark) (0.52 ± 0.07) was significantly different from mean

Table 1. Media used for rapid shoot induction in apple (cv. Gale Gala) and pear (cv. Bartlett).

		Composition (μM)			
Species	Medium	BAP	TDZ	Kinetin	Gelling agent (%)
Apple (cv. Gale Gala)[z]	GM1	6.6	---	---	Phytagel (0.25)
	GM2	6.6	---	---	Agar (0.8)
	GM3	---	9.08	---	Phytagel (0.25)
	GM4	---	9.08	---	Agar (0.8)
Pear (cv. Bartlett)[y]	BM1	13.2	---	23.2	Phytagel (0.25)
	BM2	13.2	---	23.2	Agar (0.8)
	BM3	---	2.7	---	Phytagel (0.25)
	BM4	---	2.7	---	Agar (0.8)

[z]WPM salts, B5 vitamins, cefotaxime (418.8 μM), glycine (26.6 μM), and sucrose (87.6 mM).
[y]QL salts, Staba vitamins, cefotaxime (628.2 μM), glycine (26.6 μM), and sucrose (87.6 mM).

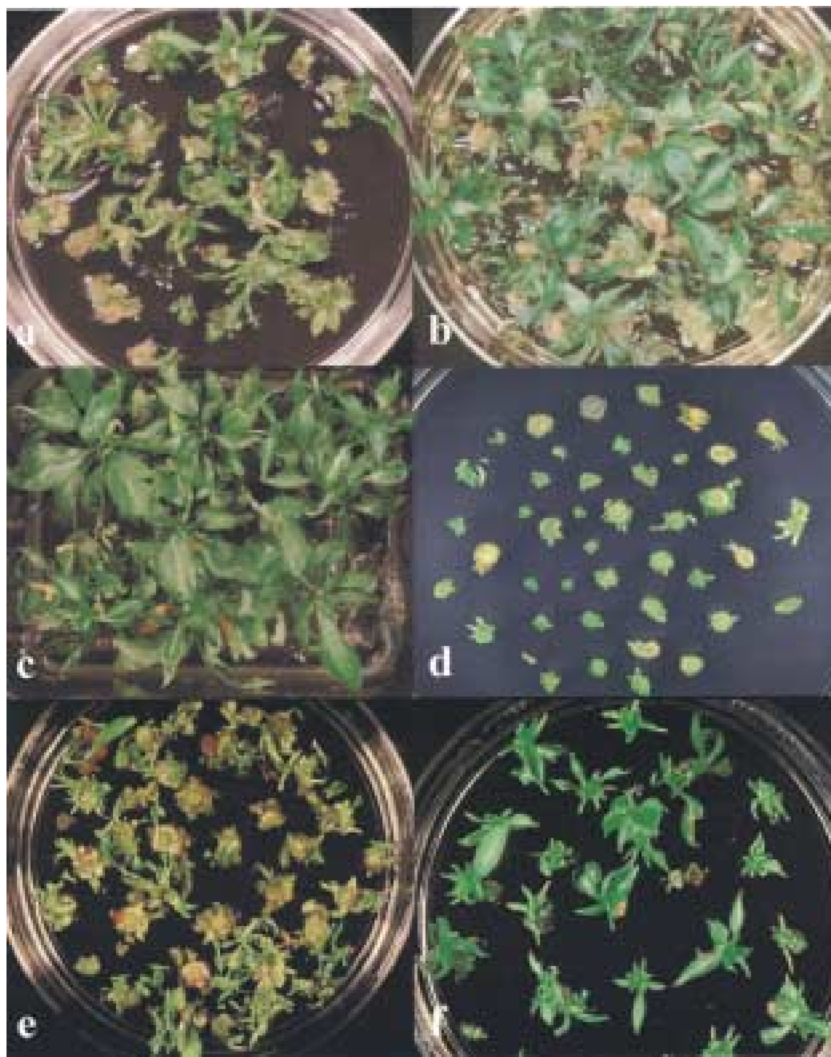

Fig. 1. Rapid multiplication and recovery of shoots from stem slices in apple and pear. Induction of apple shoots: (**a**) 2 weeks after culture in GM1 (light) medium; (**b**) 4 weeks after culture in GM1 (light) medium; and (**c**) established shoots in the micropropagation medium. Induction of pear shoots (**d**) slices at the time of culture in BM1 (light) medium; (**e**) 2 weeks after culture in BM1 (light) medium; and (**f**) 4 weeks after culture in micropropagation medium (shoots were separated and transferred to micropropagation medium).

values of shoots from other treatments. A total of 142 shoots from five source stems in GM1 (light) (i.e., 28.4 ± 4.9 shoots per source stem), and a total of 119 shoots from six source stems in GM3 (dark) (i.e., 19.8 ± 4.5 shoots per source stem) medium were obtained (Table 2).

