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Original Research Article

Efficient Micropropagation of Pear Germplasm Using Soot Tips and Nodal Explants

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Abstract - We micropropagated pear (*Pyrus* species) using shoot tips and nodal explants from three pear genotypes. The ability to establish shoot tip cultures, proliferate shoots, induce rooting, and acclimatize the resulting plantlets are all elements of *in vitro* micropropagation. Shoots were induced from shoot tips on Murashige and Skoog medium (MS) with five different plant growth regulator combinations. The highest shoot formation rates were achieved for the three genotypes using MS supplemented with 1.0 ㎎/L N^6-benzyladenine (BA) and 0.1 ㎎/L gibberellic acid (GA_3). The maximum shoot number and shoot length for the three cultivars were recorded with 2.0 ㎎/L BA and 0.2 ㎎/L indole-3-butyric acid (IBA) in multiplication medium using nodal explants produced from microshoots. Nodal explants with one or two axillary buds cultured for three weeks initiated roots on medium supplemented with various concentrations of 1-naphthaleneacetic acid (NAA) or/and IBA in half-strength MS medium for adventitious rooting. The highest rooting response was with the combination of 0.2 ㎎/L NAA and 0.2 ㎎/L IBA. A combination of NAA and IBA resulted in a significant increase in the rooting ratio over NAA or IBA alone. In this medium, the root formation rate according to ranged from 68.9% for the BaeYun No. 3 genotype to 51.8% for the Hwanggeum genotype. We also investigated the influence of the concentration the polyamine phloroglucinol in rooting medium. For all three genotypes, the highest rooting ratio, longest root length, and greatest root number were observed in the treatments with 75-150 ㎎/L phloroglucinol. Most rooted plants were acclimatized successfully.

Key words - Micropropagation, Pear, Germplasm, Shoot tip, Nodal explant

Introduction

Pear (*Pyrus* spp.) is an important horticultural germplasm that is grown worldwide. Generally, fruit tree germplasms, including those of pear, are preserved in field banks, but this approach is expensive and the materials may be exposed to pathogens; therefore, a rapid, efficient shoot tip culture method for *in vitro* propagation is desirable (Thakur *et al*., 2008). With shoot tip culture, the terminal 0.1-1.0 ㎜ of a shoot including the meristem (0.05-0.1 ㎜), together with primordial and developing leaves and adjacent stem tissue, is cultured (Pollard and Walker, 1990). Plants that are disease-free and have high metabolic activity and genetic stability can be produced using this technique because plantlet production from adventitious organs can be avoided. Micropropagation of pear was first achieved in 1979 using the pear rootstock 'OHxF51' (Cheng, 1979) and the scion variety 'Bartlett' (Lane, 1979). Since these first reports, significant progress has been made in various areas of *in vitro* pear culture (Singha, 1986; Hutchinson and Zimmerman, 1989; Chevreau *et al*., 1992). Of the methods for long-term *ex situ* conservation of clonally propagated germplasm, cryopreservation of shoot tips is the most reliable and cost- and space-effective option (Condello *et al*., 2009). Cryopreservation of pear shoot tips propagated *in vitro* has been successful (Scottez *et al*., 1992; Niino and Sakai, 1992; Suzuki *et al*., 1997; Reed *et al*., 1998; Chang and Reed, 2001). To implement cryopreservation, the establishment of an efficient micropropagation system is essential. Micropropagation methods are also a prerequisite for exploiting somaclonal variations and induced mutations and for the development of transgenic plants (Yeo and Reed, 1995; Bell and Reed, 2002; Kang *et al*., 2011).

In this study, we attempted to standardize a protocol for *in vitro* shoot formation, proliferation, rooting, and *ex vitro* acclimatization of three pear genotypes.

