Original Paper Plant Biotechnology, **20** (4), 283–289 (2003)

An Efficient and Reproducible *in vitro* Plant Regeneration from Leaf Discs in Pear Cultivars (*Pyrus* spp.).

Chandani Kumari HENNAYAKE[1], Kumara DISSANAYAKE[1], Narumi MATSUDA[2], Takeshi TAKASAKI[3] and Tetsu NAKANISHI[1,3]*

[1]*Graduate School of Science and Technology, Kobe University, Kobe 657-8501, Japan*
[2]*Horticultural Experiment Station of Yamagata Prefecture, 423 Shimaminami, Sagae, Yamagata 991-0043, Japan*
[3]*Faculty of Agriculture, Kobe University, Kobe 650-8501, Japan*
**Corresponding author* E-mail address: ntetsu@kobe-u.ac.jp

Received 4 October 2002; accepted 2 July 2003

Abstract

Leaves derived from *in vitro*-grown apical buds were used to explore the conditions required for induction of adventitious buds and regeneration of shoots. High efficiency of shoot regeneration was obtained with half-strength MS medium (1/2MS) for *Pyrus pyrifolia*, and Nitsch-Nitsch (NN) medium for *Pyrus communis* cultivars. In both species, the basal region of the leaf was more suitable than the distal region for callus and shoot bud formation. High concentration (5 mg l^{-1}) of thidiazuron efficiently induced callus formation and shoot bud initiation in both species. The size, colour and shape of calli were the important factors for successful shoot regeneration. Higher frequencies of callus production and rooting from regenerated shoots were observed with increasing indolebutyric acid concentration in rooting medium. However, there was a significant genotypic difference in adventitious shoot and root regeneration of each pear cultivar.

Key words: European pear, Japanese pear, *Pyrus*, Regeneration.

Abbreviations

BA, 6-Benzylaminopurine; GA_4, Gibberellin A4 acid; IBA, 3-Indolebutyric acid; MS, Murashige and Skoog medium (1962); NAA, Naphthaleneacetic acid; NN, Nitsch-Nitsch medium (1972); TDZ, Thidiazuron.

Introduction

The genus *Pyrus* belongs to the subfamily maloideae of the rosaceae. Pear is considered the most important temperate fruit after grapes and apple in the world. Two main species, which are genetically and morphologically different, *Pyrus communis* L., the European pear, which is grown in Europe and America, and *Pyrus pyrifolia* Nakai, the Asian pear or Nashi, which is grown traditionally in Japan, China, Korea and Taiwan, and increasingly in Europe and America.

Regeneration of adventitious shoots has been reported for several woody fruit species such as *Malus* (James *et al.*, 1988), *Prunus* (Swartz *et al.*, 1990) and *Vitis* (Stamp *et al.*, 1990). However, in woody fruit species regeneration frequencies from tissues of non-seedling origin are generally low. Studies on adventitious shoot regeneration from mature leaf tissues are still limited in pear, especially in *P. pyrifolia*.

The aim of this study is to obtain high efficiency of shoot regeneration from non-seedling materials, leaf discs of *in vitro*-grown plantlets in both *P. pyrifolia* and *P. communis* cultivars. Among many factors that are known to affect the regeneration efficiency, some were tested here: basal medium, 1/2MS or NN medium; the origin of the explant in a leaf, either basal or distal position; and plant growth regulators. Effect of IBA on root initiation from regenerated shoots was also examined.

Materials and methods

Plant materials

Young shoots of *P. pyrifolia* cultivars, 'Housui', 'Kousui', and 'Shinsei', *P. communis* cultivars, 'La France', 'Max Red Bartlett', 'Winter Nelis', 'Conference', 'Devoe', 'Seigneur d'Esperin', 'Precoce', and 'Doyenne du Comice', were used for shoot

proliferation from the apical buds. Apical buds were propagated on a 'propagation medium' consisted of MS medium, 3% sucrose, 1.1 mg l^{-1} BA and 1 mg l^{-1} GA_4 (Yotsuya *et al.*, 1984). After adjusted to pH 5.8, both 0.2% agar and 0.2% gellan-gum (personal communication) were added to the medium as a gelling agent, and then autoclaved for 20 min at 120 ℃. They were sub-cultured to fresh medium with the same composition every 30 days. Cultures were incubated at 24±1 ℃ with a 16 h/8 h (light/dark) photoperiod (cool white fluorescent tubes, 40 to 60 μmol m^{-2} s^{-1}).

