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RESEARCH ARTICLE

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Adventitious shoot regeneration from the leaves of some pear varieties (*Pyrus* spp.) grown *in vitro*

Abstract The pear (*Pyrus* spp.) is one of the most important temperate fruit crops. A complete protocol for adventitious shoot regeneration was developed from the leaves of four pear varieties grown *in vitro*: Abbe Fetel, Yali, Packham's Triumph and Aikansui, and the Chinese rootstock variety Duli. Shoot explants were collected from the field and cultured *in vitro* in Murashige and Skoog (MS) media supplemented with 1.0 mg·L^{-1} 6-benzylaminopurine (BA) and 0.1 mg·L^{-1} indole-3-butyric acid (IBA). After four weeks, leaf explants of all 5 varieties grown *in vitro* were excised and cultured in MS media supplemented with 0.0 mg·L^{-1}, 0.2 mg·L^{-1}, 0.5 mg·L^{-1}, 1.0 mg·L^{-1} and 2.0 mg·L^{-1} naphthaleneacetic acid (NAA) and 5.0 mg·L^{-1} BA or with 1.0 mg·L^{-1}, 2.0 mg·L^{-1} and 4.0 mg·L^{-1} thidiazuron (TDZ). The cultures were maintained in darkness for 21 days for shoot induction in the shoot induction medium (IM), then transferred to the shoot expression medium (EM) in 1.0 mg·L^{-1} TDZ without any auxins and kept in a growth room at (25 ± 2)°C under a 16/8 h light/dark photoperiod regime for 8 weeks. Finally, the shoots were transferred to the MS shoot elongation medium (SEM) supplemented with 0.2 mg·L^{-1} BA, 0.1 mg·L^{-1} IBA and 0.2 mg·L^{-1} gibberellic acid (GA_3). A combination of TDZ and NAA had a significant effect on the number of shoot regenerations in all 5 tested varieties. The maximum mean number of shoots and maximum number of shoots per leaf obtained from Yali variety were 11.8 ($P \leqslant 0.001$) and 22, followed by Aikansui with 6.6 ($P \leqslant 0.001$) and 4.6, and Duli with 8 ($P \leqslant 0.001$) and 12, all arising from the combination of 0.2 mg·L^{-1} NAA with 1.0 mg·L^{-1} TDZ. For Packham's Triumph and Abbe Fetel, the maximum mean number of shoots and maximum number of shoots per leaf were 5.6 ($P \leqslant 0.001$), 4.8 and 8 ($P \leqslant 0.001$), and 11, respectively, from the combination of NAA (1.0 mg·L^{-1}) and TDZ (2.0 mg·L^{-1}). Abbe Fetel was the only variety which produced significantly higher adventitious shoots from the two different combinations of 1.0 mg·L^{-1} NAA and 5.0 mg·L^{-1} BA ($P \leqslant 0.05$), and 2.0 mg·L^{-1} NAA and 5.0 mg·L^{-1} BA ($P \leqslant 0.01$). Some of the most prominent problems associated with shoot proliferation and regeneration were also observed and discussed in this paper.

Keywords adventitious shoot regeneration, *in vitro*, MS medium, *Pyrus* spp., tissue culture, phytohormones

1 Introduction

The pear is one of the oldest fruit crops widely grown in temperate and sub-tropical regions of the world and is one of the most important temperate fruit crops. It belongs to the *Rosaceae* family and *Pomoideae* subfamily (Jackson, 2003; Jules and James, 1996). Out of the 22 species of *Pyrus*, 16 originated from Asia. Mainland China is the center of origin of most Asian pears (Nee et al., 2002; Wang, 1996). The history of pear growing in China has spanned more than 4000 years (Wang, 1996).

Among the approaches used for the production of transgenic crops through tissue culture techniques, adventitious shoot regeneration through the complex process of somatic embryogenesis is the means by which many plant species are commercially micropropagated and produced by regenerating shoots and roots from transformed cells or tissues. A common problem encountered in micropropagation and plant transformation is that, within a plant species, different varieties and cultivars vary widely in their capacities to regenerate and are influenced by phytohormone types and their concentrations, genotypes, etc. (Sonia et al., 2004; Bell, 2003).

Over the past several decades, many scientists have conducted adventitious shoot regeneration using leaves

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of many plant species, including the pear. However, only a limited number of pear cultivars have been studied so far. Many aspects of adventitious shoot regeneration in European pears, including the cultivars Conferenc, Abbe Fetel, Quince rootstock BA 29 (Marino and Molendini, 2005), Bartlett, Beurre Bosc (Bell, 2003), Onward and Old Home (Sun et al., 2005) have been studied. Similarly, adventitious shoot regeneration from leaves grown *in vitro* has been studied in Asian pears, including Japanese cultivars such as Kosui, Hosui, Niitaka, Wasekaso, Okusankichi and Whangkeumbae (Lee et al., 2004); Chinese pears such as Xueqing (Shaohu et al., 2005) and Iranian pears such as Sebri (Amiri, 2002). However, very little research has been done on other cultivars like Yali and the rootstock cultivar Duli.

Growing plantlets *in vitro*, adventitious shoot regeneration from the leaf explants and the maintenance of shoots, etc, need to be investigated to determine the regeneration protocols useful for each cultivar (Ana et al., 2006). This experiment was conducted to find out the best combinations and concentrations of some phytohormones for the development of an adventitious shoots regeneration protocol from the leaves of 4 *Pyrus* species (5 cultivars) and 1 rootstock of pear as listed in Table 1. It was also designed to determine the most prominent problems associated with shoot proliferation and regeneration, and to suggest effective measures to solve these problems.

