AN ABSTRACT OF THE THESIS OF

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Title: Micropropagation of *Pyrus* Rootstocks

Abstract approved: _____

Barbara M. Reed

The availability of new pear rootstocks could be increased by development of an efficient micropropagation system. Many *P. calleryana*, *P. betulifolia* and *P. communis* rootstocks are resistant to many pear diseases and are graft compatible with leading cultivars. Explants of three potentially useful rootstock selections *P. calleryana* Decne 'OPR 157', *P. betulifolia* Bunge 'OPR 260', and *P. communis* L. 'Old Home x Farmingdale 230' were initiated from forced branches of field-grown trees. Combinations of N^6-benzyladenine (BA) at 0, 2, 4, 8 and 13 μM and Napthaleneacetic acid (NAA) at 0, 0.5, 1, 2 and 4 μM were tested on Woody Plant Medium (WPM) and Cheng medium and BA with Indole-3-butyric acid (IBA) on Cheng for shoot multiplication and elongation. Rooting was induced with NAA and IBA.

Increased shoot proliferation was observed for all three genotypes with BA concentration being the most influential factor. For OPR 260 Cheng medium with IBA produced more shoots than Cheng with NAA and increased BA concentrations improved multiplication for either auxin. Shoot proliferation was higher for shoots grown on WPM

with NAA than on Cheng with NAA and multiplication rates were lower for plants on media with NAA than with IBA. NAA concentration greater than 0.5 μM were inhibitory to shoot multiplication. Overall, the best micropropagation medium for OPR 260 was Cheng medium with 8 μM BA and 0.5 μM IBA.

For OHxF 230 NAA at 0.5 μM and IBA at 0.5 and 1 μM were stimulatory to shoot multiplication but higher levels were inhibitory. Shoots on Cheng medium proliferated better than shoots on WPM. The best medium for micropropagation was Cheng medium with 8 μM BA and 0.5 μM IBA.

Shoot multiplication of OPR 157 was better on NAA than IBA and lower auxin concentrations (0, 0.5 μM) were best with higher levels inhibiting multiplication. Shoots on either Cheng or WPM with NAA produced high multiplication rates and shoot proliferation increased with increasing BA concentrations up to 8 μM. Based on shoot proliferation and plant appearance, OPR 157 was best micropropagated on Cheng or WPM medium with 8 μM BA and 0.5 μM NAA.

Rooting (>80%) was easily achieved for OHxF 230 with all IBA and NAA treatments. The best rooting (42.9%) for OPR 260 was on Cheng medium with 10 μM IBA (dark) and for OPR 157 (23.9%) was with a 15 sec dip in 10 mM NAA. All rooted plantlets survived four weeks of greenhouse acclimatization.

Micropropagation of *Pyrus* Rootstocks

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BA (N^6-benzyladenine)

2iP (6-dimethylallylamino purine)

DMSO (Dimethylsulphoxide)

GA^3 (Gibberellic acid)

IAA (Indole-3-acetic acid)

IBA (Indole-3-butyric acid)

JA (Jasmonic acid)

MS (Murashige and Skoog)

NAA (Napthaleneacetic acid)

NGR (No growth regulators)

PG (Phloroglucinol)

SGRC (Standard growth room conditions)

TDZ (Thidiazuron)

WPM (Woody Plant Medium)

MICROPROPAGATION OF *PYRUS* ROOTSTOCKS

LITERATURE REVIEW

USES OF ROOTSTOCKS

Pear rootstocks are used to control or improve plant growth. They may impart precocity, dwarfing, influence fruit yield and quality, improve insect and disease resistance, and aid adaptation to wet or calcareous soils (Westwood and Lombard 1977, Bassi et al. 1989, Brooks 1984).

In Europe the genus *Cydonia* (quince) provides the most widely used rootstock. Quince's advantageous traits include good dwarfing, productivity and precocity but it is incompatible with leading pear cultivars such as 'Bartlett' and 'Beurre Bosc' (Bassi et al. 1989). Like many *Pyrus* clones, quince rootstocks are susceptible to fire blight and calcareous soils. In the US, *Pyrus communis*, *P. betulifolia* and *P. calleryana* cultivars are the most commonly used rootstocks. Some clones of *P. communis* and *P. betulifolia* are resistant to pear decline caused by a mycoplasma and transmitted by *Psylla pyricola* (Singha 1986, Westwood and Lombard 1977). In 1915, Reimer produced seedlings resistant to fire blight from a cross of *P. communis* cultivars Old Home and Farmingdale (OHxF). OHxF rootstocks offer size control, are resistant to fire blight and are graft compatible with many leading cultivars (Westwood et al. 1976). Evaluation of large numbers of pear genotypes for use as rootstocks resulted in selected clones of Oregon Pear Rootstock (OPR), *P. betulifolia* and *P. calleryana*, and OH x F, *P. communis* that

were resistant to pear decline (Westwood 1961, 1963, 1967, 1971). *P. calleryana* rootstocks impart dwarfing, precocity, fire blight and leaf spot resistance, high fruit yield and compatibility with many European and Asian pear cultivars (Westwood and Lombard 1966,1984, Westwood and Stebbins 1976, Westwood 1967). *P. betulifolia* is a vigorous rootstock with high yield (Westwood 1982) especially as a rootstock for 'Bartlett' pear (Fallahi and Larson 1986). *P. communis* (OHxF) rootstocks increase calcium uptake in scions which helps to control cork spot of 'Anjou' pears. In some cases OHxF rootstocks are dwarfing (Lombard and Westwood, 1987).

Several pear rootstocks were selected by Dr. Melvin Westwood at Oregon State University for their adaptability to Oregon conditions. OHxF 230 (*P. communis* L) is a semi-dwarf fireblight resistant rootstock with a smooth leaf surface and edge with medium-green leaf color. OPR 157 (*P. calleryana* Decne) is a semi-vigorous clone, compact in stature with smooth, dark entire green leaves. OPR 260 (*P. betulifolia* Bunge) is a vigorous crown-gall resistant rootstock with serrated, light-green leaves which are smooth when young but develop more trichomes with age. Each of these rootstocks has characteristics which make it useful to growers. All three selections are graft compatible with many scion cultivars including quince which is incompatible with the Asian pears. *P. betulifolia* rootstocks, while vigorous, produce high fruit yield, and do well in clay and poorly drained soils (Lombard and Westwood 1987). *P. betulifolia* rootstocks are also resistant to pear decline and fire blight, two major pear diseases (Brooks, 1984). *P. communis* rootstocks are winter hardy and are adaptable to the northern US while *P. calleryana* and *P. betulifolia* are more suited to warmer climates such as California and

southern Oregon. All three rootstock species influence fruit flavor by increasing the amount of the organic acids, malic and citric acids, in the fruits (Westwood, 1993). While clonal propagation of some *Pyrus* cultivars and species has been achieved by conventional hardwood stem cuttings, trenching or mound layering, many are difficult to propagate (Westwood 1966). The development of a micropropagation system for these rootstocks would make them more available for use by growers. Micropropagation provides a way to increase valuable selections that cannot be efficiently propagated conventionally (Gupta et al. 1981, Yu and Reed, 1993, Messeguer and Mele; 1983) or dioecious plants in which the valued male or female plants can then be perpetuated (Yang, 1977). A reliable method of producing true-to-type plantlets has been to use shoot tip and axillary bud cultures (Bhojwani and Razdan, 1992). By using the appropriate explant and culture conditions, somaclonal variations can be minimized to produce true-to-type clones (Larkin and Scowcroft, 1981). Micropropagated rootstocks can be pest and virus free, propagation rates may be higher and plantlets can be generated at any time of the year (Bhojwani and Razdan, 1992).

MICROPROPAGATION

Initiation

In pear micropropagation, most researchers use shoot tips excised either from field-grown or greenhouse mother plants to initiate aseptic cultures (Dolcet-Sanjuan et al. 1990, Hirabayashi et al. 1987, Yotsuya et al. 1980, Maarri et al. 1986). These shoot tips

are sterilized in 0.5% to 10% bleach solutions (5.25% sodium hypochlorite) with a few drops of detergent for a period of 10-20 mins (Dolcet-Sanjuan 1990, Hirabayashi et al. 1987, Bhojwani et al. 1984, Maarri et al. 1986). Calcium hypochlorite (alone or with sodium hypochlorite) in combination with 95% or 70% ethanol have also been used to surface-sterilize *P. communis* (Visuer 1987, Rodriguez et al. 1991), quince (Maarri 1986), and *P. calleryana* (Berardi et al. 1993). Contamination and oxidation of tissues may limit the establishment of explants. Singha (1982) reports a 30% loss of *P. communis* explants from greenhouse grown plants due to tissue discoloration. Hirabayashi (1987) documents a 1% contamination rate for *P. serotina* L cv. Hosui shoot tips collected from greenhouse-grown trees.

GROWTH REGULATORS

Cytokinins

BA (N^6-benzyladenine) is the most effective and frequently used cytokinin for enhancing the multiplication of *Pyrus*. The optimal BA concentration varies with genotype. Dolcet-Sanjuan et al. (1990) report optimal shoot proliferation for *P. betulifolia* OPR 266 at 10 and 20 µM BA. Nicolodi and Pieber (1989) optimized multiplication for *P. betulifolia* clones regenerated from seedling shoot meristems using BA at 17.7 µM for clone 3 and 8.8 µM for clones 5 and 9. In contrast, Pasqual (1991) achieved optimal proliferation of *P. betulifolia* with 2.22 µM BA.

Stimart and Harbage (1989) found that 2 µM BA + 0.5 µM IBA is optimal for

shoot proliferation of *P. calleryana* cv Bradford and that 10 and 20 μM BA induce maximal production of shoots but are not the best for shoot height. Dolcet-Sanjuan et al. (1990) report that 10 to 20 μM BA is the optimal range for shoot multiplication of *P. calleryana* 'OPR 191'. Berardi et al. (1993) documented that 2 μM BA was required for *P. calleryana* clones 3 and 4 while 4.4 μM BA was needed for other clones to reach maximal shoot proliferation from seedlings.

P. communis is the most common species investigated to date (Abu-Qauod et al. 1990, Singha 1980, Lane 1979, Dolcet-Sanjuan et al. 1990, Rodriguez et al. 1991 and Shen and Mullins 1984). The highest multiplication rate for 'Seckel' pear occurs with 8.8 μM BA (Singha et al. 1990), OHxF 51 with 4.4 μM BA (Cheng 1979), 'Bartlett' with 5 μM BA (Lane 1979) , 'Conference' with 8.8 μM BA (Baviera 1989) and 'Seckel' and 'Anjou' with 10 or 20 μM BA (Dolcet-Sanjuan et al. 1990).

For *P. serotina L* cv. Hosui, 10 μM BA results in the highest shoot proliferation (Hirabayashi et al. 1987). Banno et al. (1989) report the best shoot multiplication for 'Hosui' with 4.44 μM BA + 0.49 μM IBA, while Bhojwani (1984) found 6.66 μM BA + 0.1 μM NAA best.

The only other single cytokinin used was thidiazuron (TDZ) for *P.communis* at concentrations of 1.1 to 0.5 μM (Abu-Qaoud et al. 1990). Shen and Mullins (1984) incorporated a combination of 2iP (6-dimethylallylamino purine) and zeatin at 5 μM each in combination with BA to proliferate *P. communis* 'Williams's Bon Chretien', 'Packham's Triumph' and 'Beurre Bosc'.

Auxins

When auxins are incorporated these include IBA (indole-3-butyric acid), IAA (indole-3-acetic acid) or NAA (napthyleneacetic acid). Low levels of IBA (0.5 μM) (Banno et al. 1989, Stimart and Harbage 1989) and NAA (1.6 μM) (Bhojwani et al. 1984) are the most effective and commonly used auxins for *Pyrus* multiplication.