General procedure for shoot regeneration

In vitro-cultured apical buds were used as the source of explants. Basal media were supplemented with 3% sucrose and solidified with 0.4% gellan-gum. After adjusted to pH 5.8, media were autoclaved for 20 min at 120 ℃. Leaf discs were placed abaxial side down on the medium in jam bottles (8.5x8.5 cm). Cultures were incubated at 24±1 ℃ in the dark for 30 days followed by under a 16/8 h photoperiod (cool white fluorescent tubes, 40 to 60 μmol m^{-2} s^{-1}). The total number of leaf discs forming adventitious buds and the numbers of adventitious shoots were recorded. For statistics, analysis of variance (ANOVA) was performed and means were compared by *Scheffe post hoc test* for mean separation (Steel and Torrie, 1980) employing a complete random design. All experiments were conduct at least twice and similar results were obtained.

Effect of basal medium on shoot regeneration

Half-strength of MS (1/2 MS) and NN media were tested because they provided NH_4^+/NO_3^- ratio of 1:3 and 1:2, respectively. Decreasing NH_4^+/NO_3^- ratio has been reported to increase the efficiency of shoot regeneration in woody plants (Fasolo *et al.*, 1989; Predieri and Fasolo, 1989). Both media were supplemented with 5 mg l^{-1} TDZ and 0.2 mg l^{-1} NAA and 5 mm size leaf discs were used as explants.

Effect of the origin of the explant in a leaf on shoot regeneration

Fully expanded healthy leaves were excised from *in vitro*-cultured shoots and the petiole was removed. The leaves were transversely cut into halves, and 2 types of leaf discs 5 mm in length were prepared. The basal part of the leaf was referred to 'petiole position' and the distal part to 'tip position'. Explants of *P. pyrifolia* were cultured on 1/2MS and *P. communis* on NN media, supplemented with 5 mg l^{-1} TDZ and 0.2 mg l^{-1} NAA.

Effect of TDZ concentration on shoot regeneration

Three different concentrations of TDZ, 1, 3 and 5 mg l^{-1} were tested. The media were consisted of 0.2 mg l^{-1} NAA and 5 mm size leaf discs were used as explants.

Rooting of regenerated shoots

Regenerated shoots, about 3-4 cm in length, were excised from the leaf disc and transferred to 1/2MS medium containing 3% sucrose in glass tubes (12x2 cm). The medium was adjusted to pH of 5.8 and solidified with 0.2% gellan gum and 0.2% agar. Three IBA concentrations, 1, 2 and 5 mg l^{-1} in combination with 0.2 mg l^{-1} NAA, were tested for root initiation (Ahroni *et al.*, 1997). Cultures were incubated in the dark at 24±1 ℃ for one week. Then shoots were transferred to full-strength MS medium containing 3% sucrose, 0.2% gellan gum and 0.2% agar but lacking plant growth regulators, pH 5.8, and incubated at a temperature of 23/18 ℃ (day/night) under a 16/8 h photoperiod (33 μmol m^{-2} s^{-1}). After 6 weeks on full-strength MS medium, the number of roots per shoot, average root length, percentage of shoot with roots, callus diameter and percentage of shoots with calli were recorded.

Results

Callus formation and shoot regeneration from leaf discs

The first change observed with all the cultivars tested was the enlargement of leaf discs twice as their original size after 14 days of culture. After 30 days of incubation in the dark, calli appeared on the wounded and cutting edges of the explants, and some arose directly from the leaf lamina or veins. In our preliminary experiments, no callus formation was observed when explants were incubated light. Calli were whitish, compact and hard with a nodular appearance. Less frequently, globular structures formed directly on the cutting surface without callus formation. Smaller wet calli failed to develop further, turned brown and died. Generally *P.communis* more rapidly and frequently formed calli compared to *P. pyrifolia*.