Among all treatments, slices cultured on GM1 (dark) medium showed the lowest frequency of shoot induction (16.7%) and lowest mean number of shoots per cultured slice (0.17 ± 0.07) or source stem (7.0 ± 4.9). The frequency and recovery of shoots was also lower with slices cultured on agar solidified medium (GM2 and GM4), however, statistically no significant differences were observed with gelling agents or plant growth regulators (Table 2).

Multiple shoot induction in pear. Examples of stem slices at the time of culture and recovery of multiple shoots in pear are illustrated in Fig. 1d–f. Presence of multiple axillary buds on most stem slices immediately after cross-sectional incisions along a source stem is evident in Fig. 1d. Most pear stem slices showed multiple induction of shoots and very few showed no shoot induction (Fig. 1e). Representation of normal shoot growth on propagation medium in a Petri plate after separation or slice with single shoot is illustrated in Fig. 1f.

A high percentage of shoots was recovered from cultured slices in Phytagel solidified BM1 and BM3 medium (Table 2). A maximum of 97.4% of cultured slices showed shoot induction in BM3 (dark) medium, and BM4 (light) medium showed a low percentage (55.5%) of shoot induction. Significantly higher induction of shoots per cultured slice were recovered from BM1 (light) and BM3 medium [3.07 ± 0.20 shoots from BM1 (light), and 1.72 ± 0.08, and 2.02 ± 0.08 shoots, respectively from BM3 (light) and BM3 (dark) medium]. Stem slices cultured on agar solidified medium showed a relatively low induction of multiple shoots (Table 2). Slices that were given dark treatment initially showed a higher average number of shoots per slice than those directly incubated in light.

Results on shoots per source stem indicate that a high recovery of shoots per source stem is obtained in BM3 medium with mean value of 71.2 ± 17.2 (light) and 79.0 ± 12.1 (dark). Slices from four source stems cultured in BM3 (light) produced 285 shoots, and slices of four stems cultured in BM3 (dark) generated 316 shoots. Stem slices initially cultured 2 weeks in dark in BM1 medium show higher recovery of shoots than source stem slices that were directly incubated in light (Table 2).

Rooting of propagated shoots and transfer of plants into soil. Agar solidified medium showed higher percentage of rooting both in apple (91%) and pear (74%) while apple showed higher percentage of soil establishment (98%) than pear (56%) in the greenhouse (Table 3). A total of 22 'Gale Gala' and 23 'Bartlett' plants were transferred to the nursery (Van Well Nursery) for evaluation under field conditions. These plants established well in the nursery with normal growth and developmental characteristics, compared to conventionally grown plants at the nursery. These plants that were planted in 1999 growing season may flower and fruit in the year 2003.

Table 2. Rapid recovery of shoots using stem slice method in apple cv. Gale Gala and pear cv. Bartlett following preconditioning treatment of micropropagated shoots.

Medium/ light +/–[z]	Total no. of Stems	Slices	Slices with shoots (%)	Mean no. of shoots per[y] Slice	Stem
			Apple cv. Gale Gala		
GM1 +	5	279	74 (26.5%)	0.50 ± 0.06 ab	28.4 ± 4.9
GM1 –	5	198	33 (16.7%)	0.17 ± 0.07 c	7.0 ± 4.9
GM2 +	6	220	37 (16.8%)	0.30 ± 0.07 bc	11.0 ± 4.5
GM2 –	5	185	52 (28.1%)	0.40 ± 0.07 b	15.0 ± 4.9
GM3 +	4	181	63 (34.8%)	0.46 ± 0. 07 b	21.0 ± 5.5
GM3 –	6	228	84 (36.8%)	0.52 ± 0.07 a	19.8 ± 4.5
GM4 +	6	176	33 (18.7%)	0.39 ± 0.08 bc	13.1 ± 4.5
GM4 –	3	149	47 (31.5%)	0.24 ± 0.08 bc	12.0 ± 6.4
			Pear cv. Bartlett		
BM1 +	2	27	22 (81.5%)	3.07 ± 0.20 a	41.5 ± 17.2
BM1 –	4	40	38 (95.0%)	1.47 ± 0.16 d	14.7 ± 12.1
BM2 +	2	72	47 (65.3%)	0.80 ± 0.12 e	29.0 ± 17.2
BM2 –	2	74	52 (70.3%)	1.05 ± 0.12 e	39.0 ± 17.2
BM3 +	4	164	148 (90.2%)	1.72 ± 0. 08 cd	71.2 ± 12.1
BM3 –	4	156	152 (97.4%)	2.02 ± 0.08 b	79.0 ± 12.1
BM4 +	2	36	20 (55.5%)	1.56 ± 0.17 cd	28.0 ± 17.2
BM4 –	3	107	68 (63.5%)	1.89 ± 0.10 bc	67.0 ± 14.0

[z]Stem slices were directly placed under cool-white fluorescent lights ("+"); or in dark for first two weeks and then transferred to light conditions ("–").
[y]Mean separation within column (slice) by the same letter is not significantly different at $P \leq 0.05$. Mean number of shoots per slice was obtained by dividing total number of slices that showed shoot induction with total number of slices that were cultured.