Table 1 Pear varieties used in the experiment

varieties	pear types	Latin names
Yali	Asian pear (Chinese pear)	*Pyrus bretschneideri*
Aikansui	Asian pear	*P. pyrifolia*
Whangkeumbae	Asian pear	*P. pyrifolia*
Abbe Fetel	European pear	*P. communis*
Packham's Triumph	European pear	*P. communis*
Red Bartlett*	European pear	*P. communis*
Duli (Birch Pear)+	Asian pear rootstock	*P. betulaefolia*

Note: + represents this variety was not included during browning and shoots proliferation experiment; * represents this variety was not used for shoot regeneration.

2 Materials and methods

2.1 Plant materials

Six different cultivars of pears and one rootstock from 4 different *Pyrus* spp. were selected for the study (Table 1). Newly grown fresh shoots about 10 to 15 cm in length of all 7 varieties were taken from the pear orchard located on the West Campus of the Agricultural University of Hebei in Baoding, China, as explant samples. One hundred newly grown shoots were collected from each variety from the field during the first week of June in 2006 for shoot proliferation.

2.2 Preparation and sterilization of explants, utensils and medium

Immediately after being detached from the trees, all the explants were placed under running tap water for one hour. After being cleaned with running water, the explants were again cleaned with distilled water three times. The surfaces of the samples were sterilized with 0.1% mercuric chloride ($HgCl_2$) for 7 min and 75% ethyl alcohol for 30 s. The explants were then cleaned with sterile distilled water another 3 times. Finally, after proper surface sterilization, the base and tips of the explants were cut off and divided into 3 parts — the tip, middle and bottom — each 1.5 to 2.0 cm long. These were then were excised for *in vitro* shoot culture. All the glassware and utensils were sterilized in an autoclave at 0.12 MPa pressure at 121°C for 45 min, while the medium was sterilized for 20 min.

2.3 Shoot proliferation

One hundred explants of each variety were initially cultured in conical flasks (4 explants each), for shoot proliferation in a 25 mL MS-based shoot proliferation medium (PM) as specified by Marino and Molendini (2005). Some modifications to the medium were implemented, including supplementation with different kinds of growth hormones and chemicals (Table 2). Later, the number of flasks was increased according to the proliferation capacity of each variety for subcultures. All the culture and subculture materials were kept in a growth room at (25 ± 2)°C under a 16/8 hours light/dark photoperiod regime.

2.4 Effect of hormones on shoot regeneration

The medium without any hormone (T-1 and T-2), the medium with only 5.0 $mg \cdot L^{-1}$ BA (T-3) and the medium with only 1.0 $mg \cdot L^{-1}$ TDZ (T-4) and the combination of NAA and BA or TDZ as in T-5 to T-12 were tested for the role of phytohormones in adventitious shoot regeneration (Table 3).

2.5 Effects of BA on shoot proliferation

Two different treatments of BA — 0.5 $mg \cdot L^{-1}$ and 1.5 $mg \cdot L^{-1}$, along with 0.1 $mg \cdot L^{-1}$ IBA — were tested to determine the effect of BA on shoot proliferation. Varieties were classified under Group I which produced a high number of shoots from 1.5 $mg \cdot L^{-1}$ BA, and Group II which produced a low number of shoots. The total number of shoots regenerated from each treatment (5 replicates with 5 explants in each replication, totaling 25 explants) was recorded and the results from the two different treatments of BA were compared to the effects on shoot proliferation during subculture.

Table 2 Culture medium (pH 5.8) and their compositions used during shoot proliferation and adventitious shoot regeneration

items	minerals, hormones and vitamins used	PM/ mg·L^{-1}	IM/ mg·L^{-1}	EM/ mg·L^{-1}	SEM/ mg·L^{-1}
minerals	microelements	MS	MS	MS	MS
	macroelements	MS	MS	MS	MS
organic supplements	vitamin B_1	0.1	0.1	0.1	0.1
	vitamin B_6	0.5	0.5	0.5	0.5
	nicotinic acid	0.5	0.5	0.5	0.5
	glycine	2.0	2.0	2.0	2.0
	inositol	100	100	100	100
growth regulators	BA	1.0	5.0	–	0.2
	IBA	0.1	–	–	0.1
	GA_3	–	–	–	0.2
	TDZ	–	1, 2 and 4	1.0	–
	NAA	–	0.2, 0.5, 1.0, 2.0	–	–
gelling agent, sucrose and PVA	agar	6000	6000	6000	6000
	sucrose	30000	30000	30000	30000
	PVA	–	–	–	1000

Note: Modified from Marino and Molendini (2005). PM = Proliferation medium; IM Induction medium; EM = Expression medium; SEM = Shoot elongation medium.

2.6 Effect of browning

One hundred newly cultured explants from 25 conical flasks were randomly selected for observing the browning problem. The varieties were classified according to the intensity of browning. Varieties with more than 66% of explants infected by browning were classified under Group I as high browning problem varieties. Varieties with more than 33% but less than 66% of the explants infected by browning were classified under Group II as moderate browning problem varieties. Finally, varieties with less than 33% of the explants infected by browning were classified under Group III as less browning problem varieties. Observation of the browning problem was conducted thrice in April and May 2007 and July 2006. Several trials were conducted to determine the appropriate solution to the browning problem.

