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Original Research Article

Plant Regeneration from Anther Culture of *Panax ginseng*

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Abstract - The research concerned of the regeneration of plants from embryos obtained from anther cultures of ginseng (*Panax ginseng* C. A. Meyer). The aim was to determine the influence of the regeneration medium on the efficiency of the regeneration process. We conducted to determine the optimum conditions such as cold pretreatment, plant growth regulators and carbon sources on anther culture of *P. ginseng*. Highest callus formation rate was obtained when flower buds pretreated at 4℃ for 1 day. Among the treated growth regulators with various degrees of concentration in Murashige and Skoog`s (MS) medium, 4.53 μM of 2.4-dichlorophenoxyacetic acid and 4.44 μM of 6-benzylaminopurine gives the most responsive callus with the frequency of 73.89% and 129.53 g of fresh weight. When we used 3-9% of sucrose and maltose among the different kinds and various concentrations of carbohydrates, callus was formed highest 67.29% in the medium with 3% of sucrose. Shoots induced from callus supplemented with 28.9 μM of gibberellic acid and rooted in Gamborg`s B5 medium supplemented with 14.7 μM of indole-3-butyric acid.

Key words - Anther, *Panax ginseng*, Cold pretreatment, Plant growth regulators, Carbohydrates

Introduction

Panax ginseng C. A. Meyer is one of the most economically important medicinal plants in the world that has many bioactive effects such as adaptogen, antiaging, antistress, antitumor, and immune enhancement, etc (Sugaya *et al.*, 1998; Kim *et al.*, 2010). It has been reported that *P. ginseng* contains various polysaccharides, saponins, antioxidants, peptides, alkaloids (Zhang *et al.*, 2011). The improvement of any cultivar through conventional breeding requires several years and adequate facilities. Anther culture can be great value in cultivar improvements, although there are a number of reports on callus induction and on plant regeneration via somatic embryogenesis (Chang and Hsing, 1980; Tang, 2000) or organogenesis (Lim *et al.*, 1997). Callus induced from leaves, stems or root parts of ginseng successfully and regenerated plants from root-derived callus (Chang and Hsing, 1980) and cotyledons (Choi *et al.*, 1997; Choi *et al.*, 1998). Lee *et al.* (2009) reported that tetraploid roots induced from anther of ginseng. However, the regeneration frequency still needs to be improved and also no reports on plant regeneration from anther-derived callus so far.

Medium composition has been identified as a key factor influencing initial callus induction and subsequent plant regeneration in some model plants such as cereal crops and brassica (Keller *at al.*, 1975; Chen *et al.*, 2005; Gorecka *et al.*, 2009). An auxin has been reported to be absolutely required to initiate and promote microspore embryogenesis and a small amount of cytokinin in addition to auxin improves the yield of embryoids in anther culture of oilseed rape (Loh and Ingram, 1982). The effect of different combination of plant growth regulators on callus induction and plant regeneration in anther culture of ginseng has not previously been determined.

Sucrose concentration has been reported to influence androgenesis in anther culture of *Brassica campestris* (Keller *et al.*, 1975) and *Hordeum vulgare* (Solvari and Schieder, 1987). Maltose has recently been found to be superior to sucrose for embryoid induction and plant regeneration in anther/microspore culture of barley and wheat (Finnie *et al.*, 1989; Navarro-Alvarez *et al.*, 1994). The type and concentration of sugar on callus induction and shoot regeneration in anther culture of ginseng has not previously been investigated. Therefore, the effects of several combinations of cold

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pretreatment, culture media and saccharides were investigated to improve anther culture efficiency.

The present work attempted to determine of factors which influence ginseng anther culture in order to elaborate an efficient method of plant regeneration. The parameters investigated were the effects of growth regulators, carbohydrate sources and cold pretreatments on response in anther culture of ginseng.

Materials and Methods

Plant material and cold pretreatment

Flower buds of *P. ginseng* collected from Pochoen in Gyeonggi-do, Korea (provided by Ginseng Genetic Resource Bank) in May of 2010. The flower buds were cold pretreated before the anthers were plated on the induction culture medium. The flower buds were cooled (4℃) inside polyethylene bags in darkness for 1 to 14 days. Panicles without cold pretreatment were used as control.

