Plant regeneration from *in vitro* leaves of four commercial *Pyrus* species

H. Tang, Y. Luo, C. Liu

Department of Horticulture, College of Forestry and Horticulture, Sichuan Agricultural University, Sichuan, P. R. China

ABSTRACT

An efficient shoot regeneration from *in vitro* leaf sections of *Pyrus communis* Bartlett, *P. pyrifolia* Shenbuzhi, *P. bretschneideri* Zaosu and *P. ussuriensis* Manyuanxiang was successfully developed for use in future transgenic studies. On the basis of regeneration frequency and average shoot numbers, optimal shoot regeneration was obtained on leaf sections of *P. communis* Bartlett when cultured on Murashige and Skoog complete medium containing 6.0 mg/l BA (6-benzyladenine) and 0.1 mg/l NAA (α-naphthaleneacetic acid), while Quoirin and Lepoivre complete medium supplemented with 1.0 mg/l TDZ [thidiazuron (N-phenyl-N[1]-1,2,3-thiadiazol-5-ylurea)] and 0.1 mg/l NAA was found best for *P. pyrifolia* Shenbuzhi, and Nitsch and Nitsch complete medium containing 3.0 mg/l TDZ and 0.1 mg/l NAA or 0.2 mg/l IAA was suitable for *P. bretschneideri* Zaosu or *P. ussuriensis* Manyuanxiang, respectively. After cutting the leaves into three sections perpendicular to the midrib and culturing under the equivalent conditions, regeneration occurred more frequently on basal sections than middle sections, and no shoots formed on apical sections. A ratio of NH_4^+-N/NO_3^--N of 1:2~1:7 was found beneficial for shoot regeneration. 75.0–87.5% of proliferating shoots formed roots after 4 weeks of transfer to 1/4 strength of Murashige and Skoog complete medium supplemented with 2.5 mg/l IBA (indole-3-butryric acid) and 30.0 g/l sucrose. Regenerated plants were successfully established under greenhouse conditions.

Keywords: adventitious shoot; leaf explant; organogenesis; pear; caulogenesis; *Pyrus*

Pear (*Pyrus*) is the third most important temperate fruit in world production, next to grapes and apple, and can be consumed fresh as well as cooked, dried or preserved. In all temperate areas of the world, *Pyrus communis*, *P. pyrifolia*, *P. bretschneideri* and *P. ussuriensis* are the main commercially cultivated species and cultivar improvement is still continuous (Richard et al. 1996). Pear breeding by conventional methods, largely based on intra- and inter-specific hybridization is difficult, because pear is highly heterozygous and the traits that constitute an elite cultivar are polygenic in their inheritance and the long juvenile period of seedling trees. Genetic improvements of pear cultivars are possible through two approaches: one is to exploit preexisting or induced mutations resulting in genetic variability in somatic cells and the other is that of genetic engineering or gene isolation and transfer (Richard et al. 1996). A general prerequisite for the two approaches is to establish an efficient plant regeneration system from *in vitro* cultures. Although the use of *in vitro* culture for recovery of somatic variants was successfully performed (Duron et al. 1987, Brisset et al. 1988, Abu-Qaoud et al. 1990), attempts to transform pear genetically are still needed, as the genetic engineering enables a one-step integration of foreign gene(s) in existing cultivars without major modification of their genetic background (Hammerschlag and Litz 1992). Several pathways were used for regenerating plants from *in vitro* cultures of pear (Chevreau and Skirvin 1992). However, large gaps still exist in fundamental methodologies, including efficient protocols for re-

Supported by the Program for New Century Excellent Talents in University in P. R. China, Project No. NCET-04-0905, and by the Program for Excellent Ph.D. Thesis of University in P. R. China, Project No. 200253.

generating plants from matured tissues, especially leaves, of an existing pear cultivar, which could ensure that their genotype is identical to that of the parental material. Most of the previous works on plant regeneration from leaves of pears focused on *P. communis* (Chevreau et al. 1989, Predieri et al. 1989, Abu-Qaoud et al. 1991, Leblay et al. 1991) with only a few reports on *P. bretschneideri* (Chevreau et al. 1989) and *P. pyrifolia* (Lane et al. 1998, Lee et al. 2004). Recently, adventitious shoots have been regenerated from leaves of *P. pyraster* (Palombi et al. 2007) and *P. betulaefolia* (Poudyal et al. 2008) as well as *P. bretschneideri*, *P. pyrifolia* and *P. communis*. Shoot organogenesis was never reported for *P. ussuriensis*, the most cold hardy of all *Pyrus* species grown in Siberia, Manchuria, Korea and northeastern China (Richard et al. 1996). Furthermore, it was widely found that the ability to regenerate plants from leaf explants is genotype-dependent, but, as to our observations, rare comparison of plant regeneration ability with different *Pyrus* species is made. The objective of the present study was therefore to develop an efficient protocol for plant regeneration from leaves of all the four commercially grown *Pyrus* species and to compare their regeneration ability in the same culture conditions.