2.7 Adventitious shoot regeneration

Healthy and fully developed leaves together with their petioles were collected from 4-week-old sub-cultured plantlets grown in vitro. A small amount of sterilized distilled water was added to prevent dehydration of the leaves before cutting. Three horizontal cuts along the midribs of the leaves were made for the culture in MS induction media (IM) with 12 combinations of NAA-TDZ, NAA-BA and control, as shown in Table 3. Five combinations of NAA with 5 mg·L^{-1} BA along with one control (without any hormone) and 5 mg·L^{-1} BA were tested. Five other combinations of NAA with 1 mg·L^{-1}, 2 mg·L^{-1} and 4 mg·L^{-1} TDZ and one control were tested for their effects on shoot regeneration in the six varieties. The total number of adventitious shoots regenerated in each treatment and the maximum number of shoots from one leaf were recorded immediately after transformation in the shoot elongation medium (SEM). All the leaves were kept abaxial side up in the MS medium prepared in a 50 mL conical flask containing 25 mL of induction medium (IM). After being cultured in the medium, all the treatments were kept for 21 days in continuous darkness for shoot induction.

Table 3 Combination of hormones in induction medium (IM)

treatments	hormones used/ mg·L^{-1}		
	NAA	BA	TDZ
T-1	0.0	0.0	–
T-2	0.0	–	0.0
T-3	0.0	5.0	–
T-4	0.0	–	1.0
T-5	0.2	5.0	–
T-6	0.2	–	1.0
T-7	0.5	5.0	–
T-8	0.5	–	1.0
T-9	1.0	5.0	–
T-10	1.0	–	2.0
T-11	2.0	5.0	–
T-12	2.0	–	4.0

Twenty-one days after dark treatment for shoot induction, the samples were transferred to the auxin-free shoot expression media (EM) with only 1 mg·L^{-1} TDZ. After the shoots regenerated from the leaves, they were transferred to the shoot elongation medium (SEM) composed of 0.2 mg·L^{-1}, 0.1 mg·L^{-1} and 0.2 mg·L^{-1} BA, IBA and GA_3 (Table 2). 1 g·L^{-1} polyvinyl alcohol (PVA) was also added to control vitrification in SEM. Cotton was used to cover the mouths of the conical flasks to reduce vitrification. The leaves were kept in the EM for up to 2 months to regenerate shoots and the newly grown shoots were maintained in the SEM continuously. Newly grown shoots were transferred to the SEM by detaching them from the leaves with a small piece of old leaf still attached and the remaining leaves were again transferred to the EM for further shoot regeneration.

2.8 Maximum and mean number of shoots and regeneration percentage

The maximum number of shoots per treatment, the maximum number of shoots per leaf, the mean number of shoots of each variety in each treatment and the leaf regeneration percentage were recorded during the transformation of the shoots from EM to SEM.

2.9 Treatment and replicates

Five replicates of each pear variety were made consisting of 5 leaves and their petioles in a 50 mL conical flask containing 25 mL IM, EM and SEM. Each flask was considered one replicate. Twenty-five leaves in a treatment of each variety were used for 5 replicates. All the replicates and treatments were arranged in a completely randomized manner and all the experiments were conducted only once for the shoot regeneration trial.

2.10 Statistical analysis

Different computer-based methods were used to analyze the data. The mean difference among the different treatments was compared with that of CK for the adventitious shoot regenerating experiment. A Tukey test with the help of statistical package SPSS (version 12) was used for the significance test at $P \leqslant 0.05$. The data collected in the experiment included the total number of shoots per treatment in each variety for statistical analysis. The results of different treatments of different varieties were compared with each other.

2.11 Definition

The percentage of shoot regeneration was defined as the percentage of leaves which regenerates at least one adventitious shoot from one leaf.

3 Results and discussion

3.1 Shoot proliferation and maintenance

Fifteen days after the culture of explants in the proliferation medium (PM) with 1 $mg \cdot L^{-1}$ BA and 0.1 $mg \cdot L^{-1}$ IBA, bud breaks began growing from the nodes. Five weeks later, the shoots of all varieties were ready for subculture. Thereafter, newly grown shoots were ready for subculture every 4 weeks. Liu (2003) also used the same medium and hormone and concluded that the best sub-culture medium for the in vitro proliferation of Bartlett, Comice, Zaosu and Shenbuzhi pear cultivars was MS containing 1.0 $mg \cdot L^{-1}$ BA and 0.1 $mg \cdot L^{-1}$ IBA, which was the same medium we used in our experiment.

3.2 Effects of BA on shoot proliferation

BA greatly influenced shoot proliferation. On average, Group I varieties (Yali, Whangkeumbae and Aikansui) could produce new shoots during subculture from one explant (7 to 8 new shoots per explant) treated with 1.5 $mg \cdot L^{-1}$ BA and 0.1 $mg \cdot L^{-1}$ IBA at a rate of about 2 times those of Group II varieties (Abbe Fetel, Red Bartlett and Packham's Triumph) from the same amount of medium and hormone (Fig. 1), which produced a maximum of only 4 new shoots per explant. On the other hand, of all the varieties in both groups, only 3 (a maximum of 4.5) new shoots were developed from one explant treated with 0.5 $mg \cdot L^{-1}$ BA and 0.1 $mg \cdot L^{-1}$ IBA. This showed that BA concentration had a very distinct effect on shoot proliferation. According to Fig. 1, among the six varieties, Group I was found to be more sensitive to the BA concentration than the Group II varieties Abbe Fetel, Red Bartlett and Packham's Triumph. From this finding, it is clear that Asian pear varieties like Yali may respond to BA more effectively than European pear varieties. Tang et al. (2005) reported that about 7 times more shoots proliferated in the Xueqing pear in the MS media with 2 $mg \cdot L^{-1}$ BA + 0.1 $mg \cdot L^{-1}$ IAA in every subculture during one to six subcultures. Masanori and Yoshiji (2004) found that 2.5 $mg \cdot L^{-1}$ BA and 0.1 $mg \cdot L^{-1}$ IBA, with sorbitol as a carbon source, was the best medium for shoot proliferation (3.5 shoots per explant) of the Hosui pear.