Adventitious bud formation was observed two months after cultivation. Most of the buds developed from the calli that were exposed and contacted with the medium. In some cases, it arose directly from petiole tissues. Cultural treatments with higher callus formation frequencies tend to induce more buds per regenerating leaf disc. Regenerated shoot buds were proliferated on a 'regeneration medium',

Table 1 Effect of basal medium on regeneration of shoots in *P.pyrifolia* and *P.communis* cultivars.

Cultivars	No of leaf discs[1]	1/2MS medium			NN medium		
		Leaf discs regenerating shoots		No.of shoots per leaf disc	Leaf discs regenerating shoots		No.of shoots per leaf disc
		No.	%[2]	Mean[3] ± SE[4]	No.	%[2]	Mean[3] ± SE[4]
P. pyrifolia							
Housui	30	17	57	5.6 ± 1.1	0	0	0
Kousui	20	7	35	2.4 ± 1.6	3	15	2.3 ± 0.4
Shinsei	20	4	20	8.5 ± 0.9	4	20	3.5 ± 0.3
P. communis							
La France	30	0	0	0	5	17	1.4 ± 1.0
Max Red Bartlett	20	2	10	1.5 ± 0.9	9	45	1.8 ± 1.0
Winter Nelis	20	1	5	1.0 ± 1.1	13	65	4.1 ± 0.7
Conference	30	11	37	4.4 ± 0.5	22	73	4.4 ± 0.6
Devoe	14	0	0	0	5	36	4.8 ± 0.6
Seigneur d'Esperin	30	4	13	2.3 ± 0.7	9	30	3.4 ± 0.7
Precoce	30	7	23	1.9 ± 0.8	23	77	3.6 ± 0.7
Doyenne du Comice	30	6	20	2.8 ± 0.7	13	43	1.3 ± 1.1

Both media were supplemented with 5 mg l^{-1} TDZ and 0.2 mg l^{-1} NAA and 5 mm size leaf discs were used as explants.

[1] Initially inoculated leaf discs per treatment in each cultivar.

[2] Regeneration rate (%) counted as a percentage.

[3] The mean number of shoots per leaf disc regenerating shoots.

[4] The standard error of the mean (SE).

Table 2 Shoots regeneration from basal (petiole) and distal (tip) leaf positionin of *P.pynfolia* and *P.communis* cultivars.

Cultivars	No of leaf discs[1]	Petiole Position			Tip Position		
		Leaf discs regenerating shoots		No. of shoots per leaf disc	Leaf discs regenerating shoots		No. of shoots per leaf disc
		No.	%[2]	Mean[3] ± SE[4]	No.	%[2]	Mean[3] ± SE[4]
P. pyrifolia							
Housui	30	11	37	3.6 ± 0.3	0	0	3.0 ± 0.5
Kousui	30	5	17	2.6 ± 0.3	2	7	3.0 ± 0.5
Shinsei	10	8	80	3.9 ± 0.3	1	10	5.0 ± 0.4
P. communjs							
La France	30	5	17	1.4 ± 1.0	0	0	0
Max Red Bartlett	20	6	30	1.7 ± 0.9	2	10	1.5 ± 1.4
Winter Nelis	20	13	65	4.1 ± 0.6	0	0	0
Conference	30	18	60	4.4 ± 0.6	4	13	5.3 ± 0.7
Devoe	14	5	36	4.8 ± 0.5	0	0	0
Seigneur d'Esperin	30	8	27	3.4 ± 0.6	1	3	4.0 ± 0.8
Precoce	30	15	50	3.5 ± 0.6	8	27	3.8 ± 0.9
Dovenne du Comice	30	9	30	2.4 ± 0.8	4	13	1.0 ± 1.7

Explants of *P.pyrifolia* were cultured on 1/2MS and *P.communis* on NN media. All media contained with 5 mg l^{-1} TDZ and 0.2 mg l^{-1} NAA.