MATERIAL AND METHODS

Stock cultures

Pyrus communis Bartlett, *P. pyrifolia* Shenbuzhi, *P. bretschneideri* Zaosu and *P. ussuriensis* Manyuanxiang were used in the present study. *In vitro* stock cultures were established (Liu 2003) from axillary buds of 25-year-old trees grown in a pear field genebank collection of Sichuan Agricultural University, Ya'an, Sichuan, P. R. China. Stock cultures (5 cultures per 280 ml container containing 35 ml medium) were grown on Murashige and Skoog (1962) complete medium (MS) containing 1.0 mg/l 6-benzyladeine (BA), 0.1 mg/l indole-3-butyric acid (IBA) and 0.5 mg/l gibberellin A_3 (GA_3). All plant growth regulators (PGRs) used in the present study were purchased from DUCHEFA, the Netherlands; they were filter-sterilized (Sigma 45 μm filter) and added to the media (pH = 5.6) after autoclaving at 121°C (1.055 kg/cm^2) for 20 min and media were dispensed as 35 ml aliquots into 280 ml (90 × 70 mm) containers (Shanghai Jiafeng Gardening Co., Ltd.). All cultures were maintained in a growth chamber at 24 ± 1°C under a 16-h photoperiod with a light intensity of 4800 lx provided by cool-white fluorescent lamps. Subculture was performed every three weeks.

Shoot organogenesis

The expanded leaves (about 20 days old) of *Pyrus* species were taken from proliferating shoots. The leaves were cut, in a way perpendicular to the midrib, into three sections (5–8 mm in length for each) that were defined as basal, middle and apical sections, respectively (Figure 1). The three sections were put together in the same dishes and cultured with abaxial side down in plastic dishes (9–12 sections per 20 × 90 mm dish) containing 30 ml MS, Nitsch and Nitsch (1969) (NN) or Quoirin and Lepoivre (1977) (QL) complete medium supplemented with different combinations of PGRs (Table 1), 30 g/l sucrose and 7 g/l Micro agar (pH 5.8). Dishes containing the explants were sealed with "Parafilm", kept in the dark for 3 weeks and then transferred to standard light conditions, in a growth chamber as described for stock cultures, for shoot organogenesis.

After a total of 50-day period of shoot induction on shoot regeneration media, shoots were already present on the leaf explants and shoots > 5 mm were excised from the leaves and transferred to a proliferation medium (PM) containing MS salts and vitamins and 1.0 mg/l BA and 0.1 mg/l IBA. Those shoots were sub-cultured at a 4-week interval onto fresh PM. If any leaf explant had shoots shorter than 5 mm 50 days after initiation, the whole culture was transferred to PM until the shoots could be removed from the cultures. Two and half months after planting, no more shoots were obtained from original leaf explants and the regeneration reaction was evaluated by regeneration frequency and average shoot numbers. Regeneration frequency was defined as percentage of explants producing shoots and average shoot numbers as numbers of shoots per explant.

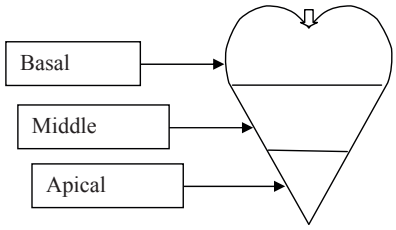

Figure 1. Schematic presentation of leaf sections

Table 1. Effects of media basal salts composition and PGRs on shoot organogenesis of basal leaf sections of *Pyrus* species