Table 3. Rooting of shoots produced by stem slice method and establishment of plants in the greenhouse for four months and then transferred to the field in the summer of 1999.

Rooting medium	No. of Shoots transferred to medium	Plants rooted (%)	Plants transferred to greenhouse	Plants surviving (%)	Plants in field
		Apple cv. Gale Gala			
GR1[z]	90	72 (80%)	54	53 (98%)	22
GR2[y]	45	41 (91%)			
		Pear cv. Bartlett			
BR1[z]	65	5 (8%)	50	28 (56%)	23
BR2[y]	98	73 (74%)			

[z]Phytagel solidified medium.
[y]Agar solidified medium.

Discussion

We describe a rapid multiplication method for the clonal propagation of apple (cv. Gale Gala) and pear (cv. Bartlett). The schematic representation of the procedure is illustrated in Fig. 2. The success of the procedure depends upon the density of leaves (i.e., axillary buds) in the source shoot material and was achieved through pre-conditioning of the in vitro propagated source shoots. Induction of multiple shoots is achieved as a result of the reorganization of the axillary meristems within a short period of time after slicing the source stem into several pieces. In maize, it was shown that the microsurgically bisected embryonic axis which contains shoot apical meristem halves of immature embryos took 6 d to reorganize into a complete meristem in the process of becoming a normal plant (Bommineni et al., 1995). Although histological studies were not carried out in apple and pear, we assume that recovery of shoots occurred through reorganization of axillary meristems after random slicing through the source stem. We obtained a maximum of 56 apple and 126 pear shoots from 1.5 cm long sliced source shoots (data not shown). Lower relative recovery of shoots in apple may be attributed to the low density of leaves achieved after the preconditioning treatment. Therefore, it would be interesting to evaluate factors that would enhance leaf density as well as recovery of shoots in different cultivars and rootstocks of apple.

Recovery of shoots from this method of rapid shoot multiplication is superior to existing methods of micropropagation. First, a high percentage of slices produced shoots, and second, an average of up to three shoots per slice (and an average of up to 79 shoots per source stem) were obtained in pear within three weeks of explantation (Table 2, Fig. 2). Shoots may be subjected to either another shoot multiplication cycle or to the rooting medium for mass production of plants (Fig. 2). Regular micropropagation methods in pear produce up to 5.4 shoots per transfer generation (e.g., Viseur, 1987). Micropropagation

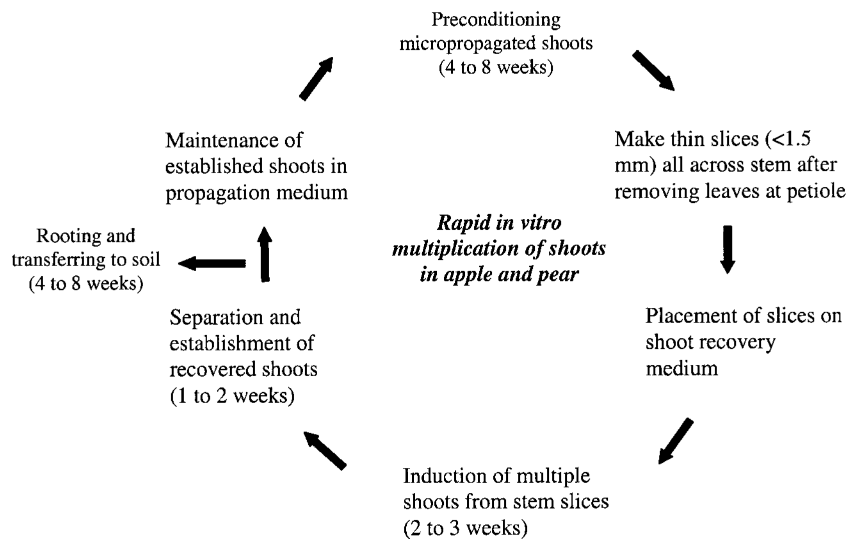

Fig. 2. Schematic representation of apple and pear in vitro clonal multiplication procedure.

