Adventitious Shoot Regeneration from Stem Segments of Whangkeumbae Pear

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ABSTRACT

The experiment was conducted to find out appropriate explant type for *in vitro* regeneration of adventitious shoots of pear (*Pyrus pyrifolia*) cv Whangkeumbae. Shoot explants were collected from the field and cultured *in vitro* in a Murashige and Skoog (MS) shoot proliferation medium (MS + 1.0 mg L^{-1} BA and 0.1 mg L^{-1} IBA) and continuously maintained for 8 months in vitro. Four types of explants (treatments) namely stems with leaves, stems without leaves, leaves with petioles and stems with leaves cultured in hormone-free medium were excised and cultured in shoot induction medium (MS + 0.2 mg L^{-1} NAA + 1.0 mg L^{-1} TDZ). Cultures were maintained in this medium for 21 days in continuous darkness for shoot induction and then transferred to a shoot expression medium (MS +1.0 mg L^{-1} TDZ) and maintained at 25 ± 1^0C with a 16/8 hours light/dark photoperiod regime for 8 weeks. Finally shoots were transferred to a MS shoot elongation media (MS +0.2 mg BA, 0.1 mg IBA and 0.2 mg $GA_{3)}$. The stem explants with leaves produced highest number of shoots (87) ($P \leq 0.001$) with 100 % shoot regeneration rate. In this treatment, some of the explants developed up to 21 adventitious shoots.

Key words: Adventitious shoot regeneration, in vitro, MS media, Whangkeumbae pear, *Pyrus pyrifolia,* tissue culture

INTRODUCTION

Pear (*Pyrus pyrifolia*) is one of the oldest fruit crops widely grown in temperate and sub-tropical regions of the world. The 'Wangkeumbae' pear, one of the popular varieties released in 1967 in South Korea, was introduced to China in 1997 (Tong et al. 2002).

In vitro Adventitious shoot regeneration from the leaves and shoot explants of any fruit variety is one of the easiest ways to regenerate a new generation of plants quickly. However, a number of limitations are also associated with this technique. The regeneration capacity is greatly influenced by varieties, explant type, types of phytohormone and their concentration (Poudyal et al., 2008). Therefore, rapid propagation of plants using *in vitro* techniques needs to be investigated to determine the regeneration protocols suitable for each cultivar (Ana et. al., 2006). Over the past several decades, numerous research works on adventitious shoot regeneration from leaves of many plant species including pear have been accomplished (Liu et al., 2007, Qui et al., 2007, Sun et al., 2004). However, very limited work has been done on adventitious shoot regeneration from the stem explants. Therefore, this experiment was conducted to find out the best explants type for *in vitro* development of adventitious shoots of 'Whangkeumbae' pear variety.