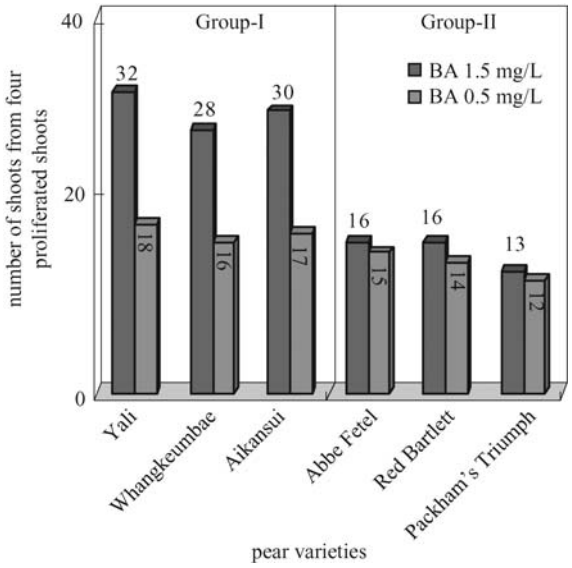

Fig. 1 Effect of BA on shoot proliferation

3.3 Browning problem

Browning of the culture medium was observed to be a severe problem in some varieties in summer (July, August). Yali and Whangkeumbae were the varieties most susceptible

to browning. Among the Group I (high browning varieties), Group II (moderate browning varieties) and Group III (less browning varieties) explants affected, 81% in Yali were infected by browning, 77% were infected in Whangkeumbae; 45% were infected in Aikansui and 34% in Packham's Triumph; and 16% were infected in Red Bartlett but only 9% in Abbe Fetel (Fig. 2).

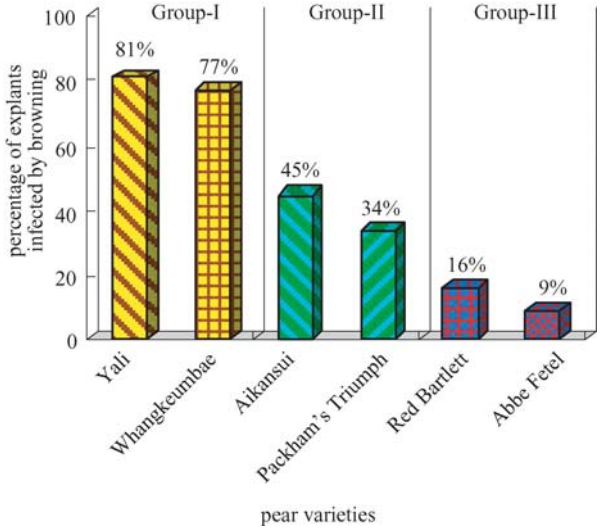

Fig. 2 Different varieties of pear affected by browning with their percentage

Some explants may leach some phenolic substances or secondary metabolites from the cut surface which will oxidize later, causing the media to brown and be toxic to the explants (Aliyu, 2005; Smith, 2000). The problem of browning during plant tissue culture has been reported in many plant species like cashew (Aliyu, 2005), litchi (Chandra and Padaria, 1999), guava (Meghwal et al., 2000), banana (He et al., 1995; http://www.comfsm.fm/library/digitallibrary/kutty.html), avocado (Castro et al., 1995), date palm (www.fao.org/DOCREP/006/Y4360E/y4360e09.htm), and pear (Gao et al., 2003; Ju, 1987; Li et al., 1994; Li and Yan, 2001; Yan and Li, 1998), etc. Within the plant species, different varieties may vary greatly from each other in terms of the browning problem (Smith, 2000). This phenomenon is very common in both Asian and European pears. In our experiment, it was observed that Asian pear varieties such as Yali, Whangkeumbae, and Aikansui were more susceptible to browning than European pear varieties such as Packham's Triumph, Red Bartlett and Abbe Fetel. In another observation, it was also found that the browning problem was much more severe during the summer months of July and August than during the spring months of April and May (data omitted). In addition, it was found that Yali pear shoots were severely infected by browning with a tendency of shoot tip >middle >shoot base (data omitted). Our experiment also showed that severe browning occurred immediately after the culture of explants from the field but the problem was not found in the subsequent subcultures. Water-soluble polyphenols were found to have played a more important role in creating browning in the cultural medium than water-insoluble polyphenols, particularly in the Yali pear. To resolve the browning problem, good shoots without browning were selected for subculture to allow us to obtain enough proliferated shoots. Taking the explants in April and May was another way to overcome the browning problem. Of the different chemicals used to control browning in the Yali pear, polyvinyl pyrrolidone (PVP) at the rate of 0.02%, 100 mg·L^{-1} ascorbic acid in the cultural medium, and 12 h cold treatment of explants prior to culturing, were found to have a more significant effect than that of the check (CK).