Media	Plant growth regulators (mg/l)					*P. communis* Bartlett		*P. pyrifolia* Shenbuzhi		*P. bretschneideri* Zaosu		*P. ussuriensis* Manyuanxiang	
	BA	TDZ	NAA	IBA	IAA	regeneration frequency (%)	average shoot numbers	regeneration frequency (%)	average shoot numbers	regeneration frequency (%)	average shoot numbers	regeneration frequency (%)	average shoot numbers
MS	3		0.1			0	0	0	0	0	0	0	0
MS	6		0.1			62.8 ± 3.5	3.04 ± 0.17	5.6 ± 2.8	0.78 ± 0.21	4.62 ± 1.7	0.41 ± 0.11	10.6 ± 1.7	0.51 ± 0.11
MS	6			0.1		24.2 ± 4.6	0.65 ± 0.28	8.4 ± 5.3	0.83 ± 0.24	6.34 ± 8.2	0.60 ± 0.14	6.23 ± 8.2	0.80 ± 0.14
MS		0.5		0.1		32.8 ± 1.2	1.60 ± 0.67	18.3 ± 5.3	1.50 ± 0.86	10.5 ± 1.7	0.86 ± 0.16	12.5 ± 1.7	2.11 ± 0.16
MS		1.0			0.2	22.3 ± 4.2	2.08 ± 0.52	56.0 ± 9.5	2.06 ± 0.53	39.1 ± 5.6	2.01 ± 0.23	39.0 ± 9.1	2.22 ± 0.51
NN		0.5		0.1		26.9 ± 4.7	1.78 ± 0.57	59.0 ± 9.1	1.66 ± 0.51	29.1 ± 5.5	1.02 ± 0.28	49.0 ± 9.1	2.62 ± 0.51
NN	6			0.1		12.6 ± 5.8	1.23 ± 0.35	35.6 ± 9.8	1.38 ± 0.32	11.9 ± 6.0	1.01 ± 0.27	43.6 ± 9.8	3.31 ± 0.32
NN		1.0	0.1			16.5 ± 7.9	2.10 ± 0.24	71.4 ± 3.9	2.32 ± 0.59	52.7 ± 0.7	1.91 ± 0.51	63.4 ± 3.9	3.32 ± 0.49
NN		1.5	0.1			35.0 ± 6.8	1.52 ± 0.74	62.6 ± 4.7	2.45 ± 0.76	29.8 ± 4.3	1.12 ± 0.62	65.6 ± 4.7	3.40 ± 0.76
NN		3.0	0.1			27.3 ± 9.4	2.03 ± 0.43	21.6 ± 8.2	1.23 ± 0.34	75.0 ± 2.2	3.22 ± 0.43	21.6 ± 8.2	1.22 ± 0.34
NN		1.5			0.2	32.0 ± 6.3	1.82 ± 0.76	52.6 ± 4.7	2.40 ± 0.76	59.8 ± 4.2	2.12 ± 0.52	69.8 ± 3.3	3.13 ± 0.62
NN		3.0			0.2	37.3 ± 9.4	2.03 ± 0.43	41.6 ± 8.5	1.25 ± 0.34	65.0 ± 2.6	4.21 ± 0.43	74.8 ± 2.2	4.41 ± 0.43
QL		1.0	0.1			10.6 ± 4.6	1.85 ± 0.21	83.6 ± 0.92	3.05 ± 0.72	71.2 ± 1.5	3.71 ± 0.51	65.6 ± 4.6	3.55 ± 0.21
QL		1.5			0.2	41.6 ± 3.6	1.80 ± 0.20	71.6 ± 0.95	3.45 ± 0.70	61.2 ± 1.8	3.52 ± 0.41	67.6 ± 3.6	3.20 ± 0.20
QL		0.5		0.1		15.0 ± 2.3	1.21 ± 0.79	25.7 ± 4.3	1.52 ± 0.21	25.3 ± 3.1	1.53 ± 0.29	45.0 ± 2.3	2.21 ± 0.79

Data were presented as means ± SD

Effect of media and PGRs on shoot organogenesis of *Pyrus* species

In our preliminary studies, we found that shoot organogenesis of *Pyrus* species varied largely with medium and PGRs (data not shown). Therefore, further experiments with *Pyrus communis* Bartlett, *P. pyrifolia* Shenbuzhi, *P. bretschneideri* Zaosu and *P. ussuriensis* Manyuanxiang were designed to select optimal medium and combination of PGRs. In those experiments, basal leaf sections were used.

Effect of ratio of nitrate/ammonium on shoot organogenesis

The effect on shoot regeneration of different ratios of nitrate/ammonium in NN medium, adjusted with KNO_3 (Table 2) and supplemented with 1.0 mg/l TDZ [thidiazuron (N-phenyl-N[1]-1,2,3-thiadiazol-5-ylurea)] and 0.1 mg/l NAA (α-naphthaleneacetic acid), was tested on basal leaf sections of *P. pyrifolia* Shenbuzhi that better underwent caulogenesis.

Rooting of shoots and plant establishment

In about 6 weeks on MS complete medium containing 1.0 mg/l BA and 0.1 mg/l IBA, well-developed shoots longer than 1.5 cm and with 4–5 expanded leaves were transferred to 1/4 MS supplemented with 2.5 mg/l IBA and 30.0 g/l sucrose with or without 1.0 g/l activated charcoal for root formation. The shoots were first incubated in dark for 10 days and then grown under light conditions in the growth chamber as described before. After rooting, plantlets were washed thoroughly with water and transplanted to plastic pots (11 cm in diameter) containing a sand:perlite:soil = 1:1:1 (v:v:v) mixture. Plantlets were covered with transparent plastic bags and placed under greenhouse conditions. After acclimatization for 3–4 weeks, the plastic bags were removed and the plants were successfully established under greenhouse conditions.

The experiments were carried out as completely randomized designs and repeated at least 3 times with 3 replicates (3 Petri dishes) for each experiment. The data were statistically analyzed using Student's *t*-test. Least significant difference (LSD) was calculated at $P = 0.05$.