[1] Initially inoculated leaf discs per treatment in each cultivar.

[2] Regeneration rate (%) counted as a percentage.

[3] The mean number of shoots per regenerating shoots.

[4] The standard error of the mean (SE).

Table 3 Effect of TDZ concentration on shoot regeneration in *P. pyrifolia* and *P. communis* cultivars.

Cultivars	TDZ (mg l^{-1})	No of leaf discs[1]	Leaf disc forming calli[2]		Leaf discs regenerating shoots[3]	
			No.	%	No.	Mean ± SE
P. pyrifolia						
Housui	1	20	20	100	0	0
	3		19	95	2	0.1 ± 0.5
	5		12	60	8	0.4 ± 0.2
Kousui	1	20	18	90	0	0
	3		18	90	1	0.1 ± 0.5
	5		20	100	6	0.3 ± 0.2
P. communis						
Conference	1	20	4	20	0	0
	3		10	50	1	0.1 ± 0.6
	5		20	100	7	0.4 ± 0.2
Winter Nelis	1	20	14	70	1	0.1 ± 0.8
	3		19	95	3	0.2 ± 0.4
	5		20	100	9	0.5 ± 0.3

5 mm size leaf discs were placed on the medium consisted of 0.2 mg l^{-1} NAA.

[1] Initially, inoculated leaf discs per treatment in each cultivar.

[2] Regeneration rate (%) counted as a percentage.

[3] The mean number of shoots per regenerating shoots.

but most shoot buds became necrotic and later died. Internode elongation of the regenerated shoots was observed only in 'Winter Nelis'. Regeneration of multiple shoots in *P. communis* was more frequently than *P. pyrifolia*. *P. communis* cultivar 'Conference' produced healthy shoots with fully expanded leaves.

Effect of basal medium on shoot regeneration

Table 1 shows the effect of 1/2MS and NN media on the shoot regeneration percentage and mean number of shoots per regenerating leaf disc in several *P. pyrifolia* and *P. communis* cultivars. Basal medium strongly affected regeneration of pear. For *P. pyrifolia* cultivars efficient regeneration was induced by 1/2MS medium, whereas for *P. communis* cultivars it was induced by NN medium. *P. communis* 'Conference', 'Precoce' and 'Doyenne du Comice' efficiently regenerated shoots on both media. *P. pyrifolia* 'Shinsei' had poor regeneration ability, but it showed good shoot proliferation rate. Based on these observations, for the subsequent experiments, 1/2MS medium and NN medium were used as basal medium for *P. pyrifolia* and *P. communis* respectively.

Effect of the origin of the explant in a leaf on shoot regeneration

The origin of the explant in a leaf had a significant effect on shoot formation. In general, higher numbers of shoots per regenerating explant and higher percentages of leaf discs with shoots were obtained in 'petiole position' than in 'tip position' in all cultivars. Generally, one shoot per regenerating explant was obtained, whereas in some cases up to 4 or 5 shoots per explant were regenerated. *P. pyrifolia* 'Shinsei' and *P. communis* 'Winter-Nelis', 'Conference', and 'Precoce' showed more than 50% of regeneration frequencies from 'petiole position' (**Fig. 1D, Table 2**).

Effect of TDZ concentration on shoot regeneration

TDZ induced shoot formation in all concentrations tested, however, the optimal shoot regeneration was obtained by 5 mg l^{-1} TDZ in all pear cultivars used (**Table 3**).

Root regeneration

The first change observed, after 7 days in the dark on IBA-containing media, was callus formation from the cutting surface of the shoots. Sometimes root primordia-like structures were observed at the cutting surface of the shoots but no further development occurred (**Fig. 1E**).