3.4 Callus formation

Callus formation was observed in all the varieties except Whangkeumbae, which did not produce any adventitious shoots with no callus formed in any of the treatments. Because of this, Whangkeumbae was excluded from further analysis. All varieties in the treatments T-1, T-2, T-3, T-4 and T-5 were also unable to produce any callus except Duli in T-5 (Fig. 3). The leaves with the highest number of shoots and the maximum number of shoots

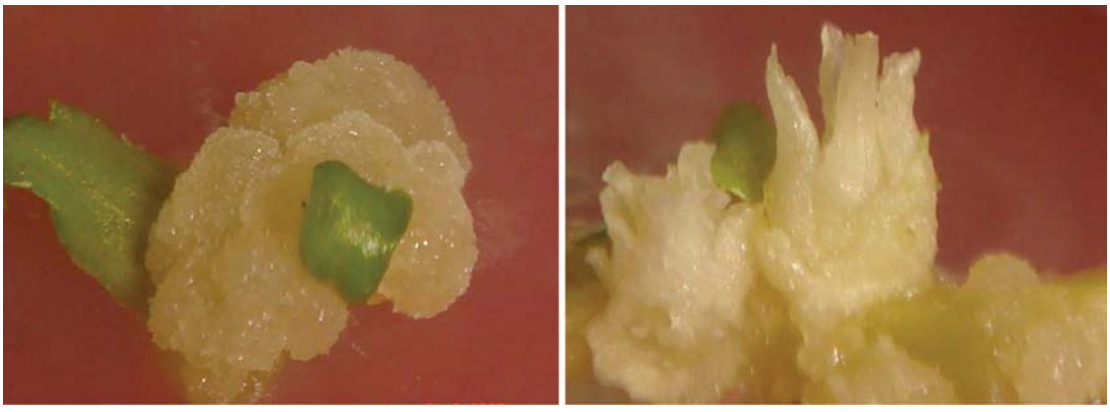

Fig. 3 Ample amount of callus in Duli and shoot initiation from compact callus

per leaf in any treatment could produce larger amounts of callus compared to others. In some cases, however, direct shoots (direct somatic embryogenesis) formed without any callus formation (Fig. 4). Such shoots formed within 21 days of dark treatment and were found to be more vigorous, healthier and taller compared to the shoots induced from an indirect process (indirect somatic embryogenesis), i.e. from the callus. Almost all newly grown adventitious shoots were regenerated from those leaves which produced an ample amount of callus within 21 days of the induction period. Callus formation was concentrated along the cut edges and the petiole tip from where the adventitious shoots regenerated (Fig. 5a). However, in some cases, the adventitious shoots could also emerge from the leaf margin and tip (Fig. 5b). Although the leaves were placed abaxial-side up in the experiment, it was found that shoots emerged from both sides of the leaves (Fig. 5a). Sometimes, the treatments T-10, T-11 and T-12 with high doses of phytohormones could produce a higher amount of callus compared to others with lower doses of hormones (T-6, T-7, T-8 and T-9) in most pear varieties (except Whangkeumbae) but could not produce enough shoots, as expected, causing some types of disorders. The presence of large amounts of callus thus could not always produce a high number of shoots but occasionally produced the disorders discussed below.

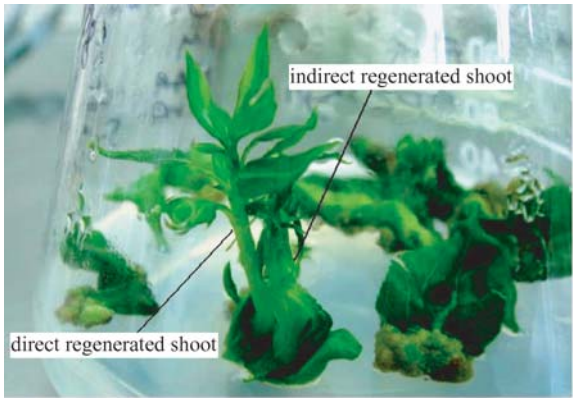

Fig. 4 Direct and indirect shoot regeneration from one leaf

3.5 Burr knots and other physiological disorders

Observation of some varieties showed that with an increase in hormone they could produce radicle structures such as burr knots (Fig. 6) which cannot develop into roots or shoots. The knots remained as such for a long time and eventually died. The burr knots were found in Packham's Triumph in T-10 (1.0 $mg \cdot L^{-1}$ NAA and 2.0 $mg \cdot L^{-1}$ TDZ) and T-12 (2.0 $mg \cdot L^{-1}$ NAA and 4.0 $mg \cdot L^{-1}$ TDZ), and in Abbe Fetel in IM with only 1 $mg \cdot L^{-1}$ NAA. Some abnormalities also existed in Packham's Triumph in T-11 (2.0 $mg \cdot L^{-1}$ NAA and 5.0 $mg \cdot L^{-1}$ BA). The petiole of cultured leaves thickened, turned upright and eventually died without forming any shoots, as shown in Fig. 7. Until now, such an abnormality has not been reported. The Duli in T-11 (2.0 $mg \cdot L^{-1}$ NAA and 5.0 $mg \cdot L^{-1}$ BA) and T-12 (2.0 $mg \cdot L^{-1}$ NAA and 4.0 $mg \cdot L^{-1}$ TDZ) produced excessively big callus which later became dark brown and hard, and eventually died without forming any shoots (Fig. 8). As in Packman's Triumph, this phenomenon was observed and described here for the first time. The burr knots and upright petioles in Duli appeared within 21 days of the induction period, lasted for 3 months and eventually turned brown and died.