RESULTS AND DISCUSSION

Shoot organogenesis from leaf sections

Shoot organogenesis from leaf sections of *Pyrus* species was presented in Figures 2–4. Leaf sections, especially the midribs, became swollen and a little white callus appeared along the wounded edges of the explants 15 days after plating (Figure 2a–c). 10 to 15 days later, adventitious buds or shoots could be observed under a dissecting microscope (Figure 3). Adventitious buds or shoots formed as nodules with a glossy surface as compared with the more diffuse appearance of other filamentous cells that occurred simultaneously along the leaf segments. They developed mostly on the midribs or in association with vascular tissues (Figure 4a, d). Although some segments formed a large portion of callus, shoots seemed to arise directly from the leaves (Figure 4c, d).

Table 2. Effects of NH_4^+-N/NO_3^--N ratio on shoot organogenesis of basal leaf sections of *P. pyrifolia* Shenbuzhi

Ratio	Total N	NH_4^{+*}	NO_3^{-*}	K^{+*}	Regeneration rate (%)[1]	Average shoot numbers[1]
0:1	18.40	0.00	18.40	18.90	0^c	0^c
1:2	27.40	9.00	18.40	9.90	68.2 ± 3.4^b	2.00 ± 0.47^b
1:3	27.40	6.90	20.50	14.10	70.6 ± 3.1^b	2.06 ± 0.35^b
1:4	27.50	5.50	22.00	17.00	72.1 ± 6.1^b	$2.23 \pm 0.29^{a, b}$
1:5	27.00	4.50	22.50	18.50	76.4 ± 3.3^b	2.85 ± 0.25^a
1:7	28.00	3.50	24.50	21.50	97.4 ± 2.4^a	2.87 ± 0.73^a

*concentration at mmol/l was used

[1]data with different letters in the same column are significantly different at $P < 0.05$ (Student's *t*-test)

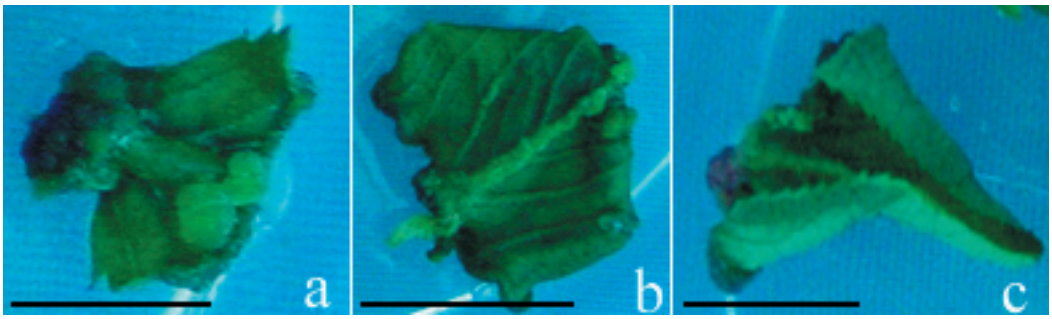

Figure 2. Callus formation along the wounded edges of the basal sections of leaves (a) or on the midribs, or in association with vascular tissues of the middle and apical sections of leaves (b and c) of *P. ussuriensis* Manyuanxiang on medium NN + 3.0 mg/l TDZ + 0.2 mg/l IAA 15 days after plating (scale bar 0.5 cm)

Effects of different sections of leaves on shoot regeneration

The apical leaf sections formed little callus along the wounded edges and some turned brown without any regeneration (Figure 4e). The middle leaf sections produced abundant callus along the wounded edges. Occasionally, shoots could be found from the proximal sides of the sections that produced little callus. The basal sections also produced callus, but shoots formed directly from both proximal and distal sides of the sections (Figure 4a–d). Table 3 showed the regeneration capability of different leaf sections using *P. ussuriensis* Manyuanxiang as an example, so that regeneration results reported in Table 1 refer to basal leaf sections, and only basal sections were used in further experiments.

Effects of medium, basal salts composition and PRGs on shoot organogenesis of *Pyrus* species

Shoot organogenesis varied largely with *Pyrus* species when cultured on the same medium (Table 1). All four *Pyrus* species showed no response to shoot organogenesis when cultured on MS medium supplemented with 3.0 mg/l BA and 0.1 mg/l NAA. About 62.8% regeneration rate with an average of 3.04 shoots were obtained on the basal leaf sections of *Pyrus communis* Bartlett when cultured on MS + 6.0 mg/l BA + 0.1 mg/l NAA. This medium however appeared not suitable for *P. pyrifolia* Shenbuzhi, *P. bretschneideri* Zaosu and *P. ussuriensis* Manyuanxiang, since only 5.60–10.6% basal leaf sections regenerated an average of 0.41–0.78 shoots on such medium. When using NN or QL medium instead of MS medium and TDZ instead of BA, the regeneration rates and regenerated shoot numbers of the basal leaf sections of *P. pyrifolia* Shenbuzhi, *P. bretschneideri* Zaosu and *P. ussuriensis* Manyuanxiang were improved significantly. The maximum regeneration frequency of 83.6%, 75.1% and 74.8% with average shoot numbers of 3.05, 3.22 and 4.41 were recorded for *P. pyrifolia* Shenbuzhi, *P. bretschneideri* Zaosu and *P. ussuriensis* Manyuanxiang, respectively. These values were obtained on QL medium containing 1.0 mg/l TDZ and 0.1 mg/l NAA, NN medium containing 3.0 mg/l TDZ and 0.1 mg/l NAA and NN containing 3.0 mg/l TDZ and 0.2 mg/l IAA, respectively, although other media with different combinations of PGRs could also induce adventitious bud formation.