Table 4 shows the effect of various concentrations of IBA on root formation from regenerated shoots of several *P. pyrifolia* and *P. communis* cultivars. Higher percentages of rooting and callus formation were obtained with higher IBA concentrations. In most of the treatments, there was direct association between high frequencies of root forma-

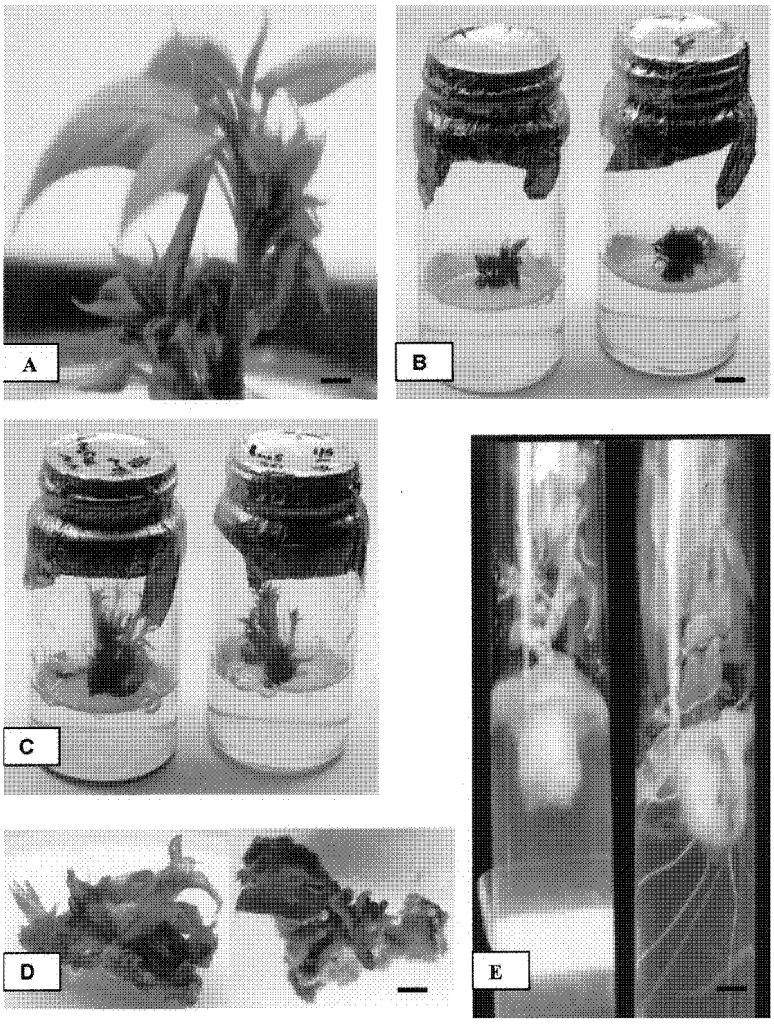

Fig. 1 Plant regeneration of several *P. pyrifolia* and *P. communis* cultivars. (A) Shoot proliferation of cultivar 'Kousui' from apical bud. (B and C) Development of adventitious shoots after 1 month on regeneration medium; (B) 'Housui', (C) 'Winter-Nelis'. (D) Adventitious bud formation from leaf discs 6 weeks after culture initiation (left-petiole position, right-tip position). (E) Callus and root formation after 1 month of culture in *P. pyrifolia*, 'Kousui' (left) and *P. communis*, 'Devoe' (right). Bar = 3 mm (in the A) or 2.5 mm (in the D) or 8 mm (in the B, C and E).

tion and callus production. Most of roots developed from the base of calli, which were initially produced from the cutting surface of the shoots. The first adventitious roots were observed 7 days after transfer of the shoots to a medium lacking plant growth regulators. Generally 3-10 healthy roots were produced per shoot and lateral roots also developed from these roots. Shoots and leaves of *P. pyrifolia* cultivars were deteriorated and turned to yellow in color very quickly.

Based on the number and the length of roots, it was obvious that *P. communis* had good root regeneration ability. All the cultivars of *P. pyrifolia* had poor rooting ability.

