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Effects of Cold Pretreatment and Medium Composition on Anther Culture Initiation in Strawberry

Haeyoung Na[1], Dae-young Kim[2], and Changhoo Chun[1,3]*

[1]*Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Korea*
[2]*National Institute of Horticultural & Herbal Science, Suwon 440-706, Korea*
[3]*Department of Horticultural Science, Seoul National University, Seoul 151-921, Korea*

Abstract. Callus culture initiation of strawberry (*Fragaria* × *ananassa* Duch.) was investigated at different Murashige and Skoog (MS) medium strengths, types and concentrations of plant growth regulators, and incorporating a cold pretreatment period to determine the optimal nutritional and environmental conditions. No high quality callus was induced on MS media without auxin regardless of medium strength. When 6-benzylaminopurine (BA) was combined with indole acetic acid (IAA), naphthalene acetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D), high quality callus were highly induced compared to medium supplemented with auxin alone. When 0.5 mg·L^{-1} BA was combined with IAA, NAA, and 2,4-D, high quality callus induction was more effective than the medium supplemented with the other BA concentrations. The best combination of auxin and cytokinin for high quality callus induction was 1.0 mg·L^{-1} NAA and 0.5 mg·L^{-1} BA. Although the differences in callus induction were not significant, high quality callus induction at half strength MS medium was more effective than at full strength medium. When 30 g·L^{-1} sucrose was added to the half strength MS medium, the rate of high quality callus induction increased. The optimum cold pretreatment temperature and period for high quality callus induction were 4°C and 72 h, respectively. Regeneration rate of high quality callus increased in MS medium supplemented with thidiazuron.

***Additional key words*:** auxin, callus quality, cytokinin, plant growth regulator, regeneration

Introduction

Cultivated strawberry (*Fragaria* × *ananassa* Duch.) is a highly heterozygous, octoploid species (2n = 8x = 56) (Svensson and Johansson, 1994). The powerful inbreeding depression expressed after selfing prohibits the production of homozygous lines by traditional crossing methods. Traditional breeding efforts to improve strawberry quality and yield are labor intensive, costly, and time-consuming since many generations of crossing and selection are routinely required for cultivar development.

Genetic variation is an essential component of any conventional crop-breeding program. Conventionally, plant breeders recombine the desired genes from crop varieties and related species by hybridization to develop new cultivars with desirable traits such as high yield, fruit quality, and resistance to disease, insects, pests, and drought (Radhakrishnan and Kumari, 2008). Genotype is the most important factor affecting yield and quality. 'Seolhyang' strawberry is a domestic cultivar with excellent traits such as fruit flavor and high yield and has been the leading cultivar grown in Korea during the last few years and has great potential as a parent for genetic hybridization (Jun et al., 2011). Therefore, it might be so valuable to enlarge the extent of somatic variations in strawberry breeding program.

The anther culture technique is a successful method that can be used to achieve variation either as material for breeders or as productive material to be used for commercial varieties (Jain, 2001). Strawberry anthers have been used as the initial explant to produce callus from which plants exhibiting variation can be regenerated. In an anther culture, useful variation like earliness of ripening and mildew tolerance has been obtained (Simon et al., 1987). Callus formation from strawberry anther culture is affected by media components (Infante et al., 1998; Owen and Miller, 1996). Among the factors affecting anther culture response in many plants, culture medium composition (plant growth regulator and carbon source) and temperature shock seem to be critical for the effective induction of callus and plant regeneration (Xie et al., 1995, 1997). The temperature shock has been reported to be critical for inducing the division of microspores in rice (Trejo-Tapia et al., 2002) and strawberry (Svensson

***Corresponding author:** changhoo@snu.ac.kr
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and Johansson, 1994), however, the optimum temperature and duration of this pretreatment varied according to genotype (Kiviharju and Pehu, 1998). Auxins are essential plant growth regulator for the induction of callus from anthers (Zheng and Konzak, 1999). The formation of high quality callus is regulated by the types and levels of auxins presented in the culture medium (Ball et al., 1993). The effects of carbohydrate are associated with maintaining high proportions of swollen microspores and increasing their division rate (Xie et al., 1995).