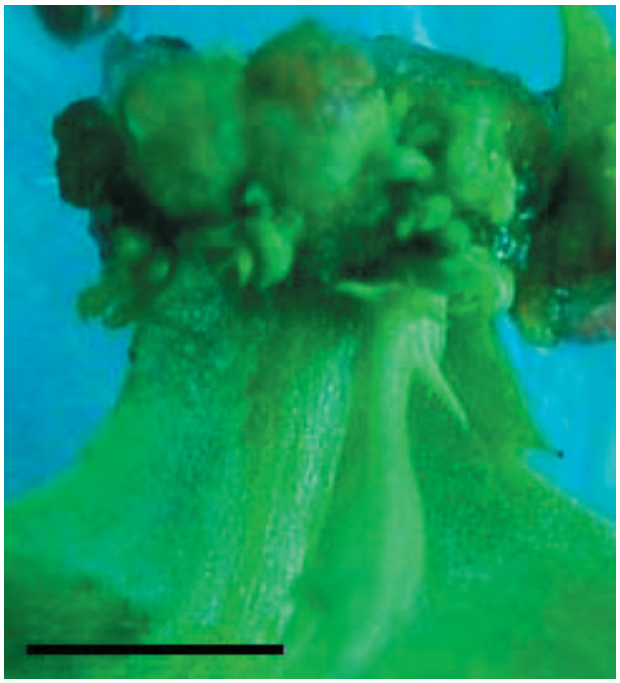

Figure 3. Adventitious bud formation from basal leaf sections of *P. ussuriensis* Manyuanxiang on medium NN + 3.0 mg/l TDZ + 0.2 mg/l IAA 25 days after plating (scale bar 0.5 cm)