Discussion

The present study demonstrates that *in vitro* adventitious shoot regeneration is possible in several cultivars of *P. pyrifolia* and *P. communis* (**Fig. 1A-C**). Although leaves of shoot cultures have been used as an explant in *P. communis*, Chinese pear (*P. bretschneideri*) (Chevreau *et al.*, 1989), and apple (*Malus domestica*) (James *et al.*, 1988; Welander, 1988) to induce adventitious shoots *in vitro*, this is the first report on regeneration of whole plant from leaves of shoot cultures of *P. pyrifolia* cultivars.

The callus formation and shoot regeneration abilities were completely different among pear cultivars. Other than the genotypic effect, some factors af-

Table 4 Effect of IBA concentration on callus and root formation of regenerated shoots of *P.pyrifolia* and *P.communis* cultivars.

Cultivars	IBA (mg l^{-1})	Callusing		Rooting		
		%	Diameter (mm)	%	Avg. No.	Length (mm)
P. pyrifolia						
Housui	1	20	1	0	0	0
	2	80	2	0	0	0
	5	100	2.8	0	0	0
Kousui	1	60	5	0	0	0
	2	100	7.2	0	0	0
	5	100	9.6	20	1	8
P. communis						
Comice	1	40	15.5	20	7	48
	2	100	11.6	40	4	30
	5	100	24.6	60	3	57
Conference	1	100	4.8	0	0	0
	2	100	12.2	20	5	97
	5	100	12.6	40	3	28
Devoe	1	60	16.7	20	11	27
	2	60	7	20	7	48
	5	100	7.2	40	9	15

Explants were initially cultured in the dark on IBA containing 1/2MS and then transferred to plant growth regulator-free, full strength MS medium.

fected callus and shoot formation in pear. The balance between NH_4^+ and NO_3^- has been reported to induce shoot regeneration in pear (Leblay *et al.*, 1991). In the present study, we also found macro-elements composition is an important factor for regeneration. *P. pyrifolia* showed efficient regeneration on 1/2MS media with a NH_4^+: NO_3^- ratio of 1:3, whereas *P. communis* did on NN medium with a NH_4^+: NO_3^- ratio of 1:2 (**Table 1**). Ammonium ion promotes the penetration of anions into the plant at the expense of cations, while NO_3^- leads to the reverse process. Similarly, the balance between the two types of nitrogen ions in the nutrient medium may regulate the differential absorption of the other ions by the leaf.

In pear cultivars, whitish, compact and hard calli showed relatively high shoot regeneration ability, whereas soft, smooth, wet-looking calli did not. So it is likely that both the vitality and the size of the calli are important factors for successful regeneration. Attempt to micro-propagate the regenerated shoots using 'regeneration medium' was not very successful since most shoot buds became necrotic and subsequently died. Further studies are necessary to establish an efficient system for shoot proliferation.

Leaf 'petiole position' was more suitable than the 'tip position' for efficient regeneration of adventitious shoots in pear cultivars (**Table 2, Fig. 1D**). This is probably due to the more vascular tissues present in the petiole based leaf discs than tip based leaf discs.

There is substantial evidence that TDZ is probably the most potent kind of cytokinin currently available for tissue culture of many woody species (Huetteman and Preece, 1993). This study illustrated the effect of TDZ concentration on shoot regeneration from leaf discs of several *P. pyrifolia* and *P. communis* cultivars, and among the different concentrations tested, 5 mg l^{-1} seemed to be the most effective (**Table 3**). However, at least 3 mg l^{-1} of TDZ seemed to be sufficient to induce adventitious buds in pear cultivars. The high concentration of TDZ induced good callus formation as well as good regeneration rate. Perhaps, this indicates that the optimal auxin and cytokinin levels were reached at this point because the balance of endogenous and exogenous auxin and cytokinin is very important for regeneration.