of 13 different cultivars and rootstocks of apple produced 1 to 11.6 shoots per transfer generation, with total mean values ranging from 3.5 to 6.3 (e.g., Yepes and Aldwinckle, 1994). Another study found that an average of 8 to 10 shoots from subculture of each original culture was achieved through multiplication of isolated shoot apices in apple (Abbott and Whiteley, 1976). An average of five shoots per transfer generation was also routinely achieved with our micropropagation culture of apple and pear (data not shown). However, the benefit of the rapid shoot multiplication method as compared with existing in vitro propagation methods is in the greater number of plants that are generated in the same period of time. As compared to in vitro propagation methods, results of rapid shoot multiplication resulted in 4-fold production in apple and over 15-fold production in pear (Table 2).

Besides preconditioning treatment, two other factors seem to be important for efficient recovery of shoots. A combination of phytagel and TDZ resulted in a high percentage of shoot recovery as compared to solidifying with agar with the addition of BAP and kinetin (Table 2). In addition, we noticed both in apple and pear that shoots from BAP and kinetin on agar medium are slower growing than shoots from phytagel or TDZ supplemented shoot multiplication medium. Similar observations with agar were also noted with our micropropagation culture (data not shown). Earlier reports indicated that BAP was an important plant hormone for proliferation and growth in apple micropropagation (Yepes and Aldwinkle, 1994). Our data indicated that addition of TDZ in shoot induction medium significantly enhanced the recovery of shoots in comparison to BAP supplemented medium (Table 2).

In contrast to shoot induction medium, root induction was enhanced on agar solidified medium compared to phytagel. After examining auxins such as NAA, IBA, IAA, and 2,4-D (2,4-dichlorophenoxy acid) (data not included), we concluded that IBA in pear and IAA in apple were more suitable for optimal induction of roots (Table 3). Pear had a low percentage of survival and acclimatization after transfer to the soil, perhaps since most of the roots were induced through callus instead of originating directly from the base of the main shoot. Similar results were reported with NAA in apple, and lack of vascular connections was implicated as one of the reasons for low plant survival (Yepes and Aldwinckle, 1994). Although callusing at the basal portion of shoots was inevitable, reports in pear showed that NAA and other auxins (e.g., IAA and IBA) were successfully used to induce roots in several cultivars and accessions of pear with 50% to 73% plant survival after transferring into soil (e.g., Reed, 1995; Viseur, 1987).

All plants that were transferred to the orchard showed normal growth after a year. Unlike somatic embryogenesis in which tissues go through a cycle of de-differentiation and re-differentiation, these plants rapidly recovered through manipulation of axillary meristems or preformed meristematic cells at the leaf axils and therefore, we would not anticipate any somaclonal variation. In maize, it was reported that genomic rearrangements are less prevalent in meristem-derived plants as compared to plants regenerated from callus (Walden et al., 1989). However, several reports on field performance of micropropagated plants indicated poor anchorage (Larsen and Higgins, 1990), delayed flowering and fruiting, and low yield in the first few years of fruit production (Larsen and Higgins, 1990; Zimmerman and Miller, 1991). In addition, all self rooted micropropagated trees were larger than trees on a rootstock (e.g., M7a) (Larsen and Higgins, 1993). It was also noted that cumulative yield per tree after five years of production varied among self-rooted and budded trees—e.g., 'Rome' and 'Jonathan' showed higher yields with microprogated trees than budded ones, and reverse results were obtained with 'McIntosh'(Larsen and Higgins, 1993). Therefore, these studies showed that performance of micropropagated trees was highly cultivar dependent. Recent studies showed that very little morphological and reproductive somaclonal variation with axillary and adventitious bud derived in vitro 'Gala' and 'Royal Gala' trees (McMeans et al., 1998). Overall, these studies were concluded that tissue culture would be beneficial to propagate the tree cultivars. These studies found no obvious differences within a cultivar on fruit color but however, because of age difference (control trees were 18 months older), tissue culture derived trees showed more vegetative growth and low fruit set in the early years of fruit production (McMeans et al., 1998). Although micropropagated 'Gale Gala' and 'Bartlett' trees from our method are normal in one year growth, the performance of these plants in the coming years will determine the potential application of this procedure at a commercial level.

A rapid shoot multiplication procedure could have an enormous impact on our ability to rapidly multiply and maintain desirable tree fruit cultivars and rootstocks, while at the same time allowing plant availability throughout the year. As illustrated in Fig. 2, some of the shoots could be transferred to rooting medium for recovery of plants and remaining shoots could be subjected to preconditioning treatment for continuous production of shoots using the rapid in vitro shoot multiplication procedure. This method could also be applied in genetic engineering and biotechnology research for rapid improvement of tree crops.

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