3.6 Vitrification

Vitrification or hyperhydricity is also commonly found in newly grown adventitious shoots, especially in shoots developed from the indirect organogenesis of Yali and Aikansui in EM with 1 $mg \cdot L^{-1}$ TDZ. When 1.5 $mg \cdot L^{-1}$ BA with 0.1 $mg \cdot L^{-1}$ IBA was used in PM, symptoms of vitrification appeared in Duli. However, after the shoots were transformed in lower doses of BA (0.5 $mg \cdot L^{-1}$) with 0.1 $mg \cdot L^{-1}$ IBA and the bottle mouth was covered with cotton, the symptoms did not appear. 1 $g \cdot L^{-1}$ PVA may have also effectively controlled vitrification in both PM and SEM. According to Masanori and Yoshiji (2004), a high-carbon source concentration in media may produce the lowest water potential. Conversely, explants cultured in the medium with a lower carbon concentration under a

Fig. 5 Adventitious shoots regenerated from leaves (a), mid and tip petioles (b), and clump of shoots (c) of Yali

Fig. 6 Burr knots in Packham's Triumph

Fig. 7 Abnormal petiole in Packham's Triumph

Fig. 8 Hard and extra big callus in Duli

suboptimal light may grow rapidly because they may absorb inorganic and organic nutrients excessively, which may thus cause hyperhydricity. Lee et al. (2004) found the same vitrified shoots in the medium containing TDZ in some Japanese pears as we found in our experiment.

3.7 Direct and indirect somatic embryogenesis

Direct and indirect somatic embryogenesis can be observed in some varieties, such as Aikansui. Shoots induced from indirect somatic embryogenesis are considered vitrified, abnormal and stunted. Two such types of shoot regeneration were found in the same leaf of the Aikansui in T-6 (0.2 $mg \cdot L^{-1}$ NAA and 1.0 $mg \cdot L^{-1}$ TDZ), shown in Fig. 4. This result confirmed that direct somatic embryogenesis performed better than indirect. It also showed that the number of shoots from direct organogenesis was lower than those generated from indirect somatic embryogenesis. Caboni et al. (2000) also found that shoots could regenerate from callus as well as directly from the leaves in some pears and apples. Onward a European pear variety, regenerates shoots from its leaves through direct organogenesis, whereas Old Home, another European pear variety, regenerates shoots from indirect organogenesis (Sun et al., 2005).

3.8 Effect of hormones on shoot regeneration

The combination of phytohormones, particularly with auxin and cytokinin, plays a vital role in shoot regeneration, without which it is impossible to regenerate shoots from leaf explants. In this experiment, it was found that the varieties in the media without any hormone (T-1 and T-2), the medium with only 5.0 $mg \cdot L^{-1}$ BA (T-3), and the medium with only 1.0 $mg \cdot L^{-1}$ TDZ (T-4) were unable to produce any shoots.

3.9 Adventitious shoot regeneration

All six varieties including Whangkeumbae did not regenerate any shoots in T-1 and T-2 (without any hormones), T-3 (5.0 $mg \cdot L^{-1}$ BA), T-4 (1.0 $mg \cdot L^{-1}$ TDZ), and T-5 (0.2 $mg \cdot L^{-1}$ NAA and 5.0 $mg \cdot L^{-1}$ BA), except for Duli in T-5, which produced 12 shoots. On the other hand, almost all varieties (except Packham's Triumph) in T-6 (0.2 $mg \cdot L^{-1}$ NAA with 1.0 $mg \cdot L^{-1}$ TDZ) could produce shoots. The highest number of shoots Yali, Aikansui and Duli produced was 59 ($P \leqslant 0.01$), 33 ($P \leqslant 0.001$) and 23 ($P \leqslant 0.001$), with the highest number of shoots per leaf being 22, 8 and 12 in T-6, respectively (Table 4). The highest number of shoots recorded for Abbe Fetel and Packham's Triumph in T-10 was 24 ($P \leqslant 0.001$) and 28 ($P \leqslant 0.001$), with the highest number of shoots per leaf being 11 and 8. Although Duli has the lowest number of shoots, T-10 (1.0 $mg \cdot L^{-1}$ NAA with 2.0 $mg \cdot L^{-1}$ TDZ) has proven to be the best treatment, in which all the 5 varieties can produce shoots; followed by T-6, T-8, T-9 and T-12, in which 4 varieties can regenerate shoots. Only 2 varieties — Packham's Triumph and Duli —can regenerate shoots in T-7. One variety each in T-5 (Duli) and T-11 (Abbe Fetel) can regenerate shoots. However, the combination of NAA and BA does not result in any shoot regeneration for Aikansui and Yali, except for Yali in T-9 (1.0 $mg \cdot L^{-1}$ NAA with 5.0 $mg \cdot L^{-1}$ BA).

This experiment made clear that adventitious shoot regeneration was not possible without the use of phytohormones, especially the combinations of NAA

Table 4 Number of adventitious shoots regenerated from different treatments

varieties	treatments	total No. of shoots per treatment	maximal No. of shoots per treatment	mean No. of shoots per treatment	regeneration rate/%	significance level
Yali	T-6	59	22	11.8	80	**
	T-8	26	6	5.2	52	
	T-9	29	6	5.8	64	
	T-10	22	5	4.4	40	
	T-12	19	10	3.8	36	
Abbe Fetel	T-6	14	4	2.8	32	
	T-8	18	5	3.6	36	*
	T-9	23	5	4.6	60	***
	T-10	24	11	4.8	60	***
	T-11	21	8	4.2	44	**
	T-12	4	3	0.8	8	
Aikansui	T-6	33	8	6.6	56	***
	T-8	20	3	4.0	40	**
	T-10	10	4	2.0	20	
	T-12	8	5	1.6	16	
Packham's Triumph	T-7	3	1	0.6	12	
	T-9	12	3	2.4	32	
	T-10	28	8	5.6	52	***
	T-12	16	5	3.2	40	*
Duli	T-5	12	4	2.4	36	
	T-6	23	12	4.6	44	***
	T-7	4	2	0.8	16	
	T-8	8	3	1.6	24	
	T-9	5	4	1.0	12	
	T-10	7	3	1.4	20	

Note: *, ** and *** represent significance by Tukey test at $P \leqslant 0.05$ or $P \leqslant 0.01$ or $P \leqslant 0.001$ respectively; $n = 60$.

and BA, and particularly, NAA and TDZ in the five tested pear cultivars excluding Whangkeumbae. Different cultivars responded differently to different combinations of hormones and each variety had a different ability to regenerate shoots. Similar findings were recorded by other scientists such as Xu (2001) and Liu (2005), etc.