In the present study, some factors including cold pretreatment and medium composition affecting anther culture initiation of 'Seolhyang' were evaluated to initiate an efficient callus culture from anther in strawberry.

Materials and Methods

Plant Material

'Seolhyang' strawberry cultivar was used as anther donor plants and grown in a greenhouse at the National Institute of Horticultural & Herbal Science located in Suwon, Korea. The anthers of the second and third flower clusters were used for the anther culture.

Callus Induction

Closed flower buds without sepals were rinsed in 70% ethanol for 5 s, surface sterilized in 1% sodium hypochlorite for 15 minutes on a shaker at 70 rpm and washed 3 times in sterile, distilled water for 3 min (Fig. 1A). All petals from the closed flower buds were removed on a clean bench and then the uninucleate anthers (1 mm) were detached (Fig. 1). For callus induction, five anthers were plated in a petri dish (20 × 90 mm) and cultured on MS semi-solid medium (Murashige and Skoog, 1962) including vitamins, various plant growth regulators, 3% sucrose, and 0.8% agar at 25 ± 1°C under darkness for 90 days (Fig. 1E). Induced calli from the anthers were separated into high quality callus and low quality callus groups. Only high quality callus were used for regeneration. The pH of all the media was adjusted to 5.8 using NaOH or HCl before autoclaving.

Pretreatment by Cold Temperature

Anthers were pretreated by exposure to cold temperature after the anthers were inoculated onto petri dishes containing semi-solid MS medium with 3% sucrose. Exposures with 0, 24, 48, 72, 96, 120, and 144 h at 4°C were tested to investigate the effect of cold pretreatment on high quality callus induction. After cold pretreatment, all cultures were incubated at 25°C in darkness for 90 days.

Culture Media and Plant Growth Regulators

After the best temperature of cold pretreatment for high quality callus induction was established, and the effects of medium strength and type and concentration of plant growth regulators were investigated. Anthers were cultured in half strength and full strength semi-solid MS medium supplemented with IAA (0, 1.0, 2.0, and 4.0 $mg \cdot L^{-1}$). Anthers were cultured in full strength semi-solid MS medium with 2.0 $mg \cdot L^{-1}$ 2,4-D containing 0, 3, 5, 7, and 10% sucrose, respectively. Each treatment set of experiment was made by the combination of four different concentrations (0.5, 1.0, 2.0, and 4.0 $mg \cdot L^{-1}$) for one of three auxins (NAA, IAA, and 2,4-D) with three different concentrations (0, 0.5, and 1.0 $mg \cdot L^{-1}$) for BA. Semi-solid full strength MS medium without any plant growth regulator was used as a control. BA, NAA, and IAA were prepared in sterile water and sterilized via filtration through a 0.22 μm filter, and 2,4-D was autoclaved at 121°C for 15 min.

Shoot Regeneration

For the conversion to plantlets, calli were separated into high and low quality callus. The high quality callus were transferred directly to MS medium containing thidiazuron (0, 0.5, 1.0, 2.0, and 4.0 $mg \cdot L^{-1}$), 3% sucrose and 8% agar. They were incubated at 25 ± 1°C, and 16 h photoperiod with 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of photosynthetic photon flux for 4 weeks.