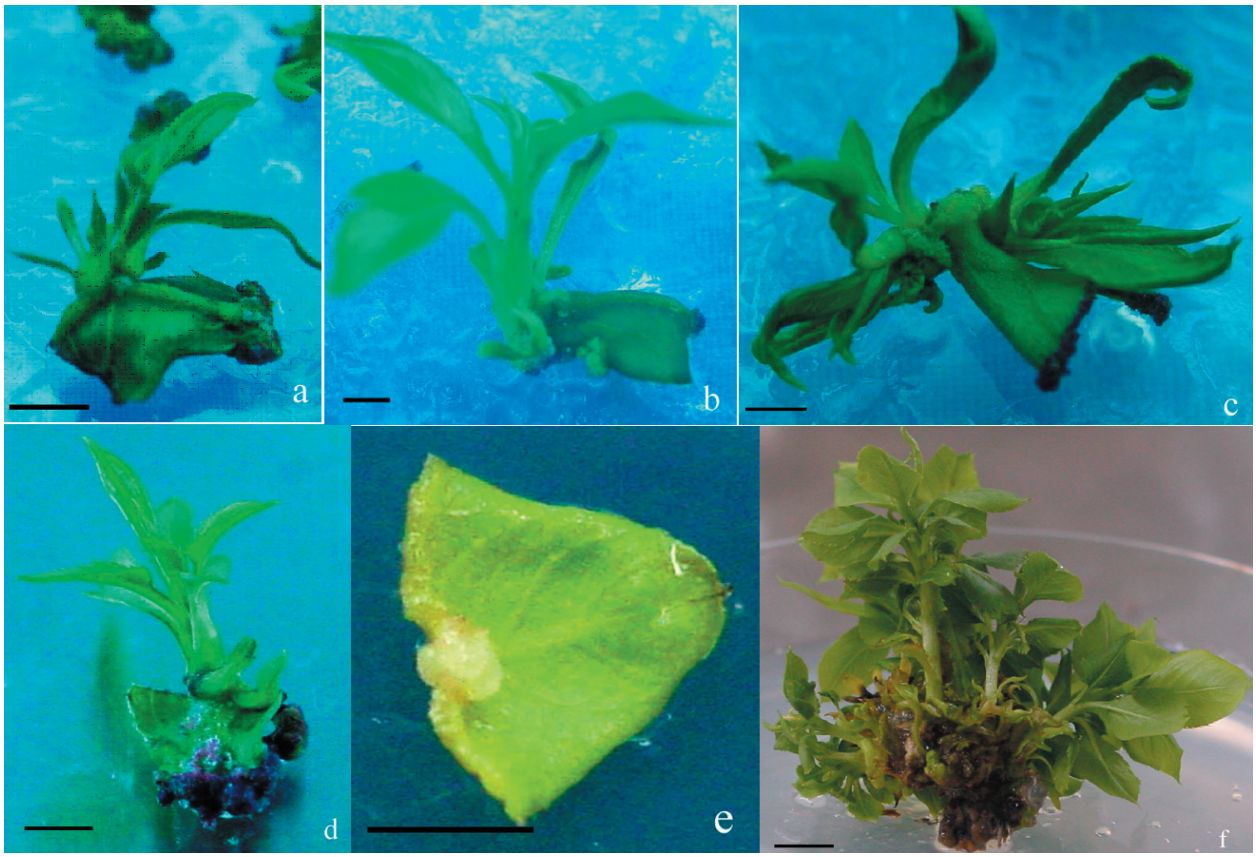

Figure 4. Shoot organogenesis from different leaf sections of *Pyrus* sp. (a) Direct shoot formation along the midribs of the proximal side of the basal section of leaves of *P. pyrifolia* Shenbuzhi on medium QL + 1.0 mg/l TDZ + 0.1 mg/l NAA 30 days after explanting; (b) adventitious shoots regeneration from the proximal side of the basal section of leaves of *P. communis* Bartlett on medium MS + 0.5 mg/l TDZ + 0.1 mg/l IBA 30 days after explanting; (c) direct shoot regeneration from the proximal side of the basal section of leaves of *P. bretschneideri* Zaosu on medium QL + 1.0 mg/l TDZ + 0.1 mg/l NAA 30 days after explanting; (d) adventitious shoot formation from the distal side of the basal section of leaves of *P. ussuriensis* Manyuanxiang on medium NN + 3.0 mg/l TDZ + 0.2 mg/l IAA 20 days after plating; (e) the apical leaf sections of *P. pyrifolia* Shenbuzhi without any regeneration on medium QL + 1.0 mg/l TDZ + 0.1 mg/l NAA 30 days after explanting; (f) proliferation of regenerated shoots of *P. pyrifolia* Shenbuzhi on medium MS + 1.0 mg/l BA + 0.1 mg/l IBA (scale bar 0.5 cm)

Effects NH_4^+-N/NO_3^--N ratio on shoot organogenesis

Basal leaf sections of *P. pyrifolia* Shenbuzhi were not able to regenerate shoots when cultured on the medium without nitrate (the ratio of NH_4^+-N/NO_3^--N = 0:1), but adventitious shoots were obtained when cultured on medium containing nitrate (Table 2). Shoot organogenesis increased with a decrease in ratio of nitrate/ammonium and the best results were obtained at the ratio of 1:7.

Rooting of shoots and plant establishment

After 6 weeks of culture on rooting medium with or without AC, 87.5% or 75.0% shoots, respectively, produced roots (Figure 5a, b). After acclimatization for 3–4 weeks, 82.5–85.5% plantlets were successfully established under greenhouse conditions (Figure 5c).

Table 3. Effects of different leaf sections of *P. ussuriensis* Manyuanxiang on shoot formation

Leaf sections	Rate of shoot regeneration (%)	Average shoot numbers
Basal sections	70.12 ± 4.10[a]	3.86 ± 0.26[a]
Middle sections	18.12 ± 2.13[b]	2.21 ± 0.45[b]
Apical sections	0[c]	0[c]

Data with different letters in the same column are significantly different at $P < 0.05$ (Student's *t*-test)

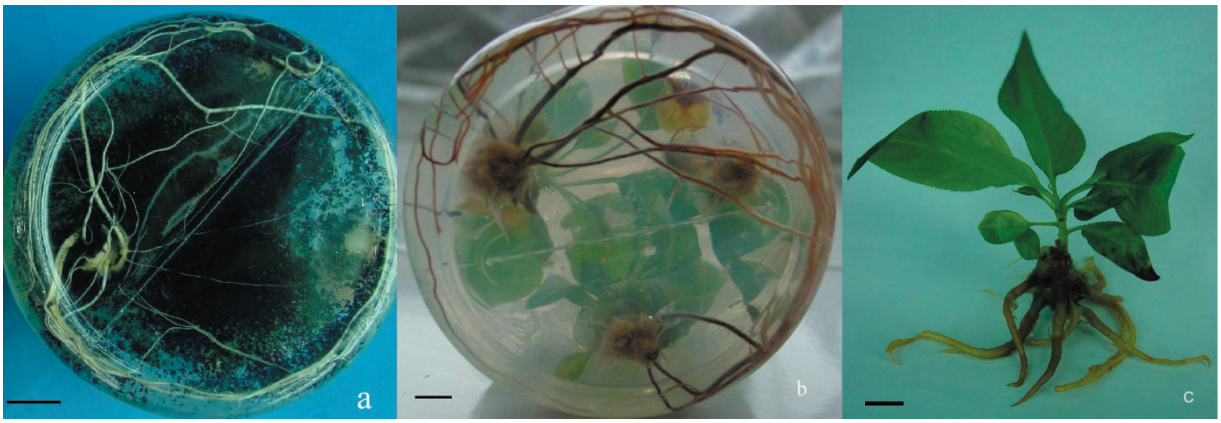

Figure 5. Rooting of leaf-derived shoots of *P. pyrifolia* Shenbuzhi on MS medium supplemented with 2.5 mg/l IBA and 30.0 g/l sucrose with (a) or without (b) 1.0 g/l activated charcoal and a well-developed plantlet (c) (scale bar 0.5 cm)