The experiment results also showed that NAA and TDZ was a better combination of hormones for shoot regeneration in all the five tested pear varieties compared to the combination of BA and NAA. Similar results were also reported by other researchers (Sun et al., 2005). They concluded that TDZ was much more effective than BA in promoting shoot regeneration of European pear cultivars including Onward and Old Home. Sun et al. (2004) also concluded that TDZ was the best plant growth regulator for the Duck pear. His group discovered that BA was more effective than TDZ in inducing shoot regeneration of *P. communis* and *P. pyrifolia* as we obtained some adventitious shoots from European cultivar Abbe Fetel in treatments T-9 and T-11. Bell (2003) similarly suggested that increasing concentrations of auxins could result in a linear increase in the number of adventitious shoots of Bartlett, while increasing concentrations of NAA could result in a decrease in adventitious shoot regeneration of Beurre Bosc. Although the combination of NAA and BA was generally not beneficial, T-9 (1.0 $mg \cdot L^{-1}$ NAA and 5.0 $mg \cdot L^{-1}$ BA) proved to be highly significant ($P \leqslant 0.001$) in the case of Abbe Fetel. T-11 (2.0 $mg \cdot L^{-1}$ NAA and 5.0 $mg \cdot L^{-1}$ BA) ($P \leqslant 0.01$) had a significantly different effect from other treatments, except for T-8 ($P \leqslant 0.05$) and T-10 ($P \leqslant 0.001$) in the same variety. In contrast to the combination of NAA and BA, the combination of NAA and TDZ had significant effects on all the five tested varieties in our case.

The induction of adventitious shoots depends on many factors including phytohormone types and their concentrations, especially on the combination of auxin and cytokinin. Many scientists have used the combinations of BA and NAA or IBA and TDZ or NAA and IBA in different concentrations and each pear variety responds differently to each combination. Lee et al. (2004) used the MS media supplemented with 0.1 to 1.0 $mg \cdot L^{-1}$ TDZ and 0.1 to 1.0 $mg \cdot L^{-1}$ IBA or NAA in six different Japanese pear cultivars.

In the experiment, we also noticed that within 21 days of the induction period in the dark, the leaves became paler, drier, more fragile and brittle compared to the fresh leaves. Leaf size was also observed to increase both in the induction medium (IM) and the expression medium (EM). Cao (2005) also observed an increase in leaf size and shrinkage in the sand pear 5 days after dark treatment.

3.10 Maximum number of shoots and regeneration rate

As far as Yali, Duli and Aikansui in T-6 were concerned, the maximum number of shoots per leaf was 22, 12 and 8 respectively. Pakham's Triumph and

Abbe Fetel regenerated a maximum of 8 and 11 shoots per leaf (Fig. 9a, b, c, f) but this number was quite small compared to that of the other varieties. The total number of shoots and the maximum number of shoots per leaf always coincided with the shoot regeneration percentage rate. In general, as shown in Fig. 9, the more the leaves produce shoots, the more chances there are of obtaining a high shoot regeneration rate as well as a