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Materials and Methods

Plant material

The starting material for establishing pear (*Pyrus* spp.) *in vitro* culture consisted mostly of shoot tips from grafted plants maintained at the National Institute of Horticultural and Herbal Science of the Rural Development Administration (NIHH, RDA; Jeonju, Korea). Current-season shoots (12-15 ㎝) were excised and 2-3 ㎜-long shoot tips were prepared. The explants were surface sterilized with 2.5% sodium hypochlorite for 15 min and rinsed several times in sterilized water for 20 min before the shoot tips were excised. Shoot tip cultures of three pear (*Pyrus* spp.) genotypes, BaeYun No. 3, Bartlett, and Hwanggeum, were established and propagated *in vitro*. In these three genotypes, regeneration rates were much higher than other genotypes we tried. The basal medium used for all of the trials was Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% sucrose and adjusted to a pH of 5.7 before autoclaving at 121℃ for 15 min. All cultures were conducted under white fluorescent light (2000 lux) with a 16-h day photoperiod at 25℃ and a light intensity of 40 μmol/㎡/s provided by cool white fluorescent tubes.

Shoot formation and proliferation

In vitro propagated cultures, obtained from single axillary buds of pear genotypes, were established according to Caboni *et al*. (1999). Five different combinations of plant growth regulator treatments were added to MS medium containing 3% sucrose for shoot induction. After six weeks in culture, nodal segments (> 0.5 ㎝) of the established shoots were subcultured for shoot multiplication on MS medium containing 3% sucrose and various concentrations of N^6-benzyladenine (BA) and indole-3-butyric acid (IBA). Shoots in the proliferation phase were subcultured at three-week intervals. To evaluate the influence of plant growth regulator treatment on shoot multiplication, both shoot number and shoot length were investigated.

Rooting and ex vitro acclimatization

To induce rooting, shoots raised *in vitro* (2.0-2.5 ㎝) were cultured on half-strength MS medium supplemented with 3% sucrose and various concentrations of 1-naphthaleneacetic acid (NAA) or IBA. To investigate the effect of a single application on root initiation, NAA (0.1 or 0.2 ㎎/L) or IBA (1.2 or 2.5 ㎎/L) was applied, and to test the effect of combined application on *in vitro* rooting, three plant growth regulator combinations were applied. The influence of phloroglucinol (PG) on *in vitro* rooting of microshoots was also studied using concentrations of 0, 75, 150, and 300 ㎎/L. In all of the rooting experiments, the percent *in vitro* rooting, number of roots per shoot, and root length were observed. Data were collected 30 days after beginning the root induction cultivation.

Plantlets grown *in vitro* with well-developed roots were removed after 30 days, washed thoroughly with running tap water to remove residual culture medium, planted in plastic pots containing 2:1 (v/v) sand:soil mixture followed by 1:1 (v/v) sand:perlite mixture, and placed in an environmentally controlled mist house under natural photoperiod conditions. After two weeks, the acclimatized plants were successfully transferred and established in a greenhouse.

Statistical analysis

Each treatment was repeated in three replicated experiments with 20 explants per treatment. All data were analyzed using analysis of variance (ANOVA) and means were separated using the Duncan's multiple range test (DMRT) at $P \leq 0.05$ (R ver. 3.1.3).

Results and Discussion

Effects of plant growth regulators on shoot induction and proliferation

Explant establishment and the shoot proliferation technique were standardized for three pear genotypes. Table 1 shows the effects of various plant growth regulator combinations on adventitious shoot formation. Shoots from the established cultures were cultured on MS medium supplemented with various concentrations of N^6-benzyladenine (BA), indole-3-butyric acid (IBA), gibberellic acid (GA_3), and 1-naphthaleneacetic acid (NAA). The combination of 1.0 ㎎/L IBA and 0.1 ㎎/L GA_3 induced the highest shoot formation rates from shoot tips of all three cultivars (Fig. 1A). In this

Table 1. Effects of plant growth regulator combinations on the shoot formation rate and number from axillary shoot tips of pear (*Pyrus* spp.) after culture for eight weeks in MS medium