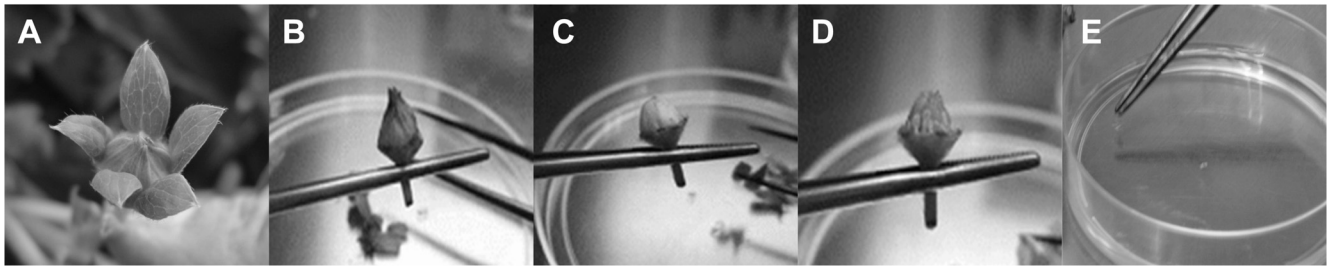

Fig. 1. Experimental process for anther-derived callus induction. Closed flower buds (A), closed flower buds with all sepals removed (B and C), closed flower buds with all petals removed (D), anthers cultured in a petri dish containing MS medium (E).

Statistical Analysis

Each treatment of all the experiments were replicated five times. Statistical analyses were performed using the SAS statistical software, release 9.2 (SAS Institute Inc., Cary). One-way ANOVA was used to examine the significant differences in high quality callus induction among the treatments and the means of significant treatment differences were separated using Duncan's multiple range test at the 0.05 level.

Results and Discussion

Callus from anthers induced after 30 days in culture. The callus could be separated into high quality callus and low quality callus groups by color and firmness. High quality callus were firm and light yellow in color while low quality callus were soft and watery, and white or light gray in color (Fig. 2). The high quality callus rapidly produced transplants while the low quality callus has low efficiency and time consuming process to establish regeneration.

Various combinations of plant growth regulators were tested for their potential to induce high quality callus in strawberry. No high quality callus was induced when grown on MS medium without auxin (Fig. 3). The medium containing BA promoted high quality callus induction when combined with the IAA, NAA, and 2,4-D auxins, compared to medium supplemented with auxin alone. High quality callus was not induced on MS media supplemented with IAA lacking BA. Moreover, high quality callus induction in 0.5 $mg \cdot L^{-1}$ BA combined with IAA, NAA, and 2,4-D was more effective as compared to medium supplemented with other concentrations of BA. The best combination of auxin and cytokinin for high quality callus induction was 2 $mg \cdot L^{-1}$ NAA and 0.5 $mg \cdot L^{-1}$ BA, respectively (Fig. 3). Hormonal balance is a key factor in the callus induction in cultured explants. The interactions between auxins and cytokinins are complex throughout plant development, and the balance between auxins and cytokinins controls the induction of callus tissue in vitro, and BA is the most commonly tested cytokinin in strawberries (Barcelo et al., 1998; James, 1987; Nehra et al., 1989). The results that we obtained for the three different auxins (IAA, NAA, and 2,4-D) alone and in combination with BA have confirmed the important role of cytokinin activity for the induction of high quality callus in strawberry anther cultures.

The high quality callus induction rate based on medium strength was assessed. There were no significant differences in high quality callus induction at half strength and full strength MS media strength containing three different IAA concentrations (1.0, 2.0, and 4.0 $mg \cdot L^{-1}$). Although there were no significant differences, high quality callus production at half strength MS medium was slightly more effective than the full strength MS medium (data not shown). Callus induction is affected by the presence of biologically available nitrogen sources in the medium (Holme and Petersen, 1996). This finding has been confirmed in several plant species such as *Daucus carota* (Wetherall and Dougall, 1976), *Oryza sativa* (Ozawa et al., 1996), and *Sorghum bicolor* (Elkonin and Pakhomova, 2000). Na et al. (2008) reported that the low nitrate level in half strength MS medium was favorable for the callus induction in *Pimpinella brachycarpa*. Therefore, effects of nitrogen sources and levels on callus induction must be further investigated in the future.

