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Anther-derived callus induction based on culture medium, myo-inositol, $AgNO_3$ and Fe-EDTA in 'Seolhyang' strawberries

Haeyoung Na[1,2*], Bong Seong Kim[1] and Jungbeom Kim[1]

[1]Horticultural Science Department, Mokpo National University, Muan 58554, Korea.
[2]Institute of Natural Resources, Mokpo National University, Muan 58554, Korea.

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Haploid breeding is an effective method of shortening the breeding period of plants. In this study, to develop a haploid breeding technique, optimum conditions of the culture medium were investigated for anther-derived callus induction in the strawberry cultivar Seolhyang. The effects of the culture medium type, myo-inositol, auxin and cytokinin combination treatment, silver nitrate ($AgNO_3$), and ferric ethylenediaminetetraacetic acid (Fe-EDTA) on anther-derived callus induction were analyzed. Anthers were incubated in Murashige and Skoog (MS) medium, Gamborg B5 medium (B5), and Lichter medium (NLN) for 8 weeks. Each culture medium had 0.4 mg·L^{-1} of 6-benzyladenine (BA), 0.1 mg·L^{-1} of indole-3 acetic acid (IAA), and 2.0 mg·L^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D) added to it. Results showed that MS medium was most effective in callus induction. When 100 mg·L^{-1} of myo-inositol was added to each medium, the callus induction rate increased. Auxin and cytokinin combination treatment was more effective with the addition of 0.4 mg·L^{-1} of BA, 0.1 mg·L^{-1} of IAA, and 2.0 mg·L^{-1} of 2,4-D to MS medium compared to the addition of 0.1 mg·L^{-1} of BA, 2.0 mg·L^{-1} of IAA, and 0.4 mg·L^{-1} of 2,4-D. With $AgNO_3$ treatment, the highest callus induction rate was found at a concentration of 25 mg·L^{-1}. The callus induction rate increased as the $AgNO_3$ concentration increased; however, a significant decrease was seen at 30 mg·L^{-1}. In the case of Fe-EDTA, the most effective concentration for callus induction was 25 mg·L^{-1}. Therefore, supplementing myo-inositol, $AgNO_3$ and Fe-EDTA can help in anther-derived callus induction in Seolhyang strawberries.

Key words: Anther culture, auxin, B5 medium, callus induction, cytokinin, haploid breeding, NLN medium.

INTRODUCTION

Strawberries (*Fragaria x ananassa* Duch), a herbaceous perennial plant belonging to the Rosaceae family, are either consumed raw or used as a source material for jams, wines, juices, and other processed foods because of their sweetness and sourness together. Strawberries are rich in antioxidants, vitamin C, anthocyanin and, and, therefore, function as a health food (Lilia et al., 2017). In South Korea, strawberry breeding and cultivation began in the 1970s. Since then, cultivars such as Josaenghongsim have been grown.

*Corresponding author. E-mail: somerze@mokpo.ac.kr.