Singha (1980) tested NAA (2.7 μM) + GA_3 (0.3 μM) with 4.4 μM BA but achieved the highest shoot proliferation of 'Seckel' at 8.4 μM BA alone. Shen and Mullins (1984) propagated cultivars 'William's Bon Chretien', 'Packham's Triumph' and 'Beurre Bosc' on MS supplemented with NAA (3 μM) with GA_3 (0.3-1.4 μM) and BA (6-10 μM). Lane (1979) found NAA (0.05 μM) with GA_3 (1 μM) had no effect on shoot proliferation of 'Bartlett' and that 5 μM BA alone was optimal. Visuer (1987) reported the highest multiplication rate for *P. communis* cultivars 'Durondeau', 'Conference', 'Doyenne du Comice' and 'Professor Molon' when GA_3 (0.3 μM) and NAA (0.05 μM) were combined with BA (4.4 μM). In contrast, IBA (0.5 μM) and GA_3 (0.3 μM) in combination with BAP (6.7 μM) reduced shoot multiplication of *P. communis* 'Abate Fetel', 'Precoce Morettini' and 'Guyot' (Rodriguez et al. 1991).

OTHER PLANT GROWTH REGULATORS

The growth regulator GA_3 is employed by some researchers (Viseur 1987, Chevreau et al. 1989) who report increased axillary bud elongation of *P. communis* while others (Lane 1979, Singha 1980, Rodriguez et al. 1991) found no significant effect. In

most *Malus* species, the addition of GA_3 at 0.3 μM is essential in the proliferation phase (Jones et al. 1977).

GROWTH MEDIA

Formulations

Of the three most cited plant media used for explant proliferation, MS (Murashige and Skoog, 1962) and modified MS are the most successful for *Pyrus*. Banno et al. (1989) used Woody Plant Medium (WPM) (Lloyd and McCown 1981) for the optimal multiplication of *P. serotina* Rehd (cvs. Nijisseki and Osa-nijisseki) while Abu-Qaoud et al. (1990), Chevreau (1989), and Visuer (1987) employed Lepoivre medium for the successful proliferation of *P. communis* cultivars. Moretti et al. (1991) combine the macronutrients of Lepoivre and micronutrients from MS to culture 'Kaiser', 'Max Red Bartlett' and 'Williams'. Both MS (Berardi et al. 1993, Dolcet-Sanjuan et al. 1990) and WPM (Stimart and Harbage 1989) have been used to micropropagate *P. calleryana*. There have been no comparisons between the two media to determine which is optimal. In *Malus* and *Prunus*, while MS medium (Jones et al. 1977, Seingre et al. 1991) is most commonly used, others employ modified WPM (Pevalek-Kozlina 1987) and Lepoivre medium (Druart 1980)

Solid/Overlay

A double-phase culture system employing solid agar overlayed with liquid medium

was used successfully by Rodriguez et al. (1991) and Viseur (1987) for shoot proliferation of *P. communis*. Rodriguez used MS basal salts with half the concentration of nitrates and double the level of $CaCl_2$ and $MgSO_4$. Viseur (1987) on the other hand used Druart's medium supplemented with Lepoivre's salts and Magara's amino acids. Wang (1991) reported a significant increase in shoot multiplication of *P. communis* rootstock BP10030 when the double-phase culture overlayed with Quiroin's basal salts was employed.

Agar Type

Proliferation and growth of pear cultures (*P. communis* cv. Seckel) are influenced by agar concentration. Singha et al. (1985) demonstrated that optimal *Pyrus* shoot proliferation was achieved at 0.6% to 0.9% Phytagar concentrations while medium with 0.3% Phytagar produced lignified stems, translucent leaves and a decreased number of shoots but had improved shoot elongation. The improvement in elongation at lower agar levels was attributed to the increased availability of elemental nutrients. An opposite response was seen in 'Almey' crabapple where it was observed that increasing the agar concentration from the optimal 0.3% decreased shoot proliferation (Singha et al. 1985). Bacto agar was shown to contain high amounts of sodium (Debergh 1983) and an overall growth rating of both crabapple and pear found Bacto agar was the least favorable type with increasing agar concentrations (Singha 1985). Similar experiments on three agar brands at 0.8% in half strength Murashige and Skoog medium showed powder agar to be superior to crude agar and Bacto agar for both shoot multiplication and growth of Japanese pears, *P serotina* cvs. Nijisseki, Osa-nijisseki, Shinsui, Kosui, Hosui and Yagumo

(Banno et al. 1989). In spite of these reports, Bacto agar is still being used for pear by several researchers (Rodriguez et al. 1991, Dolcet-Sanjuan et al. 1990, Visuer 1987). This could be explained by the relative insensitivity of pear to higher amounts of sodium and other elements (Singha et al. 1987).

OTHER MEDIUM COMPONENTS

Carbon Source

Sucrose has been generally used as the standard carbon source for most micropropagated plants. In pear tissue culture, while sucrose is the most common carbon source, glucose and fructose are also reported to support growth of *P. communis* (Coffin et al. 1976). As in pears, apple rootstocks are usually grown on sucrose but hydrolysed sucrose and sorbitol result in the highest proliferation rates in apple rootstock EMIX (Seingre et al. 1991). In the Rosaceace, 53 of 73 species are found to utilize sorbitol (Plouver 1955). Sorbitol is an important product of photosynthesis in the *Malus* genus (Coffin et al. 1976, Pua 1985). Galactose, while not supporting multiplication, is observed to inhibit hyperhydricity in *Prunus glandulosa* Thunb var sinensis, *Malus* sp and *Pyrus communis* clone FA6 (Druart 1988).

Amino Acids

The addition of purified amino acids or casein hydrolysate in vitro stimulates growth in some species but can also cause inhibition. Incorporating serine or glycine in

pear culture medium enhanced proliferation rates for pear cultures (Nedelcheva, 1990). In *Prunus glandulosa* Thunb var Sinensis, axillary branching is stimulated by adding L-methionine (50-100 mg•l-1) but is reduced when added with L-tyrosine or glutamic acid (100 $mg \cdot l^{-1}$) (Druart 1980). The addition of casein hydrolysate (50 $mg \cdot l^{-1}$) (Ochatt et al. 1992) and the antibiotic cefotaxime (400 $mg \cdot l^{-1}$) (Predieri et al. 1989) promote shoot regenerability in leaf explants of *P. communis*.

SHOOT TIP NECROSIS

Shoot-tip necrosis in micropropagated plants has been attributed to calcium (Ca) deficiency, lack of cytokinin and use of rooting media. Shoot-tip necrosis in potato (Sha et al. 1985) and chestnut shoots (Qiguang et al. 1985) in vitro is overcome when higher levels of Ca are added to the medium. Culture at temperatures below 25 C in B5 medium buffered with MES and supplemented with calcium gluconate alleviates shoot-tip necrosis in poplar (Block-de 1990). Shoot-tip necrosis in quince is controlled by increasing the Ca level in the medium but the addition simultaneously reduces both proliferation and hyperhydricity when cultured on 1.2% Phytagar (Singha et al. 1990).

ROOTING

Auxins

Rooting of cuttings of woody plants involves physiological, chemical and environmental factors. NAA and IBA enhance rooting in many woody plants (Stimart

and Harbage 1989, Berardi et al. 1991, Visuer 1987; Rodriguez et al. 1991, Bhojwani et al. 1984, Moretti et al. 1991). Cultivars like OHxF which are difficult to root by conventional means may be rooted more efficiently by culturing micropropagated shoots in Cheng medium supplemented with 5 µM IBA (Cheng 1979). Jones and Webster (1989) report that cuttings taken from micropropagated *Pyrus communis* rootstocks (code F.12.194) which are difficult to root by conventional methods, exhibited high levels of rooting in vitro when grown for four days on modified MS with 3 mg•l-1 IBA followed by three weeks on medium with no hormone.

Singha (1980) rooted *P. communis* cv. Seckel in vitro with the application of IBA and IAA. Roots induced at 2 $mg \cdot l^{-1}$ NAA developed callus around the point of origin and had small numbers of lateral roots. Lane (1979) reported induction of roots of 'Bartlett' pear with 1 µM IBA and 10 µM NAA with root development best at 1µM IBA. The shorter roots produced with 10 µM NAA were viewed as an advantage since shorter roots were less likely to be damaged during transfer to soil. Rodriguez et al. (1991) achieved rooting with minimal callus formation by dipping the basal end of the shoot in 5 µM IBA for 1 min for 'Abate Fetal', 'Precoce', 'Morettini' and 'Guyot'. Dipping the base of in-vitro plantlets in high concentrations of IBA (10 mM) and then transferring to a medium free of hormones has also been successful (Dolcet-Sanjuan et al. 1990). Moretti et al. (1991) successfully obtained rooting at 1 $mg \cdot l^{-1}$ of IBA with 'Williams' and 'Max Red Bartlett'. Wim et al. (1992) showed the effectiveness of IBA over IAA in producing a greater number of roots per shoot. It is also postulated that IBA may actually exert its activity by converting to IAA, but that IBA itself is also biologically active (Wim

et al. 1992). Either IBA or NAA, in combination with in-vitro cultural conditions, can effectively induce rooting in some pear cultivars (Shen and Mullins 1984, Cheng 1979, Banno et al. 1989, Dolcet-Sanjuan et al. 1990).

Other Chemical Treatments

Phloroglucinol (PG) has been used to improve in vitro rooting of *Malus* (Abbott 1976, Zimmermann and Broome 1981) and *Pyrus* (Bhojwani et al. 1984, et al. 1989, Singha 1980, Rossi et al. 1991) but its effectiveness is sporadic, varying from genotype to genotype. Jones (1976) first reported that phloridzin and phloroglucinol promoted root and shoot production in apple. The positive effect of PG is enhanced when the culture is grown in darkness (Wang 1991, Pontikis et al. 1984). PG at 162 $mg \cdot l^{-1}$, the concentration most frequently used, may not be the optimal level. Pontikis and Sapoutzaki (1984) report that 89 $mg \cdot l^{-1}$ PG promotes optimal shoot proliferation of 'Troyer' citrange but increasing the concentration to 178 $mg \cdot l^{-1}$ does not enhance proliferation. PG at 0, 40, 80, 120, 160, 200, 240 and 280 $mg \cdot l^{-1}$ had no appreciable effect on rooting of *P. calleryana* sel. D6 (Rossi et al. 1991). In contrast, PG at 162 $mg \cdot l^{-1}$ in combination with NAA (1 $mg \cdot l^{-1}$) stimulated rooting in several other clones of *P. calleryana* (Berardi et al. 1991). PG is shown to markedly stimulate growth, proliferation and rooting of Malling (M.26) apple rootstocks (Jones 1977, Jones and Hatfield 1976) and *Malus pumila* (Welander 1983) while PG is ineffective for *M. domestica* Borkh (Abbott 1976, Zimmermann and Broome 1981).

Vitamins participate in numerous physiological functions of cells and many play

a prominent role as co-enzymes. Vitamins (thiamine, niacin, riboflavin, pyridoxine, and biotin) are reported to stimulate in-vitro rooting ranging from elongation to production of laterals to increasing root numbers in peas (Adinarayana and Vijayamma 1991). Vitamin D and its analogues affect rhizogenesis in *Populus* spp. (Buchala and Schmid 1979). Dolcet-Sanjuan et al. (1990) report that the incorporation of vitamins did not significantly enhance rooting of *Pyrus* in vitro.

Physiology

While the application of auxins plays an important role in root induction, there are also physiological processes that can possibly increase or decrease rooting success. Peroxidase activity may denote the inductive and initiative phases of explants in apples and prunes (Druart 1982, Monocousin and Gaspar 1982). In *Malus*, total peroxidase activity increases under light and decreases in the dark but the phenol content behaves inversely so the level of phenols may be used as a marker to determine optimal timing for rooting experiments (Druart 1982, Monocousin and Gaspar 1982). The effect of darkness at the initiative phase is more beneficial than at inductive phase for *Cynara scolymus* (Monocousin 1982) while in 'Jonagold' apple rooting is improved when darkness is applied at both phases (Druart 1982). Elongation (the inductive stage) of *Prunus* (Quoirin 1974) and *Malus* shoots (Druart 1982, Welander 1991) prior to transfer to rooting medium improves rooting. The success of rooting may be closely linked to the internal levels of phenols and peroxidases as demonstrated by Druart (1982). When phenols are oxidized, they bind strongly to peroxidases and in doing so reduce enzyme

activity (Srivastava and Van Huystee 1977). Polyphenols, like PG, added at particular physiological stages may conjugate with phenols and render them less effective (Srivastava and Van Huystee 1977).