Medium	Shoot formation rate (%)		
	BaeYun No.3	Bartlett	Whanggeum
M1[z]	100 a[y]	100 a	90.5 a
M2	93.2 b	89.1 b	83.3 b
M3	70.3 c	59.3 c	66.3 c
M4	95.4 b	89.3 b	81.7 b
M5	92.2 b	90.2 b	77.8 bc

[z]M1: BA 1.0 ㎎/L+GA3 0.1 ㎎/L, M2: BA 2 ㎎/L+IBA 0.1 ㎎/L, M3: BA 3 ㎎/L+IBA 0.1 ㎎/L, M4: BA 3 ㎎/L+NAA 0.1 ㎎/L, M5: BA 1 ㎎/L+GA3 0.1 ㎎/L+IBA 0.1 ㎎/L.

[y]Mean separation within columns by Duncan's multiple range test at 5% level by R project (R version 3.0.1) for statistical computing.

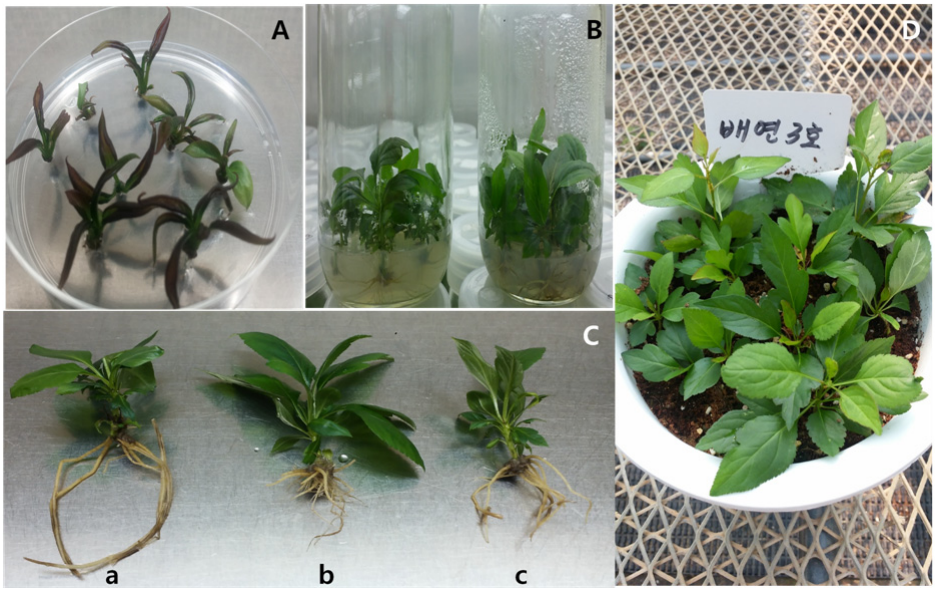

Fig. 1. Diverse stages of plant regeneration from shoot tips of pear (*Pyrus* spp.). genotypes. A, Adventitious shoot formation six weeks after shoot tip establishment; B, Proliferation of shoots three weeks after subculture in medium with BA 2.0 ㎎/L + IBA 0.2 ㎎/L; C, Rooting of microshoots 30 days after establishment on root induction medium a) NAA 0.2 ㎎/L + IBA 0.2 ㎎/L, b) NAA 0.1 ㎎/L + IBA 0.1 ㎎/L, c) IBA 2.5 ㎎/L; D, Acclimatized plantlets in greenhouse.

medium, the shoot formation rate ranged from 90.5% (Hwanggeum) to 100% (BaeYun No. 3, Bartlett). Shoot tip and meristem culture have been used to produce pathogen-free plants following thermotherapy or chemotherapy (Reed, 1995). A major advantage of working with shoot tips or meristems is the potential to exclude pathogenic organisms present in the donor plant. A second advantage is the genetic stability inherent in the technique because plantlet production from adventitious organs can be avoided (Murashige, 1974; Ancora *et al*., 1981; Chevreau *et al*., 1992).