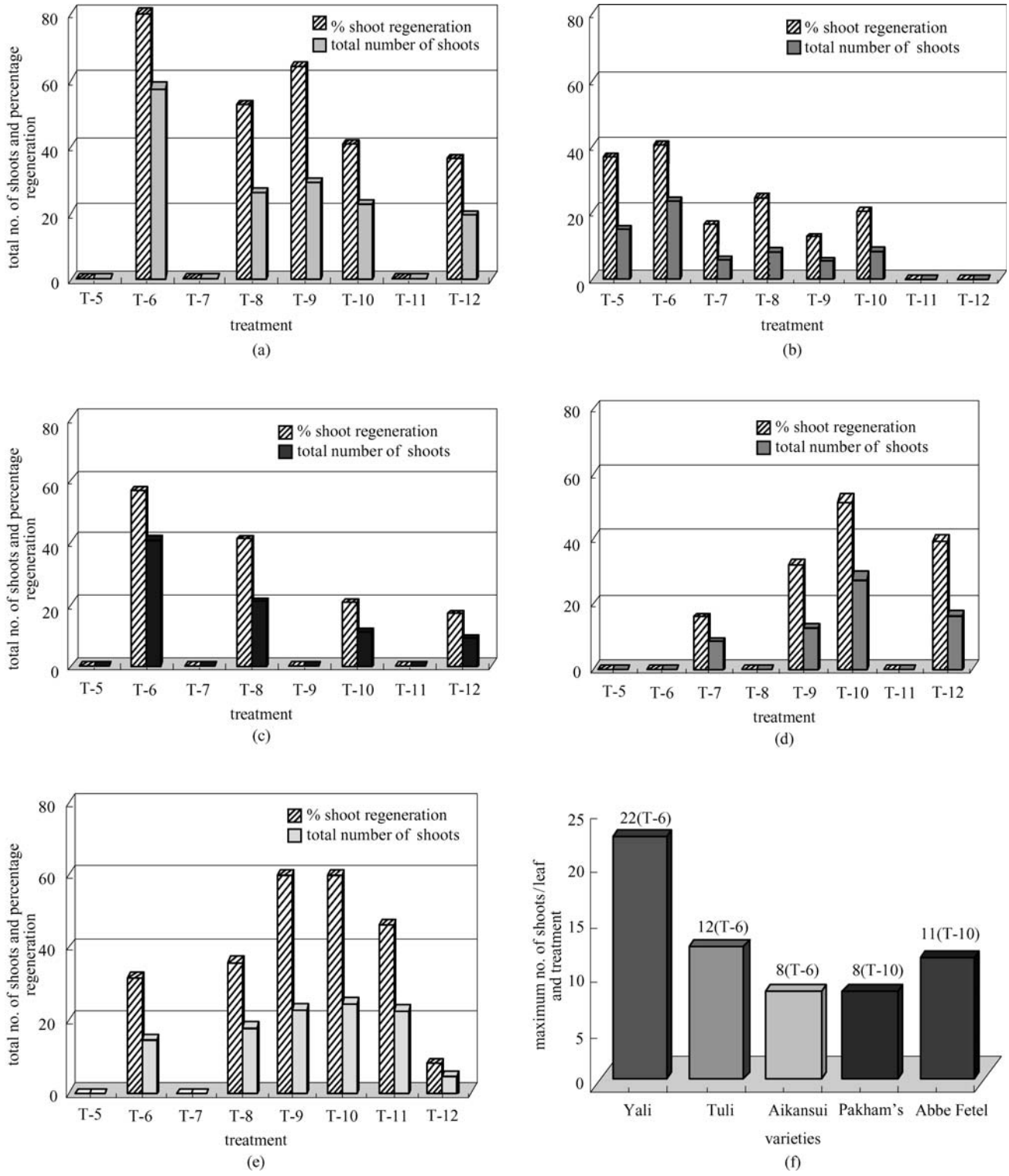

Fig. 9 Total number of shoots and regeneration percentage from different treatments in Yali (a), Duli (b), Aikansui (c), Pakhman's Truimph (d), Abbe Fetel (e) and maximum number of shoots from treatment-6 and treatment-10 of five varieties (f)

high number of leaves in specific treatments. For example, the highest shoot regeneration rate of Yali was 80% which coincided with 59 shoots in treatment T-6. Sun et al. (2004) achieved the same shoot regeneration rate of 80% in Yali by using the NN69 medium. Similarly, in the case of Abbe Fetel, a shoot regeneration rate of only 8% was achieved with T-12, with only 4 adventitious shoots obtained. Grazina and Vidmantas (2004) obtained a regeneration rate of 33.3%–43.7% by using the pear rootstock of Quince.

Lane et al. (1998) obtained a maximum shoot regeneration rate of 23% from six Japanese pear cultivars. Liu (2005) used the NN69 medium combined with 0.1 $mg \cdot L^{-1}$ IBA and 0.9 and 1.6 $mg \cdot L^{-1}$ TDZ in Bartlett and Shebazhi pears, from which he obtained 69.26% and 76.67% shoot regeneration rates, along with 66.67% in Comice pear in the MS medium combined with 0.1 $mg \cdot L^{-1}$ IBA and 4.0 $mg \cdot L^{-1}$ TDZ. Xu (2001) found 100% and 77.8% shoot regeneration from Bayue Hong and Cystal pears. Likewise, Liu (2003) found the best shoot regeneration in the MS medium supplemented with 0.5 $mg \cdot L^{-1}$ TDZ and 0.1 $mg \cdot L^{-1}$ IBA with a regeneration rate of 62.8% in the case of Bartlett and 55.6% in the case of Comic pear using MS media containing 3.0 $mg \cdot L^{-1}$ TDZ plus 0.1 $mg \cdot L^{-1}$ IBA. The cultivar Zaosu performed best in the NN69 medium supplemented with 1.0 $mg \cdot L^{-1}$ TDZ and 0.4 $mg \cdot L^{-1}$ NAA. The Shenbuzhi pear achieved 62.6%–71.4% regeneration rates using the NN69 medium supplemented with 1.0–1.5 $mg \cdot L^{-1}$ TDZ and 0.1 $mg \cdot L^{-1}$ NAA.

3.11 Leaf size and shoot regeneration

It was observed during the experiment that Yali had big leaf explants whereas Duli had the smallest leaf explants among all the cultivars. Having the size advantage of a big leaf, Yali could produce the highest total number of 59 adventitious shoots in treatment with T-6 with a regeneration rate of 80% and a maximum of 22 adventitious shoots per leaf in the same treatment (Table 4; Fig. 9a). The ranking of this cultivar was followed by Duli with the second largest number of adventitious shoots (23) in treatment with T-6, a regeneration rate of 44% and a maximum of 12 adventitious shoots per leaf in the same treatment (Table 4; Fig. 9b). Although Duli had a smaller number of adventitious shoots than Yali, it could produce more adventitious shoots than any one of the other 3 cultivars, which had much bigger leaves. This shows that leaf size does not always determine the regeneration rate of adventitious shoots. In contrast to our observation, Ana et al. (2006) concluded that, apart from the genotype and the combination of different types of phytohormones, both the leaf size and the larger leaf genotypes of *Prunus serotina* may have great influence on the adventitious shoot regeneration rate despite the same treatment.

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