Pyrus communis, *P. pyrifolia*, *P. bretschneideri* and *P. ussuriensis* are four main species commercially used for pear production (Richard et al. 1996). Since shoot organogenesis from leaf explants of pear was first reported by Laimer da Camara Machado et al. (1988), who used *Pyrus communis* as a model plant, many studies have been dedicated to the development of efficient methods for shoot regeneration in *Pyrus* species (Chevreau et al. 1989, Shibli et al. 2000, Liu and Tang 2003) as well as in important cultivars of *Pyrus communis* (Predieri et al. 1989, Abu-Qaoud et al. 1991, Caboni et al. 1999) and *Pyrus pyrifolia* (Lane et al. 1998). To date, shoot organogenesis from leaf explants was applied to *Pyrus communis*, *P. pyrifolia* and *P. bretschneideri* (Liu 2003). The present study has extended shoot organogenesis to *P. ussuriensis*, thus making transgenic studies of this pear species possible. *P. ussuriensis*, the most cold-tolerant species of *Pyrus* genus (Richard et al. 1996), consists of about 24 elite commercially-grown cultivars (Shen 1980) and is widely grown in China, Manchuria and Siberia (Richard et al. 1996). Transformation of *P. ussuriensis* is now under investigations in our laboratory.

It has been repeatedly found that when a complete organ such as leaf is cut into sections, its regenerative ability differs with its original positions on the organ. The regenerative capacity increased substantially from the distal sections toward the proximal sections of cotyledons or leaves (Tang et al. 2002, Li et al. 2004). Tang et al. (2000) found that higher concentrations of PGRs were needed for distal and medium sections of *Prunus cerasus* cotyledons to undergo morphogenesis than those for proximal sections although their regeneration percentages were similar. The difference in regenerative ability of explant source might be due to differences in the levels of endogenous hormones in the explants or an interaction between the endogenous and exogenous hormone levels in the different source explants.

Cells that have acquired competence for morphogenesis *in vitro* or that already possessed morphogenic competence at the time of explanting are prepared to express a permissive pattern of development in an appropriate *in vitro* environment. Pattern of development was determined mostly by plant species and medium constituents. James et al. (1984) reported that *Malus* rootstocks M_{25} and M_{27} could be regenerated from leaf segments on the same medium, however, M_9 and M_{26} could not be regenerated using the same protocol. In sweet and sour cherry, cultivar-medium dependence in adventitious shoot regeneration from *in vitro* leaves was well demonstrated (Tang et al. 2002). Our results with different species of *Pyrus* also confirmed genotypic specificity in morphogenesis as well as the critical effectiveness of basic media. The interactions between genotypes of pear and PGRs affecting adventitious formation of their leaf explants were demonstrated by Bell (2003) and Poudyal et al. (2008). The influence of various minerals in different media on adventitious shoot formation is not clearly understood, but the contents of nitrate and ratios of nitrate/ammonium in basic medium in triggering morphogenic progress and regulating the switch of a somatic cell from one pathway to another have been widely reported. Abu-Qaoud et al. (1991) confirmed that nitrogen amount and its form and NH_4^+-N/NO_3^--N ratios all influenced regeneration of shoots from leaves of *Pyrus communis*. NH_4^+ promoted the penetration of anions into leaves, at the expense of cations, while

NO_3^- led to the reverse process (Heller 1977). Our results showed that in the absence of NH_4^+-N, no leaves regenerated shoots even though the amount of Ca^{2+} and total nitrogen varied among the various NH_4^+-N/NO_3^--N ratios, which confirmed the assertion of Fasolo et al. (1989) that NH_4^+-N was more important for adventitious shoot formation of *in vitro* leaves of *Pyrus*.

REFERENCES

Abu-Qaoud H., Skirvin R.M., Below F.E. (1991): Influence of nitrogen form and NH_4^+-N:NO_3^--N ratios on adventitious shoot formation from pear (*Pyrus communis* L.) leaf explants *in vitro*. Plant Cell Tissue Organ Cult., *27*: 315–319.

Abu-Qaoud H., Skirvin R.M., Chevreau E. (1990): *In vitro* separation of chimeral pears into their component genotypes. Euphytica, *48*: 189–196.

Bell R.L. (2003): Interaction of genotype and auxin affecting adventitious regeneration of pear. HortScience, *38*: 750.

Brisset M.N., Paulin J.P., Duron M. (1988): Feasibility of rating fire blight susceptibility of pear varieties (*Pyrus communis*) on *in vitro* microcuttings. Agronomie, *8*: 707–710.

Caboni E., Tonelli M.G., Lauri P., D'Angeli S., Damiano C. (1999): *In vitro* shoot regeneration from leaves of wild pear. Plant Cell Tissue Organ Cult., *59*: 1–7.