Percentage of high quality callus induction was 60% at 30 $g \cdot L^{-1}$ sucrose and significantly greater than at 0, 50, and 70 $g \cdot L^{-1}$ sucrose (Fig. 4). Sucrose is the most common carbon source in plant tissue and cell culture media, since cells use sugars to store energy and regulate metabolism. Callus induction and the regeneration of plantlets in *Asparagus officinalis*

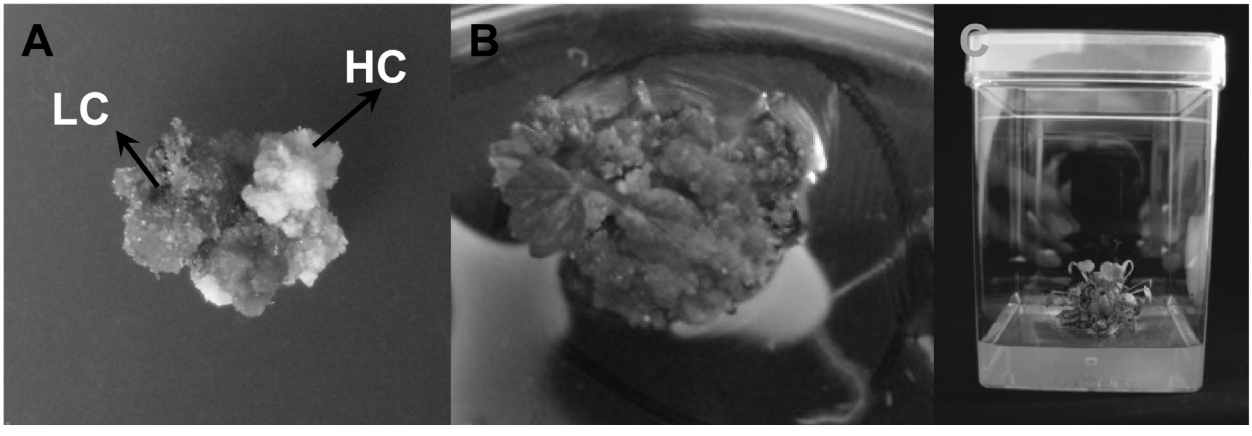

Fig. 2. Callus induction from anther (A) and shoot regeneration (B and C) on half strength MS semi-solid medium containing 1.0 $mg \cdot L^{-1}$ NAA , 0.5 $mg \cdot L^{-1}$ BA, and 30 $g \cdot L^{-1}$ sucrose after cold pretreatment for 72 h at 4°C and its regeneration. HC, High quality callus; LC, low quality callus.