Endogenous levels of carbohydrates have been implicated in increased rooting of cuttings (Stolz 1968) but others have reported no effect (Struve 1980). Niaz and Westwood (1966) observed better rooting of 'Old Home' pears when cuttings were taken prior to any chilling or rest period. The stage of development, juvenile versus adult, at which the plants are taken for rooting may influence the success and the amount of auxins required (Welander, 1983).

CALLUS

A commonly used growth hormone, 2,4-dichlorophenoxyacetic acid (2,4-D), has been applied to cultures to initiate and to increase callus production even at relatively low concentrations (Bhojwani and Razdan 1992). Callus proliferation is sometimes promoted when using certain basal media. Fukui et al. (1989) report excessive production of callus when Japanese persimmon (*Diospyros kaki*) is cultured on Gamborg salts but less is observed on MS, and the least on WPM. Ishida et al. (1989) report a significant reduction in callus with concomitant increase in peach rootstock shoot proliferation when the ratio of nitrates and ammonium in MS salts is 1:3. Nitrates alone stimulated higher callus production compared to ammonium but neither alone gave optimal shoot formation. The addition of phloroglucinol at 162 $mg \cdot l^{-1}$ minimizes callus production in *Malus domestica* (Zimmermann and Broome 1981) and in *Pyrus pyrifolia* (Bhojwani et al. 1984).

High cytokinin levels also promote callus production (Fukui et al. 1989, Druart, 1980, Kamada and Harada 1979). Inhibition of cytokinin-induced callus by jasmonic acid and methyl jasmonate at 45 μM on soybean was reported by Ueda and Katoa (1982) and in potato by Ravnikar et al. (1990). Higher concentrations of jasmonic acid and its methylester are reported to promote leaf senescence in barley with callus suppression and adventitious bud enhancement observed only at very low levels (Weidhase et al. 1987). Litz and Schaffer (1987) report suppression of callus formation in 'Keitt' mango when the polyamine, spermidine, is incorporated into the medium.

PHENOLIC PRODUCTION

Browning of explants and media is a common occurrence in tissue culture (Preece and Compton 1991). Browning frequently interferes with the growth of the explants and may result in the death of the plants (Singha 1982). Sometimes browning is initiated at the onset of callus production and progresses into the medium surrounding the plants (Preece and Compton 1991, Bhojwani and Razdan 1992). Browning can also begin at the time of intiation (Messeguer and Mele 1983, Yu and Reed (in press). Browning ofin-vitro explants may be attributed to an enzymatic oxidation of phenolic constituents (Preece and Compton, 1991). High basal-salt content was shown to induce browning but reducing MS basal salts to half or less helped control it (Werner and Boe 1980). In some genotypes browning may be controlled with the addition of citric acid, ascorbic acid, PVP (polyvinylpyrrolidone), activated charcoal and L-cysteine (Preece and Compton 1991).

The use of PVP is associated with the removal of phenolics employed in enzyme

extractions and has been added to tissue culture media to prevent media browning (Gupta et al. 1980). Saxena and Gill (1986) incorporated PVPP (polyvinylpolypyrrolidone) into cell cultures of *Cyamopsis tetragonoloba* to effectively inhibit browning. PVPP (1%), molecular weight 40,000, effectively reduced browning of the media in which *Hamamelis* shoot tips were grown (Christiansen and Fonnesbech 1975). Other compounds used to control browning include sodium thiosulfate and dithiothreitol (Liu et al. 1986), maize extract (Ilahi et al. 1987) and alpha-tocopherol (Druart et al. 1980). Diluting the agar medium (Liu 1986) has been reported to minimize oxidative browning. Sugimoto et al. (1988) reported the suppression of browning in root cultures of *Stephania cepharantha* when 1 μM GA_3 is added to the medium.

OBJECTIVE

The objective of this research was to develop a suitable micropropagation system for three potentially useful rootstocks. These genotypes were *Pyrus calleryana* Decne 'OPR 157' (local #1844), *Pyrus betulifolia* Bunge 'OPR 260' (local #1379), and *Pyrus communis* L. 'Old Home x Farmingdale 230' (local #1360).

INTRODUCTION

Pear rootstocks are used to control or improve plant growth. They may impart precocity, dwarfing, influence fruit yield and quality, improve insect and disease resistance, and aid adaptation to wet or calcareous soils (Westwood and Lombard 1977, Bassi et al. 1989, Brooks 1984). Evaluation of large numbers of pear genotypes for use as rootstocks resulted in selected clones of Oregon Pear Rootstock (OPR), *P. betulifolia* and *P. calleryana*, and OHxF, *P. communis* that were resistant to pear decline (Westwood 1961, 1963, 1967, 1971). Several pear rootstocks were selected by Dr. Melvin Westwood at Oregon State University for their adaptability to Oregon conditions. OHxF 230 (*P. communis* L) is a semi-dwarf rootstock, OPR 157 (*P. calleryana* Decne) is semi-vigorous and OPR 260 (*P. betulifolia* Bunge) is vigorous. Each of these rootstocks has characteristics which make it useful to growers. All three selections are graft compatible with many scion cultivars, including quince which is incompatible with the Asian pears. *P. betulifolia* rootstocks, while vigorous, produce high fruit yield and do well in clay and poorly drained soils (Lombard and Westwood 1987). *P. betulifolia* rootstocks are also resistant to pear decline and fire blight (Brooks, 1984). *P. communis* rootstocks are winter hardy and adaptable to the northern US while *P. calleryana* and *P. betulifolia* are more suited to warmer climates such as California and southern Oregon. These rootstock genotypes are not available because they are not easily propagated. This study established the requirements for micropropagation of these three pear rootstocks.

MATERIALS AND METHODS

GENERAL GROWTH CONDITIONS

Stock cultures were grown on Cheng medium (Cheng 1978) containing the basal salts of Murashige and Skoog (1962) (MS) but with half the nitrogen and supplemented with 250 $mg \cdot l^{-1}$ myo-inositol, 2.5 $mg \cdot l^{-1}$ thiamine HCL, and 30 $g \cdot l^{-1}$ sucrose. Growth tests employed Cheng medium as above and Woody Plant Medium (WPM) (Lloyd and McCown 1981) supplemented with 100 $mg \cdot l^{-1}$ myo-inositol, 0.5 $mg \cdot l^{-1}$ nicotinic acid, 0.5 $mg \cdot l^{-1}$ pyridoxine HCL, 1.0 $mg \cdot l^{-1}$ thiamine HCl, 2.0 $mg \cdot l^{-1}$ glycine, and 30 $g \cdot l^{-1}$ sucrose. All media were adjusted to pH 5.2 with KOH/H_2PO_4 prior to the addition of 3 $g \cdot l^{-1}$ agar (Bitek, Difco, Detroit,MI) and 1.25 $g \cdot l^{-1}$ Gelrite (Kelco, San Diego, CA) and before autoclaving at 121°C for 20 mins. Standard growth room conditions (SGRC) were a 16 h photoperiod supplied by cool-white (Watt Miser) florescent bulbs (25 $\mu mol \cdot s^{-1} \cdot m^{-2}$) at 25°C.

INITIATION

Rootstock genotypes were *Pyrus calleryana* Decne 'OPR 157' (local #1844), *Pyrus betulifolia* Bunge 'OPR 260' (local #1379), and *Pyrus communis* L. 'Old Home x Farmingdale 230' (local #1360). Ecodormant branches, after their chilling requirement was met, were collected from mature 10-13 year old field-grown trees from the National Clonal Germplasm Repository at Corvallis, Oregon in February 1992 to be forced in the greenhouse. These shoots were pruned to 30-60 cm in length, washed with Tween 20

(polyoxyethylene sobitan, Sigma, St. Louis, MO) under running tap water and placed in containers filled with 'Floralife' (Floralife, Inc., Burr Ridge, Ill) at 9 g per 946 ml of luke warm water. Each week 1-2 cm of the basal end of the branches were pruned off and the solutions replaced. Leafy shoots were collected after a period of 3-4 weeks in the greenhouse. Additional explants were collected directly from field-grown trees in April 1992.

Shoot-tips and axillary buds (2-3 cm) were stripped of leaves and washed under running tap water with 5 drops of Tween 20 for 5 mins. Explants were disinfected in 10% commercial bleach (sodium hypochlorite 0.5%) with 5 drops of Tween 20 per 500 ml, shaken on a rotary shaker for 10 min, then rinsed 3x in sterile deionized, filtered water. Single node sections were transferred to 20 x 100 mm test tubes with 10 mls of Cheng medium supplemented with 4.4 μM BA (Sigma, St. Louis, MO). Uncontaminated explants were then transferred into Magenta GA7 (Magenta, Chicago IL) boxes containing 40 ml of Cheng medium and subcultured at 3 week intervals. Data taken included the number of plants growing, the number growing but contaminated and those that failed to respond.

MULTIPLICATION

Media tested were Cheng and WPM medium with 5 x 5 factorial combinations of BA at 0, 2, 4, 8, and 13 μM and NAA or IBA at 0, 0.5, 1.0, 2.0 and 4.0 μM. All cultures were randomly arranged under standard growth room conditions (SGRC) as described in the general growth conditions. The experiment was a 3 factor randomized

complete block design. Each block consisted of 5 shoots in a Magenta box per experiment, and the experiment was done three times (total of 15 shoots). The total treatment period was 6 weeks with one transfer in the third week. Shoot proliferation was scored based on the number of useable shoots produced that were at least 1 cm in height. Shoot height of the original explant was measured from the apex to the base of the stem. Optimal shoot multiplication was based on greener leaves and minimal chlorosis, mean shoot height greater than 1.2 cm and high proliferation rate.

Data were analyzed using factoral analysis from MSTATC (Michigan State University). Significance was recorded at the $P \leq 0.05$ level and only significant effects were discussed.

ROOTING

In Vitro Rooting

Four treatments and a no growth regulator control were tested on the three genotypes:

1. 10 mM NAA Dip: The base of shoots was dipped for 15 sec in 10 mM NAA dissolved in DMSO, then planted in medium with NGR in SGRC.
2. 10 mM IBA Dip: The base of shoots was dipped for 15 sec in 10 mM IBA dissolved in DMSO, then planted in medium with NGR in SGRC.

3. 10 μM IBA (Dark): Grown in medium with 10 μM IBA in the dark for 1 week then transferred to medium with NGR in SGRC.
4. 10 μM IBA (light): Grown in medium with 10μM IBA for 1 week in SGRC, then transferred to medium with NGR in SGRC.
5. Control: Cheng medium, NGR, SGRC.

The experiment was a 2 factor randomized complete block design consisting of three blocks per treatment with 5 shoots per block (magenta box), and the experiment was done twice (total of 30 shoots). The root length, callus size, number of roots per shoot and percent rooting were measured. All treatments were grown on Cheng medium with no growth regulators (NGR) under standard growth room conditions (SGRC) unless otherwise noted. Data were analyzed using the analysis of variance (ANOVA) from MSTATC (Michigan State University). Percentage data were arcsin square-root transformed before analysis. Significance was recorded at the $P \leq 0.05$ level and only significant effects were discussed.

Ex Vitro Rooting

Treatment consisted of 15 shoots per genotype and the experiment was repeated once for a total of 30 shoots. The base of shoots (2 cm in height) were dipped in a solution of Dip'N Grow (Astoria-Pacific, Inc., Clackamas, Oregon) (1:20 water v:v) then planted directly into pots filled with peat-perlite mix. The treated plantlets were placed in the mist bed for two weeks followed by another two weeks in the greenhouse. Dip'N

Grow is a commercial rooting preparation containing IBA (1%), NAA (0.5%) and inert materials (98.5%).