Both the shoot number per culture and shoot length increased markedly in all three genotypes with the combination of 2.0 ㎎/L BA and 0.2 ㎎/L IBA (Table 2). During axillary shoot proliferation, cytokinins are used to overcome the apical dominance of shoots to enhance the branching of lateral buds from leaf axils (Hu and Wang, 1983). The effective concentration of cytokinins required to reverse apical dominance varies with the culture system (Anirudh and Kanwar, 2008a). A positive correlation between BA level and shoot multiplication in pear was reported (Dwivedi and

Table 2. Effects of BA and IBA treatment on the shoot number and length from microshoots of *Pyrus* spp. after subculture for three weeks in MS medium

Treatment BA+IBA (mg/L)	Genotypes					
	BaeYun No.3		Bartlett		Whanggeum	
	N[z]	L[y] (㎝)	N	L	N	L
PGR free	2.8 c[x]	0.9 c	1.8 c	0.6 c	1.4 c	0.7 c
1.0 + 0.1	4.3 b	1.1 bc	2.1 bc	0.9 c	1.9 c	0.6 c
1.0 + 0.2	3.8 bc	2.5 b	2.8 b	1.3 bc	2.7 bc	1.4 b
2.0. + 0.1	6.8 ab	3.3 a	5.1 a	1.9 b	3.4 b	1.7 b
2.0 + 0.2	9.2 a	3.7 a	5.3 a	2.5 a	4.1 a	2.7 a

[z]N, number of shoots per culture, [y]L, Shoot length, [x]Mean separation within columns by Duncan's multiple range test at 5% level by R project (R version 3.0.1) for statistical computing.

Table 3. Effects of plant growth regulator treatment on the percent *in vitro* rooting from microshoots of *Pyrus* spp. after culture for 30 days in half-strength MS medium

PGR Treatment (㎎/L)	Percent *in vitro* rooting (%)		
	BaeYun No.3	Bartlett	Whanggeum
NAA 0.1	7.3 e[z]	-	-
0.2	9.2 e	-	-
IBA 1.2	38.6 c	9.7 d	13.5 c
2.5	58.1 b	43.3 b	33.3 b
NAA 0.1+IIBA 0.1	45.5 bc	23.2 c	24.5 bc
NAA 0.2+IBA 0.2	68.9 a	53.4 a	51.8 a
NAA 0.5+IBA 0.5	23.4 d	31.2 bc	33.2 b

[z]Mean separation within columns by Duncan's multiple range test at 5% level by R project (R version 3.0.1) for statistical computing.

Bris, 1999) and a correlation between low auxin levels and shoot proliferation has been reported (Bhojwani *et al*., 1984; Dwivedi and Bris, 1999). Finding the best plant growth regulator combination for micropropagation of a crop is very difficult. In this study, we found that the combination of 2.0 ㎎/L BA and 0.2 ㎎/L IBA was ideal for shoot proliferation (Fig. 1B). One of the possible roles of auxin during shoot proliferation is to nullify the suppressive effects of high cytokinin concentrations on axillary shoot elongation thereby restoring normal shoot growth (Lundergan and Janick, 1980).

Browning of explants cultured *in vitro* is also a serious problem that impairs the micropropagation of woody plants (Anirudh and Kanwar, 2008a). Pear is very sensitive to phenolic compounds from explants during *in vitro* culture. A short subculture interval is critical for pear micropropagation. In this study, pear plantlets were subcultured at three-week intervals.