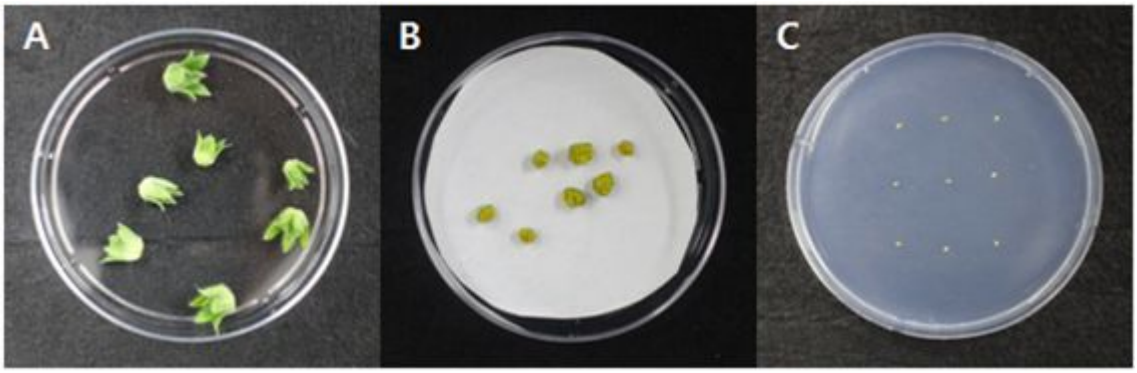

Figure 1. Experimental materials and process. (A) Closed bud, (B) closed bud with all petals removed, and (C) anther cultured in a Petri dish containing MS medium. MS, Murashige and Skoog.

However, the distribution of Josaenghongsim strawberries in farmhouses has considerably decreased compared to Japanese strawberry cultivars such as Red Pearl and *akihime*. In 2005, because of a royalty fee issue, the cultivar Seolhyang was developed in a strawberry research center. By 2015, the cultivation area for Seolhyang strawberries reached up to 80% of the entire strawberry cultivation area across South Korea. As the demand for strawberries increased in Southeast Asia, the strawberry exports continuously increased from $4.2 million in 2004 to $34 million in 2015. Seolhyang strawberries have high sugar content. In addition, they are resistant to powdery mildew, thus being convenient for cultivation as well as have a higher production efficiency compared to other cultivars. However, their texture is too soft, and they are easily spoiled and damaged when harvested. Therefore, they have low storability and cannot be exported (Reddy et al., 2000).

To meet the rising demand for strawberries in Southeast Asia, a high-quality strawberry cultivar with hard texture and good storability needs to be urgently developed. However, strawberries have high hyperdiploidy, that is, they are octoploids with 56 chromosomes. As a result, compared to other plants, inbred line production for strawberries is extremely difficult (Hirakawa et al., 2014). Therefore, it is desirable to develop haploid breeding techniques to save time and cost for the development of a new cultivar that meets the demands of the international market.

Few studies have been conducted on strawberry anther culture, although there are a few reports on meristem culture for virus-free strawberry production (Na et al., 2011; Nguyen et al., 2015). However, callus induction using anthers is an effective technique for strawberry haploid breeding. There are two ways of mass propagation using calluses, indirect embryogenesis and indirect organogenesis, both having a high proliferation efficiency (Niazian et al., 2017). In this study, optimal callus induction conditions were investigated using anthers in order to establish a haploid breeding technique by examining suitable culture types and plant growth regulators for anther-derived callus induction and regenerated plant production in strawberries.

MATERIALS AND METHODS

Plant material

Seolhyang strawberry buds were collected from a strawberry farm in Okcheon-Myeon, Haenam-Gun, South Korea, in May 2016. The bud length (calyx to flower edge) was measured and buds with a length of 10 to 14 mm were selected; however, those with visible petal differentiation were excluded (Figure 1). The collected buds were incubated at 4°C for 4 days, disinfected in 70% ethanol for 15 s, and subsequently sterilized in 1% sodium hypochlorite for 15 min. Next, they were cleansed in sterilized water on a clean bench for 3 min; this was repeated thrice.

Callus induction

To examine the optimal chemical factor for anther culture, the following rates were measured: callus induction rate based on culture medium types of Murashige and Skoog (MS) medium, Gamborg B5 medium (B5), and Lichter medium (NLN); callus induction rate based on the combination of auxin and cytokinin in MS medium; and callus induction rate based on the addition of $AgNO_3$, ferric ethylenediaminetetraacetic acid (Fe-EDTA), and myo-inositol (Gamborg et al., 1968; Lichter, 1982; Murashige and Skoog, 1962). To investigate the optimal combination of auxin and cytokinin, callus formation was induced in MS1 medium (with 0.4 $mg \cdot L^{-1}$ of 6-benzyladenine [BA], 0.1 $mg \cdot L^{-1}$ of indole-3 acetic acid [IAA], and 2.0 $mg \cdot L^{-1}$ of 2,4-dichlorophenoxyacetic acid [2,4-D] added) and MS2 medium (with 0.1 $mg \cdot L^{-1}$ of BA, 2.0 $mg \cdot L^{-1}$ of IAA, and 0.4 $mg \cdot L^{-1}$ of 2,4-D added).