ACCLIMATIZATION

All rooted and non-rooted shoots from the in-vitro rooting treatments were rinsed under tap water to remove clinging agar before transplanting to 2" pot bands filled with 50:50 peat-perlite mix. The shoots were placed in a mist bed for two weeks, then transferred to the greenhouse for acclimatization for another two weeks.

RESULTS AND DISCUSSION

INITIATION

Explants collected from forced ecodormant branches were more than 90% free of contamination in all three genotypes and most grew into plants (Table 1). Contaminants were either fungi or bacteria. Explant materials collected directly from the field were all discarded due to contamination or browning (data not shown).

P. calleryana 'OPR 157' and *P. betulifolia* 'OPR 260' were difficult to establish in culture due to heavy callusing of the basal end and leaf axils or browning of medium and explants. After six to seven subcultures, browning was greatly reduced and callusing moderated. *P. communis* OHxF 230 was the slowest to grow of the three genotypes and also had high levels of medium browning.

There are few reports of survival rates for *Pyrus* explant initiation. Singha (1982) had a 30% loss of *P. communis* L collected from greenhouse-grown plants due to tissue discoloration while Hirabayashi et al. (1987) found only a 1% contamination rate for *P. serotina* L cv. Hosui shoot tips collected from greenhouse-grown trees. Bhojwani et al. (1984) mentioned that nodal explants taken from greenhouse plants reached a 90-95% survival rate and were free of contamination. Yu and Reed (in press) reported that greater survival was obtained from nodal segments of *Corylus* sp. than from shoot tips and better survival from the greenhouse than from the field due to high oxidation rates. We observed generally in pears that nodal segment explants had better survival rates than shoot-tips. Messeguer and Mele (1983) proposed taking hazelnut explants from autumn

branches forced in the greenhouse after their chilling requirements were met to decrease contamination rates. We also had high survival rates and low contamination for all explants taken from forced ecodormant shoots for the three genotypes.

MULTIPLICATION

Optimal multiplication in a micropropagation system is based on high multiplication rates as well as many more subjective factors such as an overall healthy appearance and suitable height for easy transfer. Combinations of BA were tested with NAA on the two basal media, Cheng or WPM and with IBA on Cheng. Shoot elongation was acceptable (≥ 1.2 cm) at all auxin concentrations and at BA concentrations below 13 µM (data not shown).

P. betulifolia 'OPR 260'

Shoot Multiplication on Cheng Medium with IBA or NAA

Shoot multiplication on Cheng medium was influenced by BA concentration and auxin concentration and type (Table 2). A dosage response was seen as BA levels were increased with either auxin (Fig. 1 and 2). IBA at 0.5µM and NAA at 0 and 0.5 µM produced the best shoot multiplication at all BA levels. There was no multiplication on medium without BA. Plants on medium with IBA produced more shoots than those on medium with NAA (Table 3). Shoot multiplication was significantly lower with auxin concentrations of 2 and 4 µM than 0.5 µM.

Shoot Multiplication on Cheng and WPM media with NAA

Shoot multiplication was significantly influenced by BA and NAA concentrations and by medium (Table 4). High shoot multiplication was obtained on 8 and 13 μM BA with 0.5 μM NAA (Fig. 2 and 3). Shoots grown on WPM produced more shoots than on Cheng (Table 5). NAA concentrations of 2 and 4 μM inhibited shoot multiplication compared with 0 and 0.5 μM.

The use of Cheng medium with low levels of IBA was beneficial to the optimal multiplication of *P. betulifolia* 'OPR 260'(Fig. 1). Other studies use only BA for multiplication. Nicolodi and Pieber (1989) reported on two levels of BA in three clones of *P. betulifolia*. These clones required 8 or 16 μM BA to reach their maximum multiplication rates on MS medium with a light intensity of 3000 lux. In contrast, Pasqual (1991) achieved the best proliferation of *P. betulifolia* at 2 μM BA under 2000 lux on MS medium. Dolcet-Sanjuan et al. (1990) reported increases in multiplication of *P. betulifolia* 'OPR 266' when grown under 40 or 135 $\mu Em^{-2}s^{-1}$ (1350 lux) at 10 or 20 μM BA on MS medium. Our results showed that 8 and 13 μM BA were maximum and multiplication was improved with 0.5 μM IBA and this might be further increased with higher light intensity as shown by Dolcet-Sanjuan et al. (1990).

Based on shoot proliferation and overall appearance including expanded leaves and shoot height, the best micropropagation for *P. betulifolia* 'OPR 260' was on Cheng medium with 8 μM BA and 0.5 μM IBA.

P. communis 'OHxF 230'

Shoot Multiplication on Cheng Medium with IBA or NAA

All factors interacted in the shoot multiplication of OHxF 230 but multiplication was mainly influenced by BA and auxin concentration and the interaction of BA and auxin concentration and to a lesser extent by auxin type (Table 2). The highest shoot multiplication was obtained with 8 and 13 μM BA with 0. 5 μM auxin (Fig. 4 and 5). Shoot means were greater for plants grown on medium with NAA than with IBA (Table 3) but the best proliferation was on 0.5 μM IBA (Fig. 4). NAA at 0.5 μM improved multiplication while higher levels were inhibitory (Fig. 5). IBA at 0.5 and 1 μM were stimulatory to shoot proliferation while increased levels were inhibitory (Fig. 4).

Shoot Multiplication on Cheng and WPM media with NAA

Shoot multiplication was influenced by both BA and NAA concentrations and medium type (Table 4). There was an interaction between BA and NAA concentrations with the best multiplication at 0.5 μM NAA with 8 and 13 μM BA (Fig. 5 and 6). The main influence was due to BA levels with 8 and 13 μM BA producing the most shoots. Cheng medium produced more shoots than WPM and NAA at greater than 0.5 μM inhibited shoot multiplication (Table 5).

Cheng was better than WPM for shoot multiplication of OHxF 230. Shoots of plants grown on Cheng were also greener than those of plants grown on WPM. Cheng

is a modified MS medium and MS or modified MS were used by others for successful multiplication of *P. communis*. Moretti et al. (1991) found 3.3 μM BAP on MS medium resulted in the greatest axillary multiplication of 'Williams', 'Max Red Bartlett' and 'Kaiser'. Rodriguez et al. (1991) obtained axillary proliferation of 'Abate Fetel' with 6.7 μM BAP in a double phase system on a modified MS. Lane (1979) found 5 μM BA resulted in optimal shoot multiplication on MS medium supplemented with casein hydrolysate. We found 13 μM BA to be the best for shoot multiplication of OHxF 230 which was similar to Shen and Mullins (1984) who found 8 μM BA was best for 'Packham's Triumph' and 10 μM BA for 'Beurre Bosc'. Dolcet-Sanjuan et al. (1990) found 10 and 20 μM BA were optimal for shoot multiplication of 'Seckel' and 'Anjou'. We observed that as the level of BA increased, the leaf size decreased (data not shown) as did Dolcet-Sanjuan et al. (1990).

Our results indicated that 0.5 μM NAA or IBA produced the best shoot proliferation ($P \leq 0.05$) while 2 and 4 μM concentrations inhibited multiplication. There are no reports of IBA effects on *P. communis* multiplication. A few researchers tested NAA at concentrations lower than 0.5 μM and found no beneficial effects (Lane 1979, Singha 1980, Baviera et al. 1989) while others omit the use of auxin altogether (Dolcet-Sanjuan et al. 1990). Lane (1979) found that NAA (0.05 μM) and GA_3 (1 μM) were slightly inhibitory to shoot proliferation in 'Bartlett'. Singha (1980) tested NAA at 2.7 μM and found it was ineffective in shoot proliferation.

The best micropropagation regime for *P. communis* 'OH x F 230' based on shoot proliferation and overall appearance was with Cheng medium with 8 μM BA and 0.5 μM

IBA. Although multiplication was highest at 13 µM the shoot height and appearance were best at 8 µM.

P. calleryana 'OPR 157'

Shoot Multiplication on Cheng Medium with IBA or NAA

The greatest influence on shoot multiplication for *P. calleryana* 'OPR 157' on was from BA concentration and auxin type and with a lesser effect from auxin concentration (Table 2). Shoot multiplication was best at 8 µM BA than at other levels (Table 3). Shoot multiplication was best with no IBA treatment (Fig. 7) or with 0 or 0.5 µM NAA (Fig. 8). Overall, medium with 0 or 0.5 µM NAA produced the highest shoot proliferation (Table 3).

Shoot Multiplication on Cheng and WPM media with NAA

Shoot multiplication was mainly influenced by BA concentration but NAA concentrations and medium type were also important (Table 4). Shoot multiplication was highest at 8 µM BA and declined at 13 µM due to the production of many shoots < 1 cm in height (Table 5). Shoots grown on WPM at 0 and 0.5 µM NAA (Fig. 8) with 0.5 µM NAA on Cheng had the highest proliferation (Fig. 9). Higher NAA levels decreased shoot multiplication.

BA at 10 and 20 µM produced the highest multiplication rates for *P. calleryana* 'OPR 191' on MS medium (Dolcet-Sanjuan et al. 1990), however, BA at 2 µM was

optimal for *P. calleryana* cv. Bradford (Stimart and Harbage 1989). Berardi et al. (1993) report variations in response to BA levels (0.5 $mg \cdot l^{-1}$ for clones three and four, 1 $mg \cdot l^{-1}$ for other clones) for optimal shoot proliferation of several *P. calleryana* clones derived from seedling cultures obtained from seeds of open pollinated *P. calleryana.* Seedlings would be expected to be variable in response while shoot tips taken from mature field-grown trees are clonally derived and show little variation (Stimart and Harbage 1989, Dolcet-Sanjuan et al. 1990).

Stimart and Harbage (1989) reported that 5 μM IBA reduced the multiplicative effects of BA but lower concentrations did not affect *P. calleryana* cv. Bradford. A similar inhibitory effect on multiplication was observed at higher levels of NAA (1, 2 and 4 μM) compared with 0.5 μM in our experiments with OPR 157 (Table 5). Berardi et al. (1993) reported that NAA at 0, 0.05 and 0.1 $mg \cdot l^{-1}$ did not affect shoot proliferation of *P. calleryana.*

The best micropropagation regime for *P. calleryana* 'OPR 157' based on shoot proliferation and overall appearance was on Cheng or WPM medium with 8 μM BA and 0.5 μM NAA.

GENERAL GROWTH CHARACTERISTICS

Callus formation was confined mainly to the basal end of the shoot in all three genotypes. In *P. betulifolia* 'OPR 260', soft gritty callus was occasionally found at the leaf axils. *P. calleryana* 'OPR 157' produced the most basal callus, compared with the other two genotypes, and it was semi-hard and nodular in texture. *P. communis* 'OHxF

230' developed basal calli that were hard and globular.

Stock plants of both OPR 157 and OPR 260 exhibited shoot fasciation sporadically but none was observed in the experimental treatments. No fasciation was noted in any OHxF 230 cultures. Reports of shoot fasciation of *P. pyrifolia* (Bhojwani et al. 1984), *P. communis* (Lane 1979, Visuer 1989) and *P. calleryana* (Stimart and Harbage 1989) have been reported. Stem fasciation is reported to decrease with the addition of IBA in *P. calleryana* 'Bradford' (Stimart and Harbage 1989). It has been suggested that in-vitro fasciation is the outcome of an imbalance between auxin and cytokinin (Stimart 1989). Visuer (1989) reported that shoot-tip fasciation is induced when BAP from 0.7 to 2 $mg \cdot l^{-1}$ is used without an accompanying auxin. Our stock cultures were grown on medium with 1 $mg \cdot l^{-1}$ BA and without auxin similar to the conditions described by Visuer.