Chevreau E., Skirvin R.M. (1992): Pear. In: Hammerschlag F.A., Litz R.E. (ed.): Biotechnology of Perennial Fruit Crops. Biotechnology in Agriculture No. 8. CAB International, Wallingford: 263–276.

Chevreau E., Skirvin R.M., Abu-Qaoud H., 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.

Duron M., Paulin J.P., Brisset M.N. (1987): Use of *in vitro* propagated plant material for rating fire blight susceptibility. Acta Hort., *217*: 317–324.

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.

Hammerschlag F.A., Litz R.E. (1992): Biotechnology of Perennial Fruit Crops. Biotechnology in Agriculture No. 8. CAB International, Wallingford.

Heller R. (1977): Abrégé de Physiologie Végétale. Tome 1-Nutrition. Masson, Paris.

James D.J., Passey A.J., Malhotra S.B. (1984): Organogenesis in callus derived from stem and leaf tissues of apple and cherry rootstocks. Plant Cell Tissue Organ Cult., *4*: 256–258.

Laimer da Camara Machado M., da Camara Machado A., Hazer V. (1988): Regeneration of shoot from leaf discs and stem microcuttings of fruit trees as a tool for transformation. Acta Hort., *235*: 85–92.

Lane W.D., Iketani H., Hayashi T. (1998): Shoot regeneration from cultured leaves of Japanese pear (*Pyrus pyrifolia*). Plant Cell Tissue Organ Cult., *54*: 9–14.

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

Lee C.H., Kim S.B., Han D.H., Kim C.S., Noh Y.M., Ban S.J., Lee D.W., Lee G.P. (2004): Shoot organogenesis from leaf explants in Japanese pear (*Pyrus pyrifolia*). Acta Hort., *653*: 215–218.

Li X.M., Tang H.R., Luo Y. (2004): Adventitious shoot regeneration from the cotyledons of matured embryos in pear cv. Jinhua. J. Fruit Sci., *21*: 295–297. (In Chinese)

Liu C.Q. (2003): The system of *in vitro* culture and shoot regeneration from leaves of pear cultivars. [M.D. Thesis.] Sichuan Agricultural University, P. R. China. (In Chinese)

Liu C.Q., Tang H.R. (2003): An overview of progress on shoot regeneration from pear leaves and genetic transformation. J. Fruit Sci., *20*: 374–378. (In Chinese)

Murashige T., Skoog F. (1962): A revised medium for rapid growth and bioassays with tobacco cell cultures. Physiol. Plant., *15*: 473–479.

Nitsch J.P., Nitsch C. (1969): Haploid plant from pollen grains. Science, *85*: 169.

Palombi M.A., Lombardo B., Caboni E. (2007): *In vitro* regeneration of wild pear (*Pyrus pyraster* Burgsd) clones tolerant to Fe-chlorosis and somaclonal variation analysis by RAPD markers. Plant Cell Rep., *26*: 489–496.

Poudyal B.K., Zhang Y.X., Du G.Q. (2008): Adventitious shoot regeneration from the leaves of some pear varieties (*Pyrus* sp.) grown *in vitro*. Front. Agric. China, *2*: 82–92.

Predieri S., Fasolo F.M.F., Passey A.J., Ridout M.S., James D.J. (1989): Regeneration from *in vitro* leaves of Conference and other pear cultivars (*Pyrus communis*). J. Hortic. Sci., *64*: 553–559.

Quoirin M., Lepoivre P. (1977): Improved media for *in vitro* culture of *Prunus* sp. Acta Hort., *78*: 437–442.

Richard L.B., Harvey A.Q., Richard E.C.L., Robert M.S. (1996): Pear. In: Jules J., James N.M. (eds.): Fruit Breeding, Vol. 1: Tree and Tropical Fruits. John Wiley and Sons, Inc., Hoboken.

Shen T. (1980): Pears in China. HortScience, *15*: 13–17.

Shibli R.A., Ajlouni M.M.A., Obeidat A.A. (2000): Direct regeneration from pear (*Pyrus syriaca*) leaf explant. Adv. Hortic. Sci., *14*: 12–18.

Tang H.R., Ren Z.L., Krczal G. (2000): Somatic embryogenesis and organogenesis from immature embryo cotyledons of three sour cherry cultivars (*Prunus cerasus* L.). Sci. Hortic., *83*: 109–126.

Tang H.R., Ren Z.L., Reustle G., Krczal G. (2002): Plant regeneration from leaves of sweet and sour cherry cultivars. Sci. Hortic., *93*: 235–244.

Received on December 22, 2007

Corresponding author:

Prof. Dr. Haoru Tang, Sichuan Agricultural University, College of Forestry and Horticulture, Department of Horticulture, Ya'an, Sichuan 625014, P. R. China
phone: + 86 835 288 25 15, fax: + 86 835 288 25 78, e-mail: htang@sicau.edu.cn