Surface sterilization and induction medium

The excised unopened flower buds were surface-sterilized under a laminar flow cabinet in 70% ethanol for 30 sec and a 2% (v/v) sodium hypochlorite solution with Tween-20 (1 drop for 50 ml) for 15 min and rinsed three to five times with sterilized water. The spikelets were dissected under aseptic conditions and the anthers were plated into 60 × 10 mm Petri dishes containing 10 ml MS medium (Murashige and Skoog, 1962) supplemented with 30, 60, 90 g l^{-1} sucrose or maltose, 7 g l^{-1} agar, 4.53μM, 9.06 μM 2,4-D, 5.37 μM,10.74 μM 1-napthaleneacetic acid (NAA), 4.44μM 6-benzylaminopurine (BAP) and 4.65μM kinetin. The pH was adjusted to 5.7 before autoclaving at 121℃ for 15 minutes. The dishes were sealed with parafilm and placed in the dark at 25±1℃ for callus formation. The Petri dishes were examined daily. The frequency of callus induction (%) was estimated as the number of callus (approximately 0.5−2.0 mm diam.) per 100 plated anthers. Callus maintained on same medium with callus induction every 4 weeks.

Shoots were initiated on MS basal medium supplemented with 28.9 μM gibberellic acid (GA_3) and Gamborg's B5 medium (1968) supplemented with 3% sucrose, 0.7% agar. And 14.7 μM IBA was used for root induction and the cultures were subcultured every 6 weeks.

Statistical analysis

All experiments were completely randomized designs with 10 anthers per Petri dish and 6 dishes used for each treatment. Results were presented as an analysis of variance followed by Student`s t test. Statistically significant different values ($P<0.05$) are labeled with different superscripts.

Results

The effect of cold pretreatment

The flower buds were incubated at 4℃ for 1-14 days. Table 1 showed that pretreatment temperature and duration had a significant effect on anther response and callus yield. It showed that short-term cold pretreatments had significant effect on anther response and callus yield. 1 and 2 days cold pretreatments were more effective than unpretreated control that incubated at 25±1℃. Calli were formed a rate of 33.59% when anthers were not pretreated. While at 4℃, calli were formed at a rate of 40.9 and 39.5% when anthers were pretreated 1 and 2 days, respectively. Callus fresh weights were maximum 40.5 mg and callus size were biggest 0.45 cm when pretreated at 4℃ for 1-4 days.

The effect of plant growth regulators

In this experiment, eight different combination of NAA, 2.4-D (2.4-dichlorophenoxyacetic acid), BAP and Kin used for determine the most suitable combination and concentration for callus induction yield. The results of the effect of plant growth regulator combinations on callus induction in ginseng are summarized in Table 2. Callus induction frequencies were variable and ranged from 36.11-73.89%, respectively. However, highest frequencies (73.89 and 71.67%) of callus induction were obtained on the MS medium supplemented with 4.53 μM 2.4-D combined with 4.44 μM BAP or 4.65 μM Kin. Induced callus was transferred to shoot and root induction media.

The effect of carbohydrates

The results of the experiment in which concentrations of

Table 1. Effects of cold pretreatments on callus induction from anther of *Panax ginseng*

Cold pretreated days	No. of cultured anthers	Induced callus		Fresh weight of callus (mg)	Size of callus (cm)
		No.	%		
0	640	215	33.59	30.363 ± 8.925	0.5 ± 0.3*
1	640	262	40.94	40.525 ± 7.91**	0.45 ± 0.25**
2	640	253	39.53	36.475 ± 10.602**	0.45 ± 0.25**
4	640	229	35.78	34.838 ± 9.685*	0.45 ± 0.25**
8	565	166	29.38	11.075 ± 4.688	0.4 ± 0.3**
14	514	69	13.42	11.088 ± 1.658	0.28 ± 0.22