Browning of medium was minimal in WPM but was slightly more visible in Cheng's medium. Browning of shoots occurred sporadically and was confined to OPR 260 on 0-4 μM BA. Subculturing every 3 weeks averted medium browning altogether. Anthocyanin coloration appeared in OPR 157 leaves when shoots were cultured on WPM. These red pigments were located mainly in the callus and occasionally running up the stem in patches. In addition, in treatments where BA was excluded, the leaves of all genotypes developed areas of red coloration. Nutrient deficiency and culture conditions are known to induce anthocyanin pigmentations in some species (Rajendran et al. 1992, Hirasuna et al. 1991, Phillips 1992, Hiraoka 1990). Reduced nitrates (Rajendran et al. 1992, Hirasuna et al. 1991), increased sucrose, hormone free medium (Phillips and Darrell 1992), and gelling agents such as Gelrite (Hiraoka 1990) all contribute to anthocyanin

biosynthesis in varying amounts. Each genotype possesses a critical minimal nitrate level at which anthocyanin production is optimized (Hirasuna et al. 1991, Yamakawa et al. 1983). Omitting BA in combination with the low nitrate level in WPM (956 mg for WPM compared to 1770 mg for Cheng) resulted in treatments with increased anthocyanins. This is similar to grape cell culture where the addition of BA or kinetin drastically reduced anthocyanin biosynthesis and excluding BA increased it (Hirasuna et al. 1991). Reducing the concentration of kinetin from 2 $mg \cdot l^{-1}$ to 0.6 $mg \cdot l^{-1}$ with 2,4-D is optimal for anthocyanin production in *Vitis* cells. IAA and NAA also stimulate anthocyanin biosynthesis (Mizukami et al. 1989, Hirasuna et al. 1991, Yamakawa et al. 1983).

Our general observation was that there was less shoot tip necrosis in OPR 260 at higher levels of BA (4, 8 and 13 μM) than at lower levels. In all three genotypes, growth of shoot tips was arrested when BA was omitted. Shoot tip necrosis in quince, potato and chestnut was controlled by increasing $CaCl_2$ in the medium (Sha et al. 1985, Vieitez et al. 1989, Qiguang et al. 1985). The addition of calcium gluconate in the culture medium alleviated shoot tip necrosis in poplar trees (De-Block 1990).

Leaf chlorosis was more visible in OPR 260 than in OPR 157 and chlorosis was observed to be influenced more by BA concentration than auxin type or concentration. Interveinal chlorosis was most pronounced without BA and became less prominent as BA levels increased. For all genotypes, Cheng's medium produced larger greener plants with less yellowing of the leaves than WPM.

ROOTING

Rooting with NAA and IBA

OHxF 230 had the highest percentage (> 80%) of rooting compared to the other genotypes and rooted well on all treatments (Table 6). While 10 µM IBA (dark)(treatment 3) produced 90% rooting in OHxF 230, the number of roots per shoot (5.8) were significantly fewer than the 10 mM NAA (8.4) or IBA (6.9) dips. For OPR 260 the most rooting (42.9%) was obtained on plants grown with 10 µM IBA (dark). OPR 157 rooted poorly in all treatments with the highest percentage (23.9%) obtained with 10mM NAA dip treatment. The controls grown on basal medium did not root.

Berardi et al. (1993) found NAA (0.5 $mg \cdot l^{-1}$) promoted rooting significantly better than IBA (0.5 $mg \cdot l^{-1}$), for seedlings of *P. calleryana* cultured on half MS for six weeks. Dolcet-Sanjuan et al. (1990) induced high percentages of rooting of *P. calleryana* 'OPR 191' with a 15 sec dip in 10 mM IBA or growing the shoots on 10 or 32 µM IBA for seven days followed by transfer to medium with NGR. Stimart and Harbage (1989) were unable to induce rooting of 'Bradford' with IBA (0, 4.92, 14.8 and 39.8 mM). While we were able to induce rooting with NAA and IBA for OPR 157, the percentages were low. Comparison of these results indicates that a large amount of genotype variation occurs within *P. calleryana.*

The use of NAA to stimulate rooting of *P. communis* cultivars has been investigated at 8 µM (Visuer 1987) and 10 µM (Singha 1980, Lane 1979). Other investigators have successfully rooted *P. communis* cultivars with IBA at 1 µM (Lane

1979), 10 μM (Shen and Mullins 1984) and 10 or 32 μM (Dolcet-Sanjuan et al. 1990). Dolcet-Sanjuan et al. (1990) reported NAA at 0, 0.3, 1, 3.2, 10 and 32 μM was ineffective for rooting *P. communis* cvs 'Seckel and 'Anjou'. Lane (1979) found 10 μM NAA produced 70% more rooted shoots than 10 μM IBA which was considered toxic for 'Bartlett'. We found that either NAA or IBA easily induced rooting in OHxF 230 (Table 6).

Successful rooting with IBA at 10 or 32 (Dolcet-Sanjuan et al. 1990) and 200 $mg \cdot l^{-1}$ (Nicolodi and Pieber 1989) was reported for *P. betulifolia* cultivars. Banno et al. (1989) found 9.7 μM IBA in combination with PG, after keeping the shoots in the dark for five days, greatly stimulated rooting of *P. serotina.* We found that 10 μM IBA (Treatments 3 and 4) in light or darkness promoted the highest rooting percentage for OPR 260 (Table 6).

For OHxF 230 the largest callus induction was obtained with 10 mM IBA dip (0.9 cm) and was larger than all other treatments (Table 6). The larger mean callus size correlated with a higher percent rooting. More rooting occurred in OHxF 230 which had a larger mean callus size in all treatments, while less rooting was seen in the other two genotypes with smaller mean callus size. Calli induced by the IBA and NAA 10 mM dips developed mainly around the point of root origin.

Dolcet-Sanjuan et al. (1990) reported profuse production of callus by *P. calleryana, P. communis* and *P. betulifolia* on 10 or 32 μM IBA . NAA stimulated callus formation at 0.01 $mg \cdot l^{-1}$ (Baviera et al. 1989) and 10 μM (Lane 1979, Singha (1980) on *P. communis* cultivars. We observed that OHxF 230 developed callus at the base of the

shoots in all treatments while the other two genotypes had considerably less callus. The controls did not form any callus.

For OHxF 230 the highest number of roots per shoot was with the 10 mM NAA and IBA dips (Table 6). The largest number of roots for OPR 260 were with 10 µM IBA (dark) and 10 µM IBA (light) and were better than the 10 mM NAA and IBA dips. For OPR 157 the number of roots per shoot were low in all treatments with the most roots produced on the 10 mM NAA dip.

The longest roots were induced by 10 µM IBA (light) for OHxF 230 and OPR 157 but were not significantly greater than the other treatments while 10 µM IBA (dark) was best for OPR 260 (Table 6). The treatments on which the longest root means were obtained also had the lowest number of roots per shoot for OHxF 230 and OPR 157.

Lane (1979) observed that NAA-induced roots were shorter and had more roots per shoot than the longer IBA-induced roots in 'Bartlett' pears. Dolcet-Sanjuan et al. (1990) reported that culturing the shoots in the dark decreased root length and generally did not improve rooting in *P. calleryana*, *P. communis* and *P. betulifolia*. We did not see this trend for the genotypes tested.

The best in vitro rooting for OHxF 230 and OPR 157 was the 10 mM NAA dip for 15 sec followed by transfer to NGR medium for 4 weeks. For OPR 260 the recommended rooting regime is to culture the shoots for 1 week in the dark on medium with 10 µM IBA followed by transfer to NGR medium.

ACCLIMATIZATION

After four weeks of acclimatization in the greenhouse, the number of shoots that survived the transplant corresponded with those that had rooted in vitro (Table 7). Shoots that had no roots died. No rooting or survival was observed for the ex-vitro rooting treatment (dip and grow) and subsequent acclimatization.

CONCLUSION

Micropropagation systems for the three pear rootstocks were similar but not identical. Axillary buds of forced ecodormant branches of all genotypes grew well on Cheng medium with 4.4 μM BA. For *P. betulifolia* 'OPR 260' the best micropropagation was on Cheng with 8 μM BA and 0.5 μM IBA and rooting was best with 10 μM IBA (dark) for one week. Multiplication for *P. communis* 'OHxF 230' was best on Cheng with 8 μM BA and 0.5 μM IBA with the 10 mM NAA or IBA dip treatment for rooting. Micropropagation for *P. calleryana* 'OPR 157' was best on Cheng or WPM with 8μM BA and 0.5 μM NAA with the 10 mM NAA dip treatment for rooting. Acclimatization of rooted plantlets was successful with two weeks in the mist and two weeks in the greenhouse.

Table 1: Initiation of *Pyrus* explants taken from forced ecodormant shoots in the greenhouse. Explants were grown Cheng medium with 4.4 μM BA for four weeks

Genotype	Number of explants			
	Total Collected	Contaminated	Growing	Growing (%)
P. betulifolia 'OPR 260'	42	3	39	92.8 %
P. communis 'OHXF 230'	40	3	37	92.5 %
P. calleryana 'OPR 157'	57	2	55	96.5 %

Table 2: ANOVA on shoot multiplication of *P. betulifolia* 'OPR 260', *P. communis* 'OHxF 230' and *P. calleryana* 'OPR 157' shoots grown on Cheng medium with BA and NAA or IBA

Source	df	OPR 260		OHxF 230		OPR 157	
		MS	F Value	MS	F Value	MS	F Value
Replication	2	4.0	8.1***	2.5	7.7**	0.4	8.4**
Auxin Conc.	4	2.4	4.9**	23.7	73.6***	0.5	11.7***
BA Conc.	3	12.9	26.6***	94.5	293.4***	7.5	161.0***
Auxin x BA Conc.	12	0.2	0.5NS	6.1	19.0***	0.2	3.8**
Auxin Type	1	22.0	45.2***	2.8	8.8**	13.1	283.1***
Auxin Conc. x Auxin Type	4	0.7	1.4NS	1.8	5.5**	0.3	6.9**
BA Conc. x Auxin Type	3	0.5	1.0NS	2.9	8.6**	0.3	6.7**
Auxin and BA Conc. x Auxin Type	12	0.2	0.4NS	0.9	2.6**	0.1	1.8NS
Error	78	0.5		0.3		0.1	

NS,*,**,*** Not significant or significant at P≤0.05, 0.01, or 0.001.

Table 3: Means of shoot multiplication of *P. betulifolia* 'OPR 260', *P. communis* 'OHxF 230' and *P. calleryana* 'OPR 157' on Cheng medium with BA and NAA or IBA

		Shoot number		
Source		OPR 260	OHxF 230	OPR 157
Auxin Conc.	0	1.3ab	2.5bc	1.6a
	0.5	1.7a	4.2a	1.7a
	1.0	1.3ab	2.7b	1.4b
	2.0	1.0b	2.2c	1.3b
	4.0	1.0b	1.5d	1.4b
BA Conc.	2	0.5b	0.8c	0.7c
	4	0.9b	1.5c	1.4b
	8	1.6a	3.6b	2.2a
	13	1.9a	4.6a	1.6b
Auxin Type				
	IBA	1.7a	2.5b	1.1b
	NAA	0.8b	2.8a	1.9a

[z]Means in a column followed by the same letter are not significantly different at P ≤0.05.

Table 4: ANOVA on shoot multiplication of *P. betulifolia* 'OPR 260', *P. communis* 'OHxF 230' and *P. calleryana* 'OPR 157' shoots grown on Cheng and WPM with BA and NAA

Source	df	OPR 260		OHxF 230		OPR 157	
		MS	F Value	MS	F Value	MS	F Value
Replication	2	0.7	4.2**	2.1	6.6**	1.7	19.9**
NAA Conc.	4	1.9	11.1***	12.3	3.9***	0.9	9.8***
BA Conc.	3	9.9	56.5***	47.7	152.7***	5.9	63.0***
NAA x BA Conc.	12	0.1	0.7NS	2.2	7.0***	0.2	2.7*
Medium	1	3.4	19.5***	42.0	134.4***	0.8	9.0**
NAA Conc. x Medium	4	0.4	2.6NS	1.0	3.1*	0.1	0.8NS
BA Conc. x Medium	3	0.2	1.0NS	2.7	9.2***	0.2	2.1NS
NAA and BA Conc. x Medium	12	0.1	0.8NS	0.3	1.0NS	0.2	2.1NS
Error	78	0.2		0.3		0.1	

NS,*,**,*** Not significant or significant at P≤0.05, 0.01, or 0.001.