The callus induction rate in MS medium, B5, and NLN was investigated under the same conditions of plant growth-regulating substances. Equal amounts of plant growth-regulating substances (0.4 $mg \cdot L^{-1}$ of BA, 0.1 $mg \cdot L^{-1}$ of IAA, and 2.0 $mg \cdot L^{-1}$ of 2,4-D) were added because these agents showed the best callus induction rate in preliminary tests. To determine the callus induction rate, anthers extracted from the buds were cultured in the dark in MS medium, B5, and NLN with equal amounts of BA, IAA, and 2,4-D, both with and without 100 $mg \cdot L^{-1}$ of myo-inositol for 8 weeks.

To examine the effects of $AgNO_3$ and Fe-EDTA concentrations

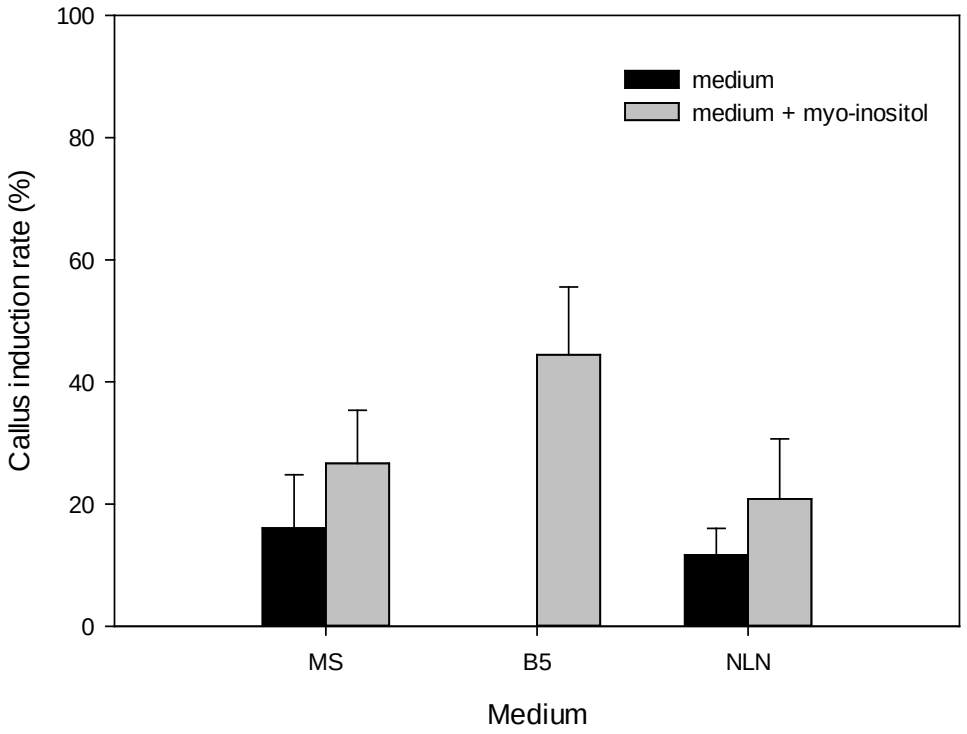

Figure 2. Effect of myo-inositol and different types of culture medium on anther-derived callus induction in Seolhyang strawberry anther culture.