Both callus and root formation frequencies were low in *P. pyrifolia* compared with *P. communis* cultivars (**Fig. 1E, Table 4**). Roots formation was observed in all of the cultivars of *P. communis* (20% - 60%), whereas only one cultivar, 'Kousui', produced roots in *P. pyrifolia* (20%). It indicates that genotypic variation mainly influenced roots initiation and calli formation of pear cultivars. In this experiment, regenerated shoots explants were ini-

tially exposed to auxin-containing medium for seven days and then transferred to plant growth regulator-free medium. It has been suggested that the high concentration of auxin is necessary for the first stage of organogenesis but it can inhibit the subsequent development of the morphogenic regions (Middleton, 1977).

This study showed the high frequency of shoot regeneration in *P. pyrifolia*, 'Housui', 'Kousui', and 'Shinsei' and *P. communis*, 'Precoce', 'Conference' and 'Winter Nelis' (**Fig. 1B, C**). The procedure presented here yields efficient adventitious shoot regeneration from leaves, derived from *in vitro*-grown apical buds. Its suitability to a variety of cultivars, and high frequency of shoot-forming explant, together with the high number of shoots formed per explants may be very advantageous for a various of purposes, including both classical and molecular breeding such as gene transformation with *Agrobacterium*, somaclonal variation or induced mutagenesis for the genetic improvement of *Pyrus* species.

References

Ahroni, A., Zuker, A., Rozen, Y., Shejtman, H., Vainstein, A., 1997. An efficient method for adventitious shoot regeneration from stem-segment explants of gypsophila. Plant Cell, Tissue Organ Cult., **49**: 101-106.

Chevreau, E., Skirvin, R. M., Abu-Qaoud, H. A., Korban, S. S., Sullivan, J. G., 1989. Adventitious shoot regeneration from leaf tissue of three pear (*Pyrus* sp.) cultivars *in vitro*. Plant Cell Rep., **7**: 688-691.

Fasolo, F., Zimmerman, R. H., Fordham, I., 1989. Adventitious shoot formation on excised leaves of *in vitro* grown shoots of apple cultivars. Plant Cell, Tissue Organ Cult., **16**: 75-87.

Huetteman, C. A., Preece J. E., 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell, Tissue Organ Cult., **33**: 105-119.

James, D. J., Passey, A. J., Rugini, E., 1988. Factors affecting high frequency plant regeneration from apple leaf tissues cultured *in vitro*. J. Plant Physiol., **132**: 148-154.

Leblay, C., Chevreau, E., Raboin, L. M., 1991. Adventitious shoot regeneration from *in vitro* leaves of several pear cultivars (*P. communis* L.). Plant Cell, Tissue Organ Cult., **25**: 99-105.

Middleton, W., 1977. Root development in cuttings of *Phaseolus aureus* Roxb. PhD Thesis. Univ. Sheffield. UK.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., **15**: 473-497.

Nitsch, J. P., 1972. Haploid plants from pollen. Z. Pflanzenzüchtg., **67**: 3-18.

Predieri, S., Fasolo, F. M. F., 1989. High frequency regeneration from leaves of the apple rootstock M26 (*Malus pumila* Mill.). Plant Cell, Tissue Organ Cult., **17**: 133-142.

Stamp, J. A., Colby, S. M., Meredith, C. P., 1990. Direct shoot organogenesis and plant regeneration from leaves of grape (*Vitis* spp.). Plant Cell, Tissue Organ Cult., **22**: 127-133.

Steel, R. G. D., Torrie, J., 1980. Principles and procedures of statistics. pp. 177-181. McGraw-Hill Book Company.

Swartz, H. J., Bors, R. M. F., Naess, S. K., 1990. The effect of the *in vitro* pre-treatments on subsequent shoot organogenesis from excised *Rubus* and *Malus* leaves. Plant Cell, Tissue Organ Cult., **21**: 179-184.

Yotsuya, T., Ichii, T., Sawano, M., Nakanishi, T., Ozaki, T., 1984. Effect of bud scales and gibberellins on dormancy of *in vitro* cultured Japanese pear leaf bud. Sci. Hort., **24**: 177-184.

Welander, M., 1988. Plant regeneration from leaf and stem segments of shoots raised *in vitro* from mature apple trees. J. Plant Physiol., **132**: 738-744.