Table 5: Means of shoot multiplication of *P. betulifolia* 'OPR 260', *P. communis* 'OHxF 230' and *P. calleryana* 'OPR 157' on Cheng and WPM with BA and NAA

		Shoot number		
Source		OPR 260	OHxF 230	OPR 157
NAA Conc.	0	1.3a	2.3b	1.8b
	0.5	1.3a	3.3a	2.1a
	1.0	1.0ab	2.0bc	1.7bc
	2.0	0.7bc	1.8c	1.5c
	4.0	0.7c	1.3d	1.7bc
BA Conc.	2	0.4c	0.8d	1.1c
	4	0.7c	1.5c	1.8b
	8	1.2b	2.8b	2.4a
	13	1.7a	3.6a	1.9b
Medium Type				
	Cheng	0.8a	2.8a	1.9a
	WPM	1.2b	1.6b	1.7b

[z]Means in a column followed by the same letter are not significantly different at $P \leq 0.05$.

Table 6: Means of root length, number of roots, callus and percent rooting of *P. betulifolia* 'OPR 260', *P. communis* 'OHxF 230' and *P. calleryana* 'OPR 157' on Cheng medium with four rooting treatments

Genotypes	Treatments[y]	Means[z]			
		Root length (cm)	No. of roots	Callus (cm)	Percent rooting
P. betulifolia	1	0.2b	0.2b	0.2a	19.8b
OPR 260	2	0.2b	0.3b	0.3a	17.5b
	3	1.2a	1.0a	0.2a	42.9a
	4	0.5b	0.5ab	0.2a	27.9ab
P. communis	1	2.0a	8.4a	0.6b	85.6ab
OHxF 230	2	1.4a	6.9ab	0.9a	81.1b
	3	1.4a	5.8bc	0.5b	90.0a
	4	2.1a	4.2c	0.6b	85.6ab
P. calleryana	1	0.1a	0.3a	0.3a	23.9a
OPR 157	2	0.1a	0.2a	0.3ab	21.9a
	3	0.1a	0.2a	0.2b	15.0a
	4	0.2a	0.1a	0.3ab	13.3a

[z]Means in a column followed by the same letter are not significantly different at $P \leq 0.05$.
[y]Treatments 1)10 mM NAA dip, 2)10 mM IBA dip, 3)10 µM IBA (dark) and 4)10 µM IBA (light).

Table 7: Percent survival of rooted and non-rooted pear plantlets from in vitro and ex-vitro treatments after four weeks in the greenhouse. Survival rate corresponded with those that were rooted in vitro prior to transfer to the greenhouse

Treatment	Percent survival		
	P. betulifolia OPR 260	*P. communis* OHxF 230	*P. calleryana* OPR 157
10 mM NAA dip	19.8	85.6	23.9
10 mM IBA dip	17.5	81.1	21.9
10 µM IBA (dark)	42.9	90.0	15.0
10 µM IBA (light)	27.9	85.6	13.3
Control	0	0	0
DIP 'N GROW[z]	0	0	0

[z]Commercial rooting preparation containing IBA (1%), NAA (0.5%) and inert materials (98.5%).

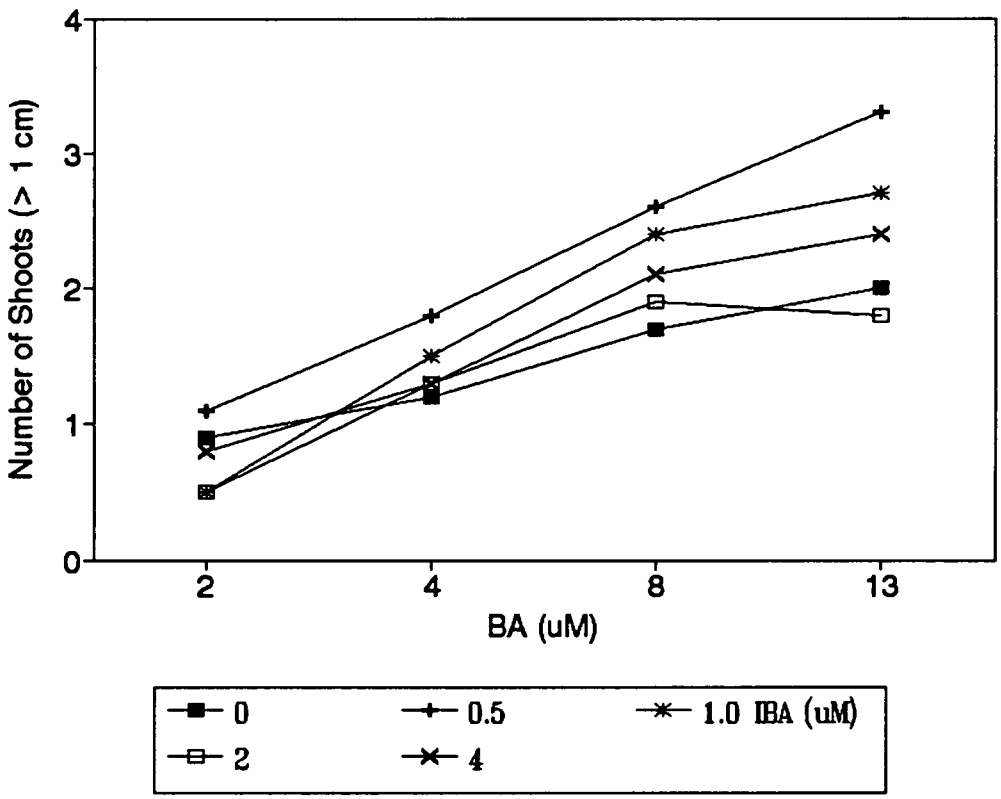

Fig 1: Shoot multiplication of *P. betulifolia* 'OPR 260' on Cheng medium with BA and IBA.

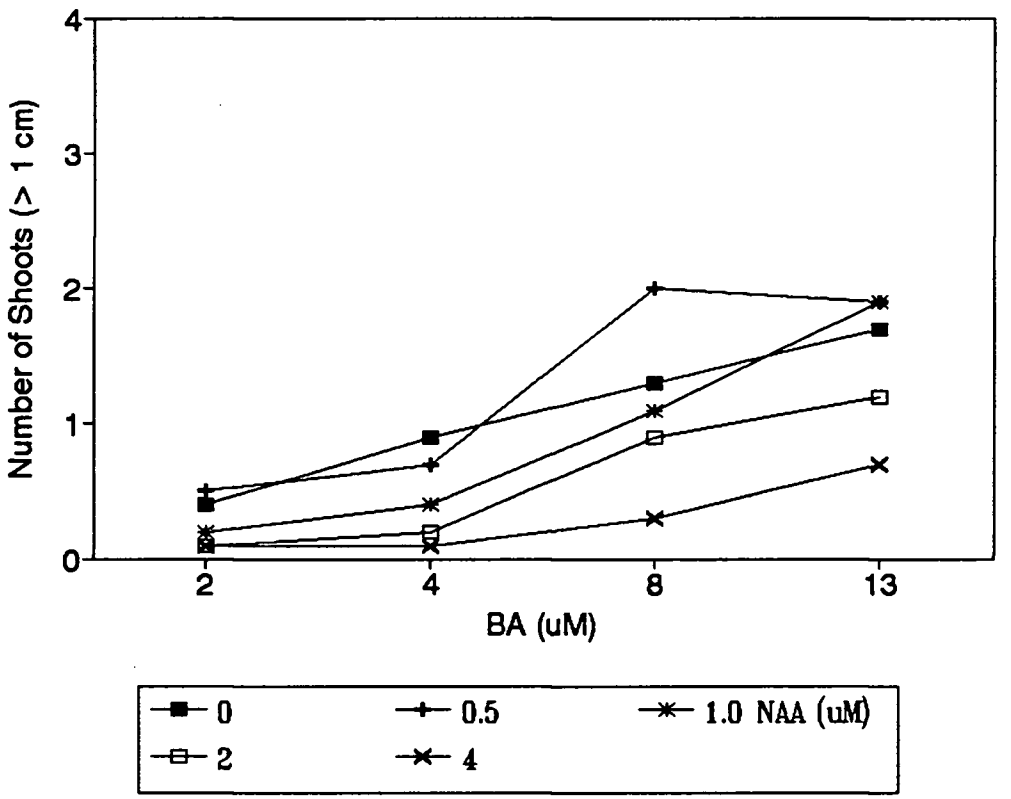

Fig 2: Shoot multiplication of *P. betulifolia* 'OPR 260' on Cheng medium with BA and NAA.

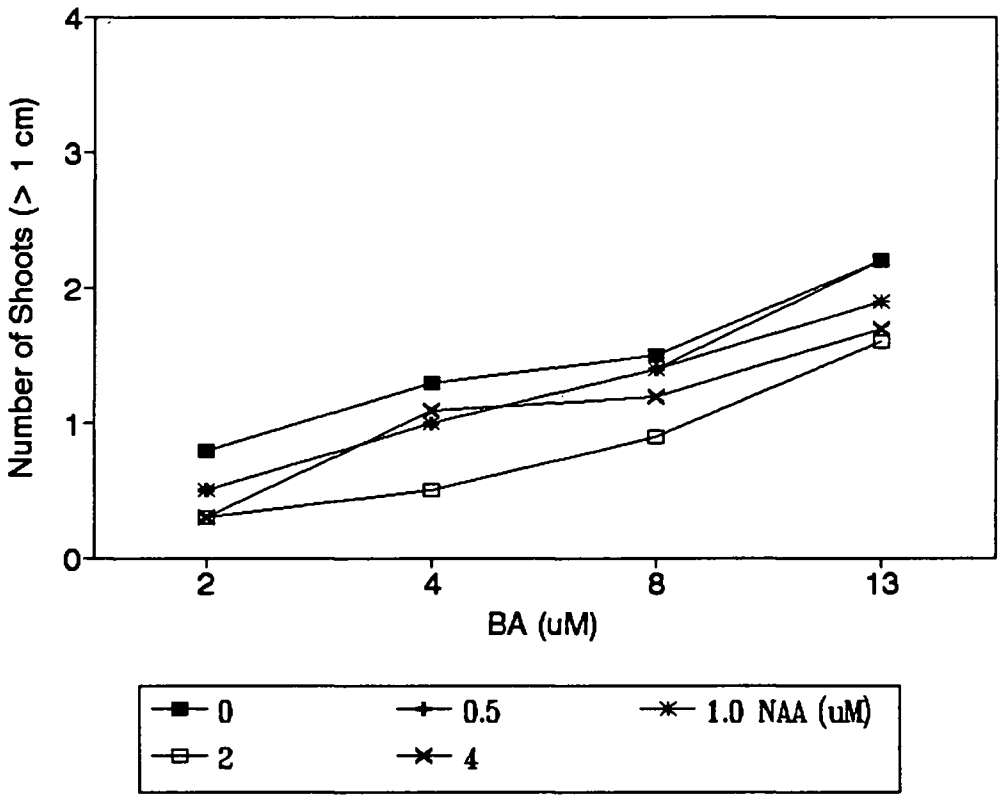

Fig 3: Shoot multiplication of *P. betulifolia* 'OPR 260' on WPM medium with BA and NAA.