on callus induction, 0, 5, 15, 25, and 30 mg·L^{-1} of $AgNO_3$ and 0, 25, 50, 75, and 100 mg·L^{-1} of Fe-EDTA were added to MS medium separately and cultured in the dark for 8 weeks before the callus induction rate was examined. In addition to 0.4 mg·L^{-1} of BA, 0.1 mg·L^{-1} of IAA, and 2.0 mg·L^{-1} of 2,4-D, 30 g·L^{-1} of sucrose and 8 g·L^{-1} of agar were also added to all culture media. After adjusting the pH of the culture media to 5.8, the media was autoclaved in a high-pressure sterilizer at 120°C and 1.5 atmospheric pressure. For all experiments, a 90 × 20 mm^2 Petri dish in which six anthers were treated was used. After heat-shock treatment at 32°C for 48 h in the dark, the culture was maintained in a dark room at 25°C for 8 weeks. Thereafter, the callus induction rate was calculated as a percentage of the number of calluses induced from the six anthers cultured in the Petri dish. All experiments were repeated 10 times. The SigmaPlot 12.0 program was used for statistical analysis.

RESULTS AND DISCUSSION

Callus induction based on culture medium type, myo-inositol, and plant growth regulators

On the basis of the culture medium type, cultures without myo-inositol showed the highest callus induction rate of 16% in MS medium as compared to 0% in B5 and 11.7% in NLN (Figure 2). On the other hand, the callus induction rate in cultures with 100 mg·L^{-1} of myo-inositol was highest in B5 (44.4%), followed by MS medium (13.7%) and NLN (9.1%) (Figures 2 and 3). The results of this study were consistent with those conducted on other plants. As a biomembrane component, myo-inositol metabolizes into UDP-xylose or UDP-glucuronic acid to be used for cell wall polysaccharide biosynthesis and combines with IAA to play an important role in cell growth regulation (Loewus and Murthy, 2000). Myo-inositol improves callus induction in immature embryos of turf-type tall fescue (Bai and Qu, 2001). Myo-inositol is also an essential factor for cell wall formation and the phosphate pathway in plants (Bohnert et al., 1996; Hegeman et al., 2001). Moreover, myo-inositol protects plants against salt stress and is suggested to function in salt tolerance in two major ways: to protect cellular structures from reactive oxidizers and to control the water pressure inside cells (Loewua and Murthy, 2000). In addition, myo-inositol acts as a growth enhancer *in vitro* and a carbohydrate source, which are good osmotica for sustained cell division (Azad et al., 2006; Eun et al., 2011). Therefore, myo-inositol is considered an important factor for increasing the callus induction rate in strawberry anther culture. Using 0.4 mg·L^{-1} of BA, 0.1 mg·L^{-1} of IAA, and 2.0 mg·L^{-1} of 2,4-D resulted in increasing the callus induction rate up to 33.3%, which is higher than the callus induction rate of 12.7% obtained with 0.1 mg·L^{-1} of BA, 2.0 mg·L^{-1} of IAA, and 0.4 mg·L^{-1} of 2,4-D (Figure 4).

The auxin and cytokinin combined treatment is a way to increase the callus induction rate. Studies have reported that the callus induction rate is higher if the concentrations