Effects of plant growth regulators and phloroglucinol (PG) on rooting

As shown in Table 3, the combination of NAA and IBA resulted in a significant increase in rooting frequency over NAA or IBA alone. Maximum rooting (68.9%) in BaeYun No. 3 was obtained on the medium with the combination of NAA and IBA (0.2 ㎎/L each) (Fig. 1C), *i.e*., the rooting ratio improved only at lower auxin levels (0.2 ㎎/L each). The improved rooting with NAA and IBA in combination, as compared to NAA or IBA alone, may be due to the complementary effect of NAA and IBA. *In vitro* adventitious root formation can be induced easily in many herbaceous species, but it is very difficult in most woody species. In particular, rooting pears *in vitro* has proven difficult and scion cultivars are more difficult to root than rootstock cultivars (Bhojwani *et al*., 1984). Nevertheless, the induction of roots on micropropagated pears *in vitro* has been reported

Table 4. Effects of the phloroglucinol concentration on the percent *in vitro* rooting and root number and length from microshoots of *Pyrus* spp. after culture for 30 days in half-strength MS medium supplemented with NAA (0.2 ㎎/L) and IBA (0.2 ㎎/L)

Genotypes	Phloroglucinol (㎎/L)	Per cent *in vitro* rooting (%)	N[z]	L[y] (㎝)
BaeYun No.3	0	68.9 b[x]	4.1 c	1.1 b
	75	89.3 a	9.1 a	2.4 a
	150	91.8 a	9.9 a	2.3 a
	300	34.5 c	5.9 b	1.4 b
Bartlett	0	53.4.b	2.9 c	1.9 b
	75	77.3 a	8.1 a	2.9 a
	150	71.1 ab	7.8 ab	3.2 a
	300	30.1 c	4.3 b	2.11 b
Whanggeum	0	51.8 b	2.1 c	1.6 b
	75	62.1 ab	7.9 a	2.9 a
	150	70.3 a	8.1 a	2.3 a
	300	28.1 c	3.8 b	2.1 ab

[z]N, root number per culture; [y]L, root length, [x]Mean separation within columns by Duncan's multiple range test at 5% level by R project (R version 3.0.1) for statistical computing.

(Reed 1995; Bell and Reed, 2002; Anirudh and Kanwar, 2008b), although the rooting responses were poor. The ability of plant tissues to form adventitious roots depends on the interactions of many endogenous and exogenous factors. Torrey (1976) reviewed the role of auxins in root development and stated that that auxins have been established as the main factors involved in root formation.

The rooting behaviors of the three pear genotypes were affected significantly by the PG concentration in the rooting medium. As shown in Table 4, PG stimulated the rooting response, *i.e*., the rooting ratio, root number per culture, and root length. The rooting ratio in BaeYun No. 3 improved to 91.8% with 150 ㎎/L PG. The number of roots per microshoot also increased with the PG concentration. The most roots (3.15) were observed with 150 ㎎/L PG in the rooting medium for Bartlett. The differences in root number and length between 75 and 150 ㎎/L PG were not significant in all three genotypes (Table 4) so supplementation with 75 ㎎/L PG was suitable for rooting medium. The stimulation or inhibition of root initiation by phenolic compounds like PG is due to their interaction with auxins (Nemeth, 1986). James and Thurbon (1979) reported that the addition of PG to the rooting medium improved the rooting percentage in the M9 apple rootstock. James and Thurbon (1981) also showed that increased levels of endogenous PG in the microshoot favor root initiation during the auxin-sensitive phase and have synergistic effects with auxin. In contrast, Zimmerman and Broome (1981) reported that PG stimulated rooting in only one of the eight apple cultivars examined. In this study, we found stimulatory effects of increasing PG concentration on the rooting response, *i.e*., increases in the percent *in vitro* rooting, root number per culture, and root length. Most well-rooted plantlets survived on 2:1 (v/v) sand:soil mixture followed by 1:1 (v/v) sand:perlite mixture (Fig. 1D). The surviving plants were hardened in a normal greenhouse.

Micropropagation under controlled conditions could be an alternative to the field production of pear cultivars because more plants can be obtained in less time with guaranteed genetic stability and phytosanitary quality (Vengadesan *et al*., 2002; Martin, 2003), which are prerequisite conditions for a mid- to long-term germplasm preservation system for pear germplasm.

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