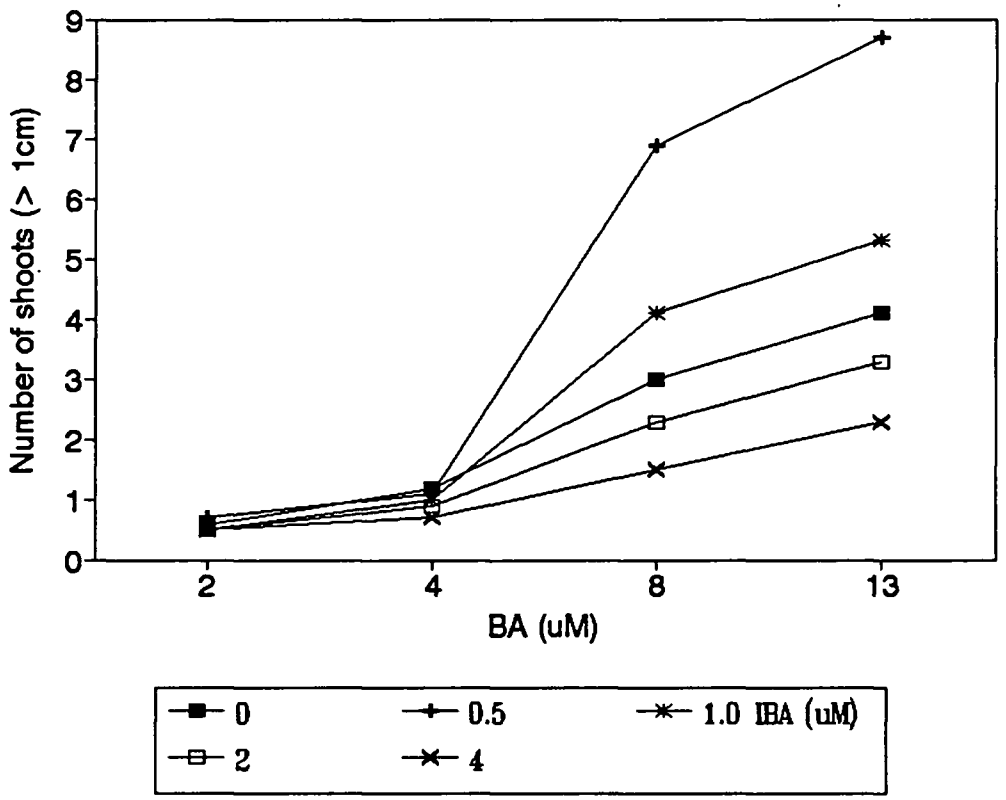

Fig 4: Shoot multiplication of *P. communis* 'OHxF 230' on Cheng medium with BA and IBA.

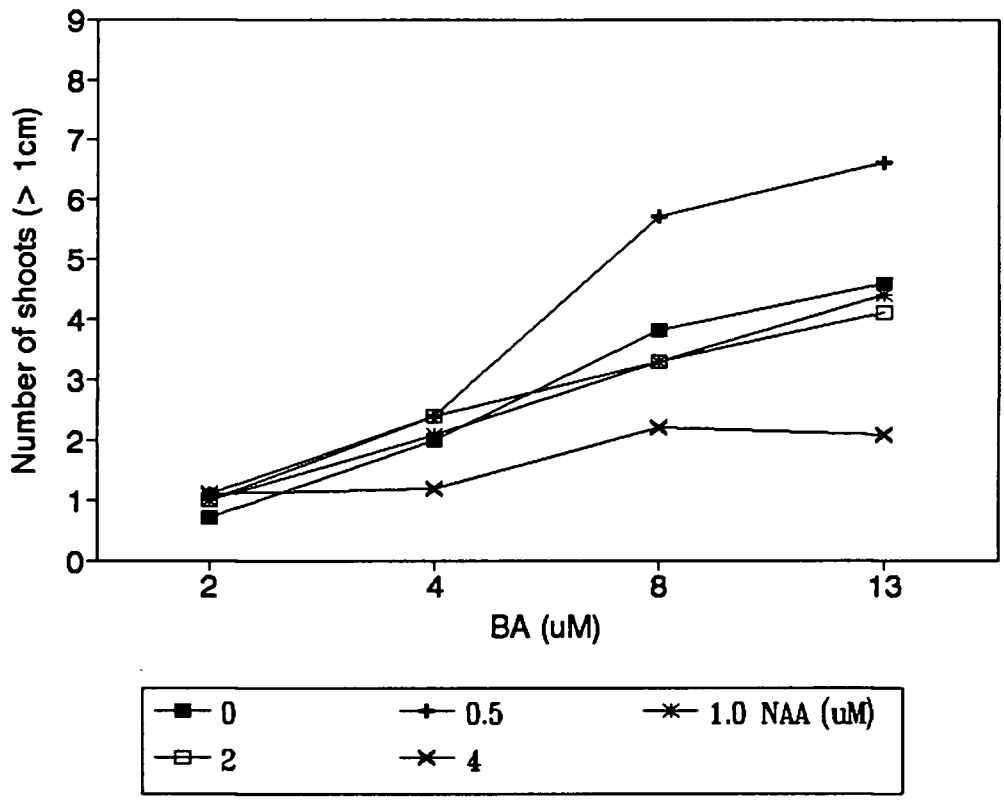

Fig 5: Shoot multiplication of *P. communis* 'OHxF 230' on Cheng medium with BA and NAA.

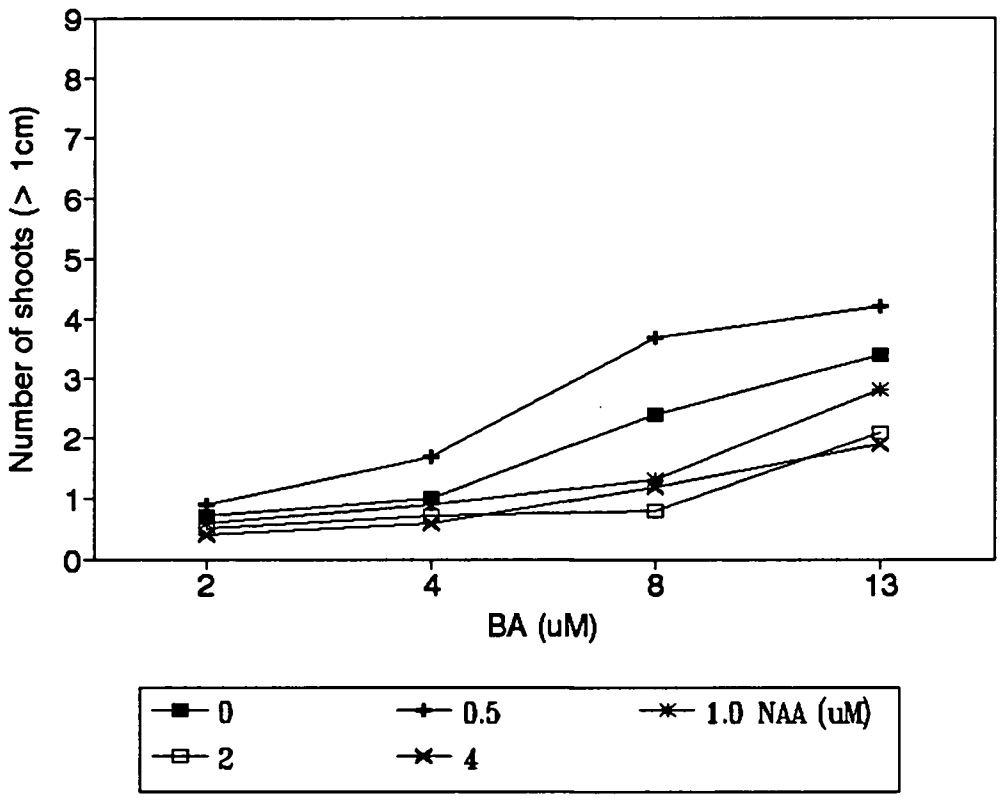

Fig 6: Shoot multiplication of *P. communis* 'OHxF 230' on WPM medium with BA and NAA.

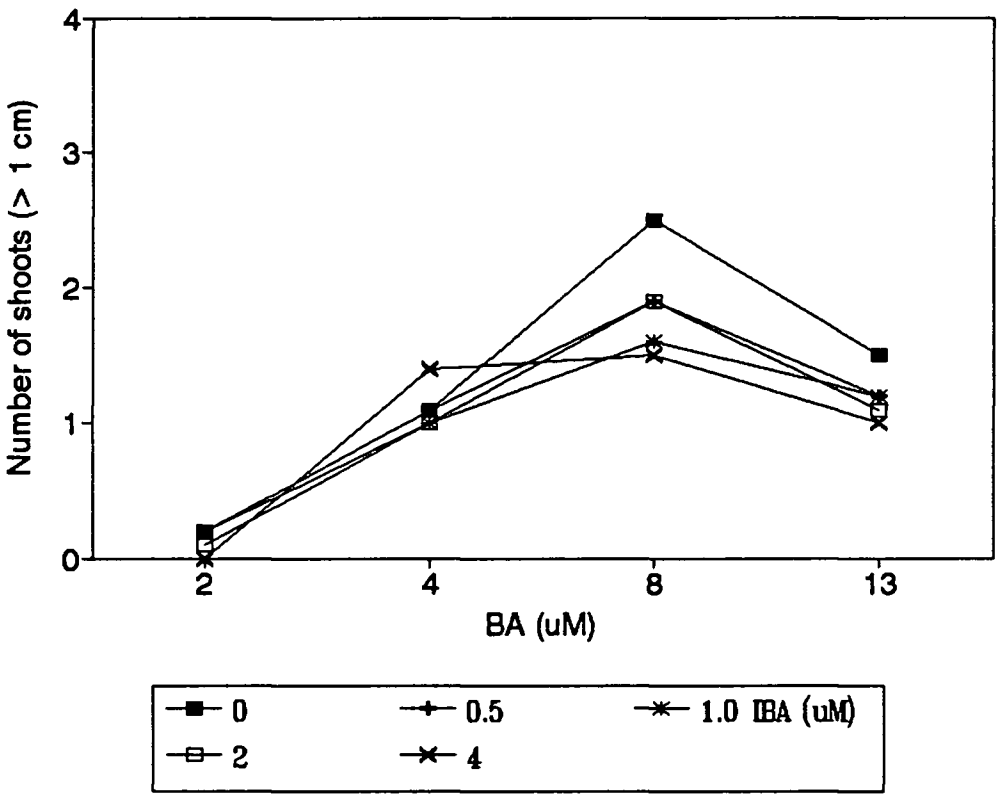

Fig 7: Shoot multiplication of *P. calleryana* 'OPR 157' on Cheng medium with BA and IBA.

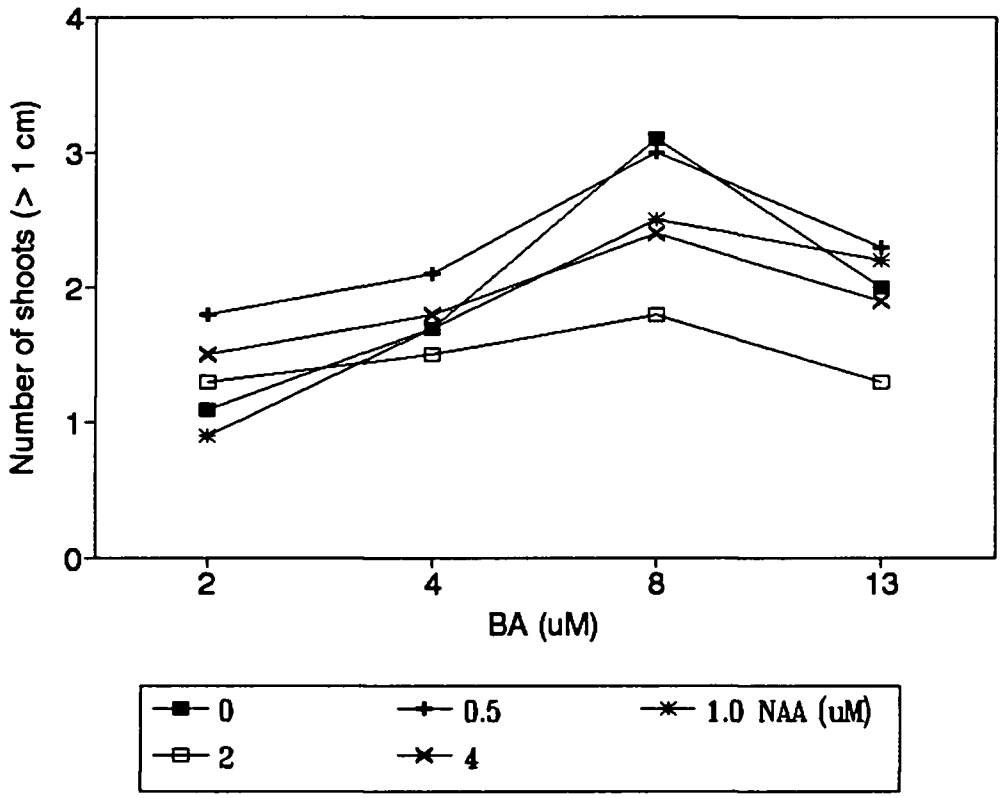

Fig 8: Shoot multiplication of *P. calleryana* 'OPR 157' on Cheng medium with BA and NAA.