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MATERIALS AND METHODS

Methods and hormones were used according to Marino and Molendini (2005) with some modifications. Initially, newly grown vigorous shoots from field grown 'Whangkeumbae' pear were collected and kept in running tap water for about an hour followed by washing them with distilled water for three times. The shoots were sterilized with 0.1 % mercuric chloride ($HgCl_2$) solution for 7 minutes and then dipping into 75 % ethyl alcohol for 30 seconds. To keep the explants free from hazardous effects of chemicals, all the explants were subsequently rinsed three times in sterile distilled water. After sterilization, they were transferred to sterilized petri dish under laminar flow hood. Tip and bottom of the shoots were trimmed to make about 1.5 cm long explants. The explants were cultured in proliferation medium (PM). The PM was composed of MS medium supplemented with BA 1.0 mg L^{-1} and IBA 0.1 mg L^{-1}. The cultures were kept in growth room $(25\pm1)^0$ C in 16/8 hours light/dark photoperiod condition and sub-cultured in every three weeks interval. After 8 months of sub-culture different types of explants (treatments) were obtained from the in vitro grown plantlets in PM. Four different types of treatments (explants) used for the experiment were: (i) stems with leaves (ii) stems without leaves (iii) leaves with petioles and (iv) stems with leaves cultured in hormone-free medium. Base and tip were cut out to make about 3 mm long stem explants from the middle portion of the shoots. In case of leafy explants fully expended leaves along with petiole were selected for culturing. A small amount of sterilized distilled water was also added to prevent dehydration of explants during excising. These explants were transferred to shoot induction medium (IM) which was composed of MS medium supplemented with 0.2 mg L^{-1} NAA and 1.0 mg L^{-1} TDZ. A small amount of sterilized distilled water was also added to prevent dehydration of explants during excising. Three horizontal cuts along the midrib of the leaves and 3-4 slight cuts in the stem of the explants were also made prior to culture. The cultures were maintained for 21 days under continuous darkness in 25 ± 1^0 C for the induction of adventitious shoots. After 21 days treatments explants were transferred to shoot expression medium (EM), composed of MS medium plus 1.00 mg L^{-1} TDZ. Cultures were maintained in EM up to two months. During this culture period new shoots from EM that attained the suitable size (3-4 cm tall) were continuously transferred to shoot elongation medium (SEM). SEM was also MS medium enriched with 0.2, 0.1, and 0.2 mg L^{-1} BA, IBA and GA_3 respectively. Polyvinyl alcohol (PVA) at the rate of 1.0 g L^{-1} of medium was also added to SEM to control vitrification of the shoots. The cultures were kept in growth room $(25\pm1)^0$ C in 16/8 hours light/dark photoperiod condition.

All the glassware and distilled water were sterilized in an autoclave at 0.12 MPa pressure and 121^0C temperature for 45 minutes and media for 20 minutes. All phytohormones were added prior to sterilization and pH of all medium was adjusted to 5.8 before sterilization.

The maximum number of shoots, mean number of shoots and regeneration percentage of each treatment were recorded during transforming shoots from the EM to the SEM. Percent shoot regeneration rate was defined as the percentage of explants which produced at least one adventitious shoot.

Explants were cultured in 50 ml conical flask containing 25 ml medium. Each flask was considered as one replication and there were 5 explants in each flask replicated five times. All experimental units were arranged in a completely randomized design and experiment was conducted only once. A Tukey test using statistical package SPSS (version 12) was used for the test of significance ($P<0.05$) and mean comparison of treatments.

RESULTS AND DISCUSSION

Callus formation

Two types of explants namely stems with leaves and stems without leaves developed callus during culture but other two treatments: leaves with petioles and stems with leaves without hormone did not produced callus except some on the tip of leaf petioles. Stems with leaves produced a higher amount of callus than stems without leaves treatment.

Figure 1. Adventitious shoot regenerated from the stem explant, shoot regeneration from the tip of Leaf Petiole and emerging shoots from the swollen stem

The explants that produced the highest number of shoots also produced a higher amount of callus compared to the others. Callus formation was concentrated on the cut surface and tip of the petiole from where the shoots regenerated. On the other hand, leaves with petioles and stems with leaves (both without hormone) produced callus neither in the induction period nor the expression period of 60 days.

Table 1. Number of Adventitious Shoots Regenerated from Different Types of Explants

Treatments	Total No. o Shoots	Maximum No. o Shoots pe explant	Regeneration percentage	Significance level
Stems with leaves	87	21	100	***
Stems without leaves	25	10	56	*
Leaves with petioles	2	1	8	
Stems with leaves cultured in hormone-free medium	9	3	32	

Note: [i)] *, *** significant by Tukey test at P≤0.05 or P≤0.001 respectively;

