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

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Additional index words: axillary buds, Malus ×domestica, micropropagation, Pyrus communis, shoot multiplication, stem slices

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. 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 phytogal solidified shoot induction medium than on agar. Cultured stem slices of both apple and pear showed maximum regeneration 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 thidiazuron (TDZ) compared to medium supplemented with BAP and kinetin. A maximum of 37% of cultured apple stem slices, and 97% of pear stem slices, showed induction of shoots. More shoots were recovered on phytogal solidified shoot induction medium than on agar. Cultured stem slices of both apple and pear showed maximum regeneration 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-y lurea (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 production of new tree fruit cultivars. As new cultivars continue to replace older cultivars, a rapid in vitro vegetative propagation system is 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 (3.9 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.

Revised for publication 8 June 2000. Accepted for publication 6 Mar. 2001. We thank the U.S. Dept. of Commerce, National Institute of Standards and Technology for providing matching funds in the tree fruit research; Van Well Nursery, Wenatchee, Wash., for supplying the plant material; Susan Bovee and Nickki Bristol for media preparation; Nancy Webb for her initial help on micropropagation; and Peter Lentz for maintenance of plants in the greenhouse. We also thank Stephanie Clendennen and Alex Liu for their valuable comments on earlier drafts and Karen Wolff on final draft of the manuscript. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertising solely to indicate this fact.

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Materials and Methods

Micropropagation of apple and pear. Micropropagation of apple (cv. Gala 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 µmol·m–2·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 µg), 6-benzylaminopurine (BAP, 4.4 µM), kinetin (13.9 µM) 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 µg), 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 to developing a rapid clonal multiplication system in both apple and pear. We have used in vitro propagated shoots to develop a rapid clonal multiplication procedure in apple (cv. Gala 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.
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).


tooting 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 \( \mu \)M), casein hydrolysate (25 mg L\(^{-1}\)), sucrose (87.6 mM), indole-3-acetic acid (IAA, 17.1 \( \mu \)M), and pH adjusted to 5.8 prior to autoclaving. Pear rooting medium consisted of QL salts, Staba vitamins, glycine (26.6 \( \mu \)M), sucrose (87.6 mM), casein hydrolysate (25 mg L\(^{-1}\)), indole butyric acid (IBA, 24.6 \( \mu \)M) 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. 1b 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

\[ \text{Table 1. Media used for rapid shoot induction in apple (cv. Gale Gala) and pear (cv. Bartlett).} \]

<table>
<thead>
<tr>
<th>Species</th>
<th>Medium</th>
<th>BAP (µM)</th>
<th>TDZ (µM)</th>
<th>Kinetin (µM)</th>
<th>Gelling agent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple (cv. Gale Gala)</td>
<td>GM1</td>
<td>6.6</td>
<td>---</td>
<td>---</td>
<td>Phytagel (0.25)</td>
</tr>
<tr>
<td></td>
<td>GM2</td>
<td>6.6</td>
<td>---</td>
<td>---</td>
<td>Agar (0.8)</td>
</tr>
<tr>
<td></td>
<td>GM3</td>
<td>---</td>
<td>9.08</td>
<td>---</td>
<td>Phytagel (0.25)</td>
</tr>
<tr>
<td></td>
<td>GM4</td>
<td>---</td>
<td>9.08</td>
<td>---</td>
<td>Agar (0.8)</td>
</tr>
<tr>
<td>Pear (cv. Bartlett)</td>
<td>BM1</td>
<td>13.2</td>
<td>---</td>
<td>23.2</td>
<td>Phytagel (0.25)</td>
</tr>
<tr>
<td></td>
<td>BM2</td>
<td>13.2</td>
<td>---</td>
<td>23.2</td>
<td>Agar (0.8)</td>
</tr>
<tr>
<td></td>
<td>BM3</td>
<td>---</td>
<td>2.7</td>
<td>---</td>
<td>Phytagel (0.25)</td>
</tr>
<tr>
<td></td>
<td>BM4</td>
<td>---</td>
<td>2.7</td>
<td>---</td>
<td>Agar (0.8)</td>
</tr>
</tbody>
</table>

\( ^{a} \text{WPM salts, B5 vitamins, cefotaxime (418.8 µM), glycine (26.6 µM), and sucrose (87.6 mM).} \)

\( ^{b} \text{QL salts, Staba vitamins, cefotaxime (628.2 µM), glycine (26.2 µM), and sucrose (87.6 mM).} \)

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).