Each value represents the mean ± SE of two repeat experiments with 30 replicates each.
The numbers in a column followed by asterisks are not significantly different at $P < 0.05$ by Student's *t* test

Table 2. Effects of plant growth regulators on callus induction from anther of *Panax ginseng*

Plant growth regulators (μM)				No. of cultured anthers	Induced callus		Fresh weight of callus (mg)	Size of callus (cm)
NAA	2.4-D	BAP	Kin		No.	%		
5.37	-	4.44	-	180	89	49.44	17.077 ± 3.607	0.392 ± 0.055
10.74	-	4.44	-	180	113	62.78	30.527 ± 5.875	0.511 ± 0.043**
-	4.53	4.44	-	180	133	73.89	129.533 ± 32.696***	0.778 ± 0.086***
-	9.06	4.44	-	180	108	60	16.413 ± 2.694	0.367 ± 0.029
5.37	-	-	4.65	180	65	36.11	17.007 ± 9.135	0.282 ± 0.059
10.74	-	-	4.65	180	86	47.78	32.642 ± 5.023	0.465 ± 0.049*
-	4.53	-	4.65	180	129	71.67	54.591 ± 14.5**	0.604 ± 0.07**
-	9.06	-	4.65	180	119	66.11	39.482 ± 12.332*	0.498 ± 0.06*

Each value represents the mean ± SE of two repeat experiments with 10 replicates each.
The numbers in a column followed by different asterisks are not significantly different at $P < 0.05$ by Student's *t* test

sucrose and maltose were varied are summarized in Table 3. 480 anthers were cultured each concentration of sugars. High percentages (52.29-67.29%) of callus were formed when used sucrose compared with maltose as a hydrocarbon source. Callus fresh weights were 64.5 and 53.4 mg and size were 0.63 and 0.5 cm in media supplemented with 3 and 6% of sucrose, respectively. But high concentration (9%) of sucrose does not give more effects to callus induction from ginseng anthers compared less percentage of sucrose.

Plant regeneration

For plant regeneration, we tried different culture media with various concentrations of plant growth regulators. Callus was subcultured in MS medium with 4.53 μM 2.4-D and 4.44 μM BAP. Friable, white-yellowish callus transferred to plant regeneration mediums (Table 4). Shoots were induced in MS medium supplemented with 28.9 μM GA_3(Figure 1). Also transferred callus to Gamborg's B5 medium with 14.7 μM indol 3-butyric acid (IBA), and 3 weeks later roots differentiated from calli. The efficiency of root induction was about 70% after 6 weeks in basal plate.

Discussion

Anther culture produce for regeneration of plants has been established in ginseng. Anther culture of ginseng has previously been attempt (Lee *et al.*, 2009) but regeneration of plants has not been achieved before. In our study, shoots regenerated successfully from callus in cultured anthers.

Several reports regard to the effect of various pretreatments such as cold and heat. Especially, cold pretreatment can increase callus induction response from anthers. The anthers

Table 3. Effects of carbohydrates on callus induction from anther of *Panax ginseng*

Carbon source	No. of cultured anthers	Induced callus		Fresh weight of callus (mg)	Size of callus (cm)
		No.	%		
3% sucrose	480	323	67.29	64.467±8.691**	0.627±0.038**
6% sucrose	480	268	55.83	53.434±14.336**	0.503±0.049*
9% sucrose	480	251	52.29	15.292±2.282	0.331±0.022
3% maltose	480	86	17.92	0.909±0.429	0.081±0.025
6% maltose	480	59	12.29	1.289±0.722	0.07±0.025

Each value represents the mean ± SE of two repeat experiments with 15 replicates each.
The numbers in a column followed by different asterisks are not significantly different at $P < 0.05$ by Student's *t* test

Table 4. Shoot induction from anther-derived callus of *Panax ginseng*

Medium composition	No. of cultured callus	% of callus induction	% of shoot induction
MS + 4.53 μM 2.4-D + 4.44 μM BAP	120	100	-
MS + 4.44 μM BAP + 2.89 μM GA3	120	-	-
1/2MS + 4.44 μM BAP + 2.89 μM GA3	120	-	-
MS + 28.9 μM GA3	120	25.83	1.67

of different crop plants responded when used cold pretreatments in culture. Lee *et al.*, (2009) reported that chilling pretreatment of umbels was more effective than unpretreated control and heating pretreatments in ginseng. Our study showed best callus induction obtained when flower buds pretreated at 4℃ for 24-48 h. It is double confirmed that ginseng flower buds give response in chilling pretreatments.