Adventitious shoot regeneration

The type of explants greatly influenced the regeneration capacity of adventitious shoots. Although all types of explants produced adventitious shoots, the numbers of shoots varied greatly according to the explant type. Explants from stems with leaves produced the highest number 87 of adventitious shoots and was highly significant ($P \leq 0.001$) compared to all the other treatments (Table-1). Stems without leaves produced the second highest number (25) of adventitious shoot and it was significantly different with the explant type: leaves with petioles ($P \leq 0.05$). From this experiment, it is clear that adventitious shoot regeneration was highest from the explants with leaves. More than 50 % of the leaves from the explants with leaves died and become black within the induction period (21 days) and the rest of the leaves also died in the EM after a month but adventitious shoot regeneration was significantly higher in the explants possessing leaves on them. This strongly indicates that the presence of leaves is very important for shoot regeneration. Attached leaves might have provided its stored nutrients and other hormones to the stem explants. The explants without leaves produced less number of adventitious shoots compared to explants with leaves. Although it was possible to regenerate shoots without the use of phytohormones, rate of adventitious shoot regeneration in hormone-free medium was significantly less compared to hormone-enriched medium. Therefore, this experiment gives clear indication that both endogenous and exogenous phytohormones are essential for adventitious shoot regeneration of 'Whangkeumbae' pear stem explants. Moreover, leaves explants with petioles produced only 2 shoots from 2 different leaves and both leaves produced shoots only in the tip of the petioles. It can be assumed that adventitious shoot regeneration at the tip of petiole was due to the result of interaction between endogenous and exogenous phytohormones. The adventitious shoots regeneration from the stem explants with leaves in hormone-free medium was found to have emerged during 21 days of induction period without the formation of callus. Those shoots might have emerged from the dormant buds within the stems when they got conducive environment in *in vitro* condition. It shows that there might be two different types of shoots regenerated from the stem explants: shoots from dormant buds and the another adventitious shoots from the callus. In apple cultivar 'Starkrimson', Liu et al. (2007) reported 74.1, 51.9 and 42.6 percent regeneration rate respectively in leaf, etiolated internode, and internode explants.

Figure 2.Shoots Explants with Leaves which are Turning Black

The regeneration rate was maximum (100 %) in stem explants with leaves, and number of adventitious shoots produced by an explant was highest (21) in this treatment (Table 1). Likewise, stems without leaves produced a maximum of 10 shoots from one stem with a 56 percent shoot regeneration rate. Leaf explants with petioles produced a maximum of only 1 shoot from the one stem with a 4 percent shoot regeneration rate. Stem explant with leaves on hormone-free medium produced a maximum of 3 shoots from the one stem with a 32 percent shoot regeneration rate.

The total number of shoots and maximum number of shoots per leaf had positive correlation with shoot regeneration rate (Table 1). For example, the highest shoot regeneration rate for explants with leaves was 100 % coincided with 87 shoots. Likewise, stems without leaves has produced second highest regeneration rate 56 % with 25 adventitious shoots. It seems that, in certain varieties like 'Whangkeumbae' pear, it is easy to regenerate adventitious shoots from stem than from the leaves explants. Wang G.P. et al. (2006) conducted an experiment on leaves and stems of jujubes for adventitious shoot regeneration. They found that, the regeneration of the stem was easier than from the leaves. Similarly, Wang G.Z. et al. (2006) studied the effects of different basal media on adventitious shoot regeneration from etiolated internode segments of the apple cultivar 'Nagafu No.2'. They found that a BA 5.0 mg l^{-1} with NAA 0.4 mg l^{-1} and CH 200 mg l^{-1} together with sucrose 30 g l^{-1} and agar 7.0 g l^{-1} had 83 % shoot regeneration rate and a mean numbers of 3.03 shoots per explant, which was less than ours in pears. The mean number of adventitious shoots as well as the regeneration percentage was lower than our findings in this case.

Sun et al. (2004) found a shoot regeneration rate as high as 27.3 % from the leaf explants of the 'Whangkeumbae' pear by using NN69 medium enriched with BA 5 mg l^{-1} and NAA 0.3 mg l^{-1}. In the same experiment, they were unable to get any shoots from the same medium containing TDZ 2 mg l^{-1} and NAA 0.3 mg l^{-1}. On the other hand, in our experiment the shoot regeneration rate was 100 % from stem explants and only 8 % from leaf explants. Therefore, shoot explants are better than the leaf explants for adventitious shoot regeneration for the 'Whangkeumbae' pear particularly in MS medium.

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