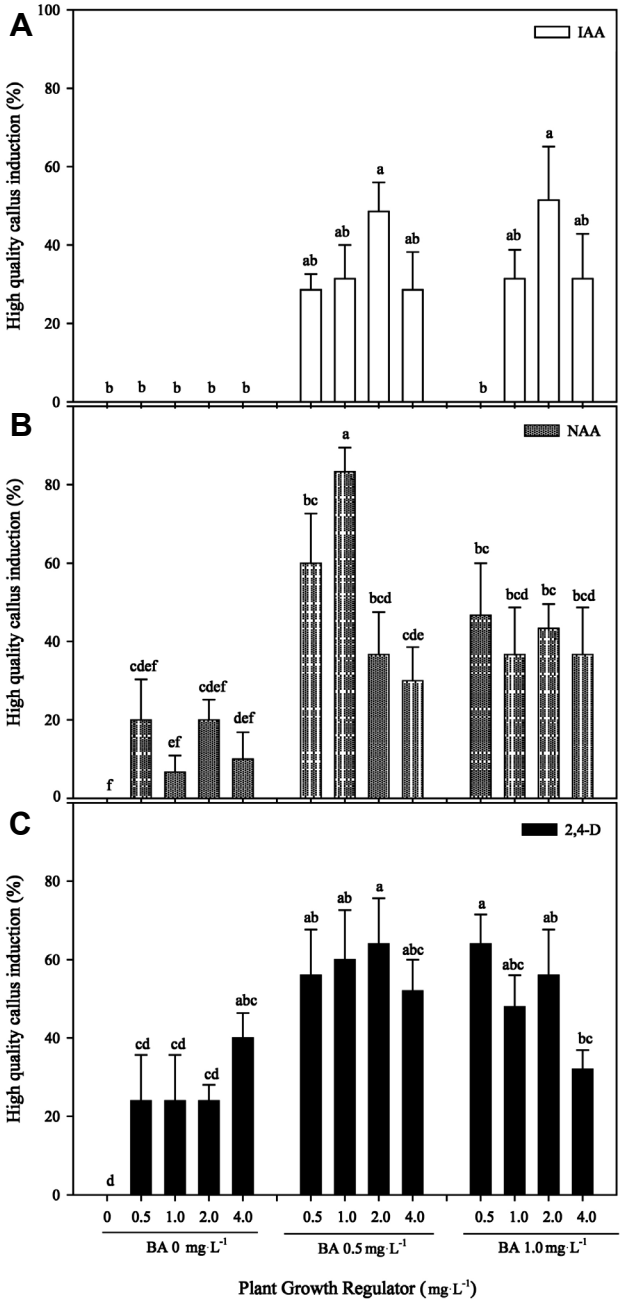

Fig. 3. Anther-derived high quality callus induction rate per petri dish of *Fragaria* × *ananassa* Duch. in anthercultures treated with various BA concentrations combined with IAA (A), NAA (B), and 2,4-D (C) auxins in half strength MS semi-solid medium. High quality callus induction (%) = (the number of high quality callus/the total number of anther) × 100. Data were collected 90 days after culture. Each value is the mean obtained from five replicates of 9 anthers each. Columns with the same letter are not significantly different according to Duncan's multiple range test at $p < 0.05$ (n = 5).

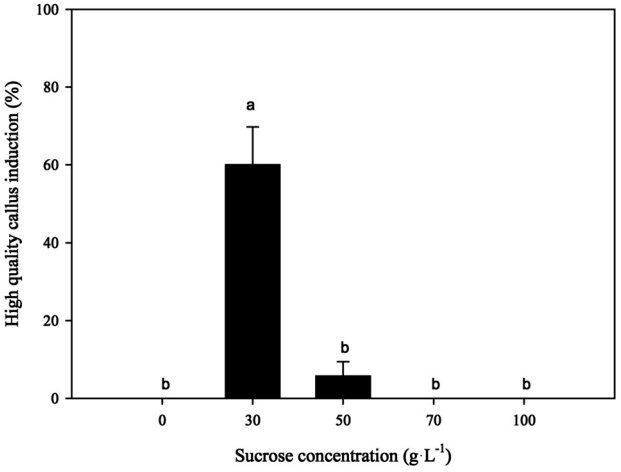

Fig. 4. Anther-derived high quality callus induction rate per petri dish of *Fragaria* × *ananassa* Duch. anther cultures treated with various sucrose concentrations in half strength MS semi-solid medium. High quality callus induction (%) = (the number of high quality callus/the total number of anther) × 100. Data were collected 90 days after culture. Each value is the mean obtained from five replicates of 9 anthers each. Columns with the same letter are not significantly different according to Duncan's multiple range test at $p < 0.05$ (n = 5).

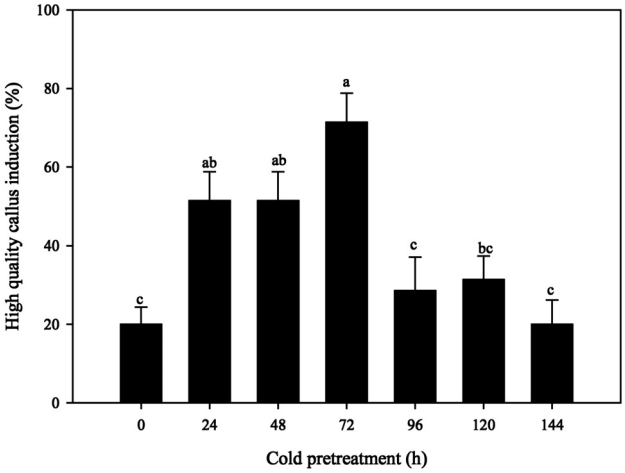

Fig. 5. Anther-derived high quality callus induction rate per petri dish of *Fragaria* × *ananassa* Duch. Anther cultures treated with various cold pretreatment lengths in half strength MS semi-solid medium at 4°C. High quality callus induction (%) = (the number of high quality callus/the total number of anther) × 100. Data were collected 90 days after culture. Each value is the mean obtained from five replicates of 9 anthers each. Columns with the same letter are not significantly different according to Duncan's multiple range test at $p < 0.05$ (n = 5).

were improved by the presence of sucrose over glucose and/or fructose (Levi and Sink, 1991). Lee et al. (2002) reported that a sucrose concentration of 30 g·L^{-1} resulted in the best callus induction in *Oenanthe stolonifera,* while callus induction at 60 g·L^{-1} sucrose has low efficiency.