HortScience, Vol. 36(6), October 2001
is obtained in BM3 medium with mean value that a high recovery of shoots per source stem than those directly incubated in light. Fied medium showed a relatively low induc-

end 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 solidi-

fied 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. Ex-

amples 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 induc-

tion (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 recov-

ered from cultured slices in Phytagel solidi-

fied 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 percent-

age (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 solidi-

fied medium showed a relatively low induc-

tion 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) gener-

ated 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 trans-

fer of plants into soil. Agar solidified me-

dium showed higher percentage of rooting both in apple (91%) and pear (74%) while apple showed higher percentage of soil establish-

ment (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 estab-

lished well in the nursery with normal growth and developmental characteristics, compared to conventionally grown plants at the nurs-

ey. These plants that were planted in 1999 growing season may flower and fruit in the year 2003.

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 de-

pends upon the density of leaves (i.e., axil-

lary buds) in the source shoot material and was achieved through pre-conditioning of the in vitro propagated source shoots. Induc-

tion of multiple shoots is achieved as a result of the reorganization of the axillary mer-

isims 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 mer-

istem in the process of becoming a normal plant (Bommireni 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 dif-

erent cultivars and rootstocks of apple.

Reciprocal shoots from this method of rapid shoot multiplication is superior to ex-

isting 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

multiple shoot induction or to rooting pro-

tocols. Therefore, it would be interesting

to evaluate factors that would enhance leaf density as well as recovery of shoots in dif-

erent cultivars and rootstocks of apple.

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

<table>
<thead>
<tr>
<th>Medium/</th>
<th>Total no. of</th>
<th>Mean no. of shoots per</th>
</tr>
</thead>
<tbody>
<tr>
<td>light</td>
<td>Stems</td>
<td>Slices</td>
</tr>
<tr>
<td>GM1 +</td>
<td>5</td>
<td>279</td>
</tr>
<tr>
<td>GM1 –</td>
<td>5</td>
<td>198</td>
</tr>
<tr>
<td>GM2 +</td>
<td>6</td>
<td>220</td>
</tr>
<tr>
<td>GM2 –</td>
<td>5</td>
<td>185</td>
</tr>
<tr>
<td>GM3 +</td>
<td>4</td>
<td>181</td>
</tr>
<tr>
<td>GM3 –</td>
<td>6</td>
<td>228</td>
</tr>
<tr>
<td>GM4 +</td>
<td>6</td>
<td>176</td>
</tr>
<tr>
<td>GM4 –</td>
<td>3</td>
<td>149</td>
</tr>
</tbody>
</table>

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.

| No. of | Rooting medium | Shoots transferred to medium (%) | Plants rooted Plants transferred Plants surviving Plants surviving Plants in field |
|--------|----------------|---------------------------------|---------------------------------|---------------------------------|
| Stems  | Slices Slices with shoots (%) | Slice Stem | green house (%) | greenhouse (%) |
| Apple cv. Gale Gala |
| GR1*  | 90 | 72 (80%) | 54 | 53 (98%) | 22 |
| GR2*  | 45 | 41 (91%) | 50 | 28 (56%) | 23 |
| Pear cv. Bartlett |
| BR1*  | 65 | 5 (8%) | 73 (74%) | 50 | 28 (56%) | 23 |
| BR2*  | 98 |

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.

*Phytagel solidified medium.

†Agar solidified medium.
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).

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

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 Aldwinckle, 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 BIA in a 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 micropropagated 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 ‘Gala 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 to 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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