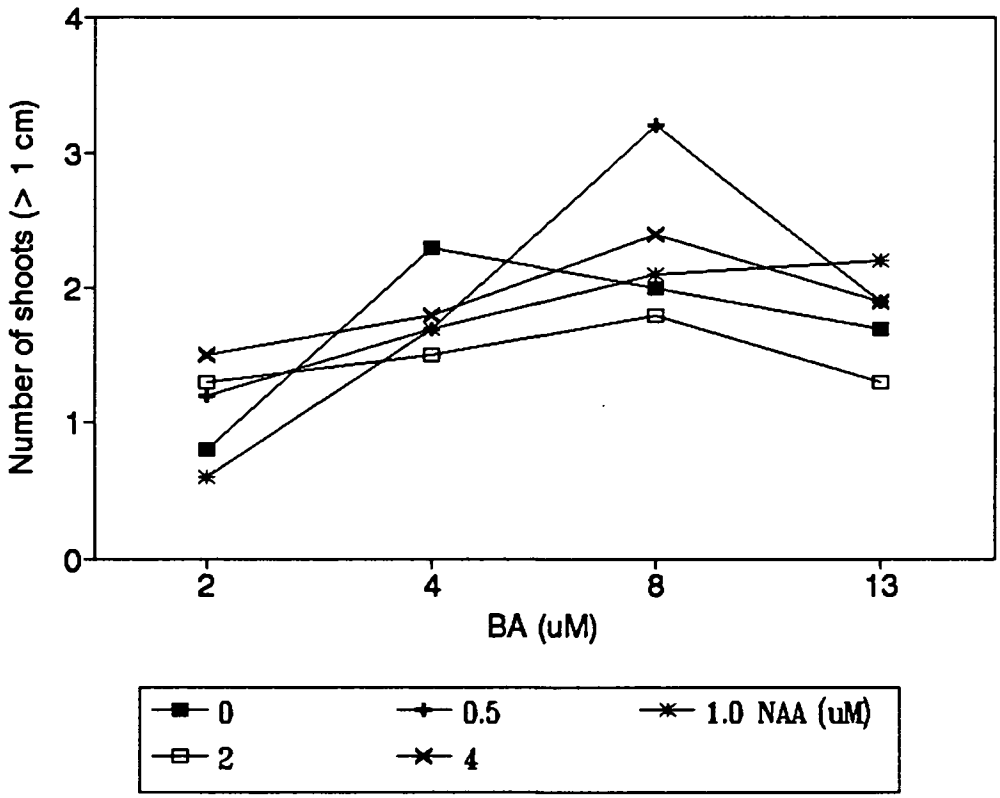

Fig 9: Shoot multiplication of *P. calleryana* 'OPR 157' on WPM medium with BA and NAA.

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APPENDICES

APPENDIX A

TRANSFORMATION OF *P. CALLERYANA* Decne 'OPR 157'

INTRODUCTION

Pear rootstocks, as well as other fruit rootstocks, are widely used to impart a variety of traits to the scion (Westwood and Lombard 1977, Bassi et al. 1989). The transfer of a gene of interest into the rootstock of interest would bypass breeding programs that would require years of cross breeding. This is especially true of woody fruit trees. Although number of pear cultivars have been regenerated (Ochatt et al. 1992, Predieri et al. 1989, Laimer et al. 1988), transformation of pear has not been reported. The objective of this work was to regenerate plantlets from transformed *P. calleryana* 'OPR 157' calli.

MATERIALS AND METHODS

TISSUE CULTURE

In-vitro shoot tips (2 cm) from *Pyrus calleryana* 'OPR 157', maintained on Cheng medium (Cheng 1978) supplemented with 1 $mg \cdot l^{-1}$ BA, were excised for callus production. All leaves were removed and shoots were cut into 0.5 cm transverse sections.

Callus was induced on medium (MSI) containing MS salts (Murashige and Skoog 1962) supplemented with filtered-sterilize B5 (Gamborg 1966) vitamins and 100 $mg \cdot l^{-1}$

glutamine, 200 $mg \cdot l^{-1}$ PVP 360, and 150 $mg \cdot l^{-1}$ $CaCl_2.2H_2O$ and 1.5 1 $mg \cdot l^{-1}$ 2,4-D for 5 weeks in the dark. Shoot regeneration medium (MSR) was MSI, but without 2,4-D, with 400 $mg \cdot l^{-1}$ cefotaxime, 40 $mg \cdot l^{-1}$ shikimic acid, 5 $mg \cdot l^{-1}$ BA and 0.2 $mg \cdot l^{-1}$ NAA. All media were solidified with 0.7% Bacto agar and adjusted to pH 5.7 except MSB0 (MSB0 is MSB without agar and growth regulators) which was at pH 5.4.

VECTOR

Agrobacterium tumefaciens strain ASE harboring a binary plasmid pMON505 containing the NPTII gene conferring kanamycin resistance was used. The bacteria were grown at 28°C overnight in YEP broth (Yeast extract 10 $g \cdot l^{-1}$, NaCl 5 $g \cdot l^{-1}$ Peptone 10 $g \cdot l^{-1}$) at pH 7.0 with 5000 µg per ml of streptomycin and measured to an absorbance of 0.4 at 595 nm. The bacteria were then resuspended in 1 ml of 75 mM $CaCl_2$ after centrifuging 1 ml of the overnight culture and removing the supernatant.

COCULTIVATION AND REGENERATION

Resuspended ASE(pMON505) (100 µl) was added to 10 ml of MSB0 at pH 5.4 in a petri-dish. Callus pieces of about 0.5 cm diameter were excised from the stems and swirled in MSB0 with the added ASE(pMON505) for 10 mins. The calli were then removed, blot dried on sterile paper towels and transferred to solidified MSB0 without any antibiotics or hormones. The calli were cocultivated with bacteria for 48 h at 25°C, then rinsed in liquid MSB0 containing 400 $mg \cdot l^{-1}$ carbenicillin and 100 $mg \cdot l^{-1}$ kanamycin

for 15 mins. After blotting dry, the calli were placed on the regeneration medium, MSR, at 25°C with under illumination. The control was subjected to the same conditions except it was not inoculated with the bacteria.

The calli were transferred to fresh MSR with 400 $mg \cdot l^{-1}$ carbenicillin and 100 $mg \cdot l^{-1}$ kanamycin each week for 3 weeks. On the 4th week, the calli were subcultured on to 1/2 strength MSR media containing 200 $mg \cdot l^{-1}$ carbenicillin and 50 $mg \cdot l^{-1}$ kanamycin.

RESULTS

Spots of anthocyanin sporadically appeared on the inoculated callus by the end of the third week of culture on MSR. Heavier red colorations were observed on the tips of protuberances of the inoculated calli while the uninoculated control had no anthocyanin production and few prominent protuberances. By the end of the sixth week, the inoculated calli had less anthocyanin formation and greener coloration was seen around the calli amidst the blackened (dead) calli. Two putatively transformed shoots grew out from the previously anthocyanin-filled protuberances on the inoculated calli by the ninth week of culture. The control had deteriorated to a blackened mass. Transformants were not confirmed with Southern blot.

APPENDIX B

JASMONIC ACID

Jasmonic acid (JA) and methyl-ester jasmonate (MeJA) are a group of cyclopentanones that induce a wide range of physiological activities in plants (Meyer et al. 1984, Yamane et al. 1982, Weidhase et al. 1987, Falkenstein 1991, Ueda and Kato 1982a, Ravnikar and Gogala 1990). MeJA is an essential oil of *Jasminum grandiflorum* L. and *Rosmarinus officinalis* L. (Meyer et al. 1984) and also occurs in anthers of *Camellia* species (Yamane et al. 1982). Responses induced by JA and MeJA include senescence in barley (Weidhase et al. 1987), root development in potato (Ravnikar et al. 1992), tendril coiling (Falkenstein 1991), gene expression of vegetative storage proteins in soybean (Anderson et al. 1989) and *Brassica napus* (Wilen 1991) and inhibition of callus induced by cytokinins in soybean and potato (Ueda and Kato 1982a, Ravnikar and Gogala 1992). In view of JA's rooting ability we decided to conduct a test to see of JA would induce rooting in pear shoots in vitro.

Jasmonic acid (Pfaltz and Bauer, Inc., Waterbury CT) was tested at 0, 0.1, 0.5, 1.0, 5, 10 and 20 μM on in-vitro shoots of *Pyrus calleryana* Decne 'OPR 157', *Pyrus betulifolia* Bunge 'OPR 260', and *Pyrus communis* L. 'Old Home x Farmingdale 230'.

Each treatment consisted of three replicates and the experiment was done twice (total of 18 shoots 2 cm in height). All treatments were placed randomly in SGRC. The total treatment period was 4 weeks. Callus size, number of roots, root length and percent

rooting were recorded.

Only OHxF 230 developed roots on jasmonic acid while the other two genotypes produced no roots at any concentration. The optimal concentration was 0.5 µM JA which produced a significantly higher percentage (66.7%) of rooted shoots and more and longer roots per explant than the other treatments (Table 8). JA from 0.1-1 µM produced longer roots but concentrations greater than 10 µM induced a compact and thickened roots in potato plantlets (Ravnikar et al. 1992). A reduction in root length with increasing concentrations was also observed in our JA treatments. There was a significant reduction in root length as the concentration of JA was increased from 0.5 µM to 20 µM. Callus formation was absent or very small at the point of root initiation. Suppression of cytokinin-induced callus by JA has been observed in soybean and potato (Ueda and Kato 1982b, Ravnikar et al. 1992).

Table 8: Means of callus, root length, number of roots per shoot and percent rooting of *P. communis* 'OHxF 230' on jasmonic acid

	Jasmonic acid (µM)						
Means[z]	0	0.1	0.5	1.0	5	10	20
Percent rooting	0b	16.7b	66.7a	27.7b	22.2b	22b	22.2b
Callus	0b	0b	0.03a	0b	0.01ab	0.01ab	0.03a
Number of roots	0b	0.2b	1.9a	0.6b	0.5b	0.8b	0.7b
Root length	0c	1.4bc	3.4a	1.7b	0.7bc	0.8bc	0.7bc

[z]Means in a row followed by the same letter are not significantly different at $P \leq 0.05$. n=18 plantlets per treatment.

After three days of culture, OPR 157 plantlets on JA developed red pigments (anthocyanin) on the lower 1-1.5 cm of the stems. By the end of the fourth week of culture all OPR 157 shoots developed deep red pigmentation in the leaves and stems. OPR 260 showed minimal and sporadic anthocyanin pigmentation in the leaves. Some yellowing of the leaves occurred at all levels of JA and was most noticeable with OHxF 230 at the end of the 6th week of culture. All three genotypes were relatively greener without jasmonic acid (control) than on any of the treatments. Senescence is just one of the several biological activities of jasmonic acid (Staswick 1992). Leaf senescence followed by degradation of chlorophyll and RubisCo activity has been documented in barley (Weidhase et al. 1987), in *Artemisia absinthium* L (Ueda and Kato 1980) and *Cleyera ochnacea* DC (Ueda and Kato 1982b).

In conclusion, only OH x F 230 responded to JA with 0.5 μM the optimal level for root induction. The lack of response from OPR 157 and OPR 260 may be genotype dependent or evidence that other concentrations of JA are required for rooting.