The effects of cold pretreatment was more effective for inducing high quality callus than omitting cold pretreatment, except for the 144 h incubation (Fig. 5). The cold pretreatment for 72 h at 4°C showed the highest high quality callus induction from anthers. These results indicate that the cold pretreatment promoted high quality callus induction from strawberry anthers. The positive effects of the cold pretreatment

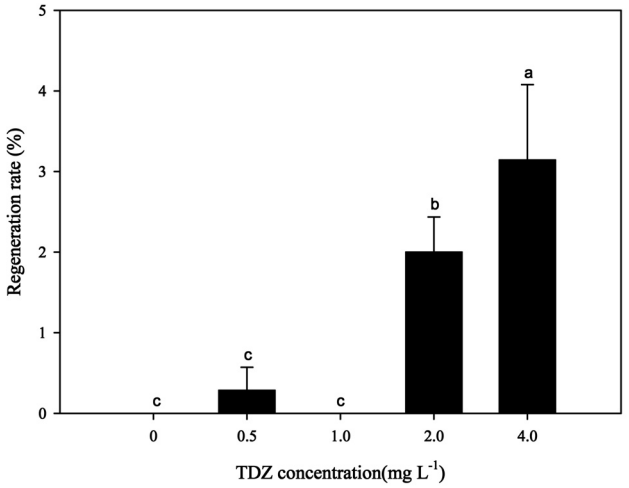

Fig. 6. Anther-derived plantlet per petri dish of *Fragaria* × *ananassa* Duch. in response to various concentration of TDZ in MS semi-solid medium. Data was collected 120 days after culture. Each value is the mean obtained from ten replicates. Columns with the same letter are not significantly different by Duncan's multiple range test at $p < 0.05$ (n = 10).

on callus induction are possibly due to a delay of pollen or anther wall senescence, an increase in symmetric divisions of pollen grains, and/or the release of substances necessary for androgenesis (Kiviharju and Pehu, 1998; Xie et al., 1997).

The conversion of high quality callus to plantlets is also associated with plant growth regulators (Jimenz, 2005). Among plant growth regulators, cytokinin may have certain regulatory functions during regeneration, as demonstrated by the positive effects of plant growth regulators either separately or in combination (Lakshmanan and Tasi, 2000). In the present study, thidiazuron was used as a source of cytokinin because it is well known that TDZ shows higher regeneration rate on strawberry rather than other cytokinin and, what is more, other cytokinin requires a much higher concentration should be used than TDZ (Steven and Johansson, 1994). Shoot regeneration was stimulated when thidiazuron concentration increased to some extent (Fig. 6). It is required to add thidiazuron on the medium to regenerate from the high quality callus. Although the addition of high concentration of thidiazuron could increase the regeneration rate, abnormal or hyperhydric plantlet could be formed also. Therefore, it might be important to identify an optimal concentration rate for producing high quality plantlet and further study was need to examine it clearly.

Callus induction was greatly influenced by cultivars, explant types, and plant growth regulators in the culture media (Biswas et al., 2007). Our results indicate that callus induction of strawberry is greatly influenced by medium strengths, sucrose concentrations, types and concentration of plant growth regulators, and a cold pretreatment period. The optimum conditions for high quality callus induction are a 1/2 MS semi-solid medium containing 1.0 $mg \cdot L^{-1}$ NAA, 0.5 $mg \cdot L^{-1}$ BA, and 30 $g \cdot L^{-1}$ sucrose. The highest high quality callus induction rate was identified under the above conditions with the addition of a cold pretreatment period for 72 h at 4°C.

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