Moreover, to finding the optimum concentration and combinations of plant growth regulators are important in anther culture. High auxin concentrations were reported to increase embryo induction from anthers (Gosal *et al.*, 1997; Hu, 1997). Addition of low level of cytokinin to the induction medium may be beneficial to obtaining callus of high morphogenetic potential (Hu, 1997). Similarly in our study, 4.53 μM 2.4-D with 4.44 μM BAP or 4.65 μM kinetin were the best plant growth combinations to give anther response in culture.

Carbohydrates are one of the important factors on the response of anthers in culture and it has been investigated in a number of species. Sucrose is the most frequently used carbon sourse for anther culture for most species (Yang *et al.*, 1992; Trejo-Tapia *et al.*, 2002). In ginseng somatic embryogenesis and organogenesis culture attempted four different kinds of carbon sources such as glucose, mannose, fructose

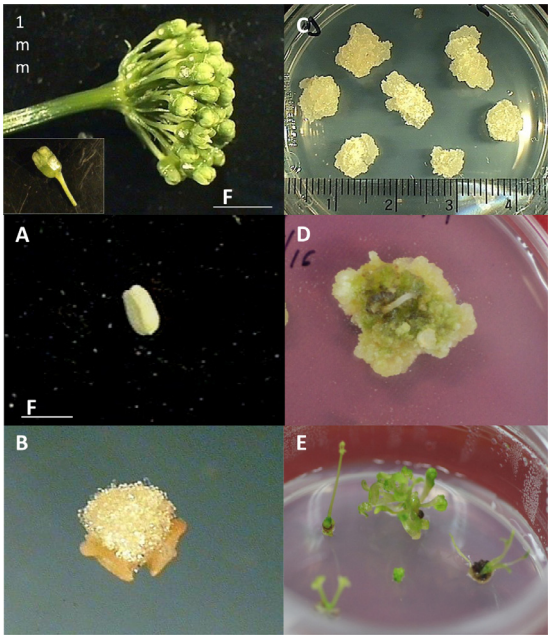

Fig. 1. Regeneration of plantlets via anther culture of *Panax ginseng*. Flower buds of ginseng and a single spikelet (A). Anthers took out from spikelets under aseptic condition (B). Callus was induced from anther on the MS basal medium added 2.4D and Kin (C). Callus was maintained every 4 weeks on MS basal medium with 2.4D and Kin (D). Shoots initiated from anther-derived callus on MS basal medium supplemented with 28.9 μM GA3 (E). Regenerated ginseng shoots from anther-derived callus (F).

and sorbose (Tang, 2000). In this case, the induction of somatic embryos and adventitious buds were over 80%. In our experiment, high callus induction rate obtained when we used 3% of sucrose as a carbon source. High concentration (6-12%) of maltose improved embryoid induction and shoot regeneration from anthers of wheat, barley and potato (Finnie *et al.*, 1989; Navarro-Alvarez *et al.*, 1994; Batty and Dunwell, 1989). However, ginseng anthers could not show high level of callus induction in different concentration of maltose compared with sucrose.

In case of medicinal plants such as ginseng it is difficult to regenerate shoots from callus and few reports mentioned that plants regenerated through somatic embryogenesis (Chang and Hsing, 1980; Tang, 2000) and organogenesis (Tang, 2000) using MS or SH (Schenk and Hildebrandt, 1972) basal medium with low concentration of 2.4-D and BA. Arya *et al.*, (1991) reported that GA_3 is suitable to produce shoots from protoplasts in ginseng. Similarly, shoots were induced in MS medium supplemented with 28.9 μM GA_3 and also roots were grown in Gamborg`s B5 medium with IBA within 6 weeks.

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