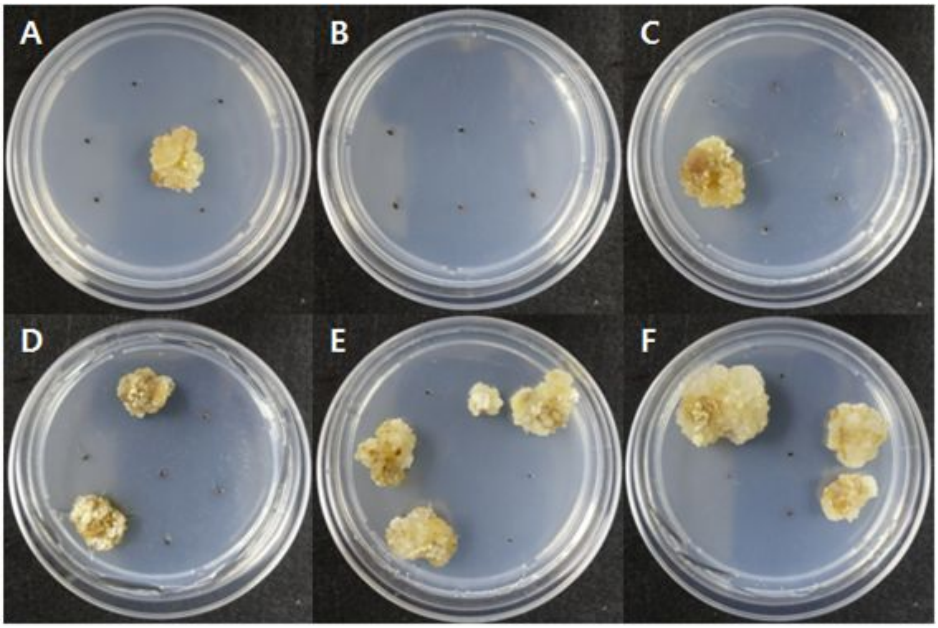

Figure 3. Morphology of anther-derived callus induction on solid media. Callus induced in a Petri dish containing (A) MS medium, (B) B5, (C) NLN, (D) MS medium + 100 mg·L^{-1} of myo-inositol, (E) B5 + 100 mg·L^{-1} myo-inositol, and (F) NLN + 100 mg·L^{-1} myo-inositol. MS, Murashige and Skoog; B5, Gamborg B5 medium; NLN, Lichter medium.

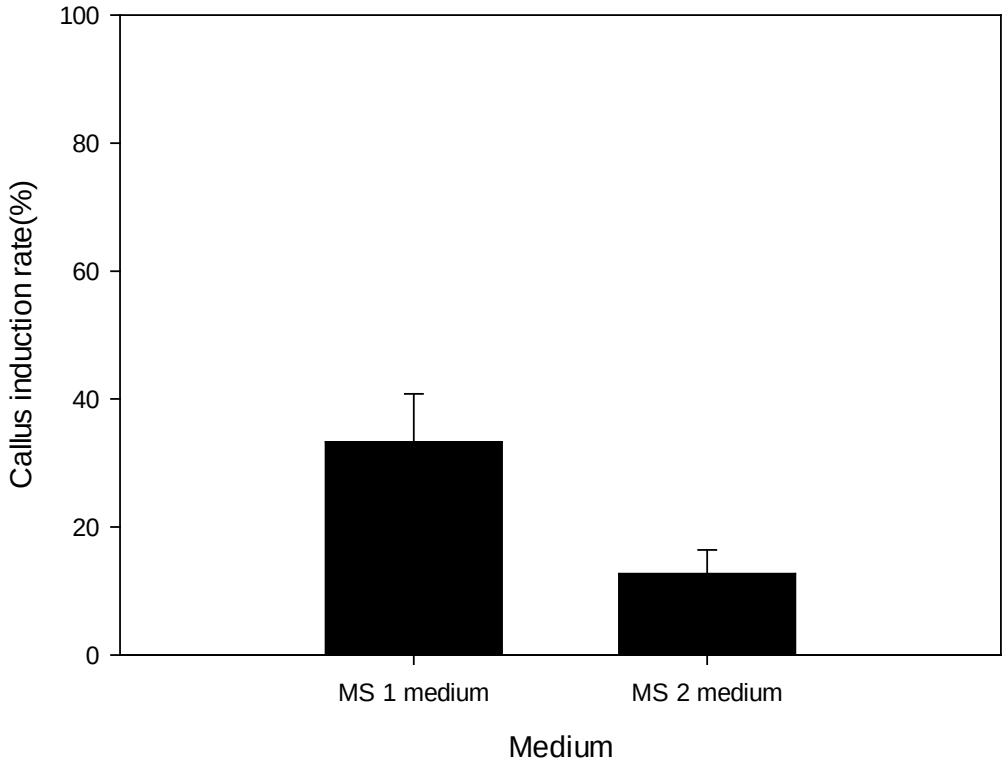

Figure 4. Effect of plant growth regulators on anther-derived callus induction in Seolhyang strawberry anther culture. MS1 medium was supplemented with 0.4 mg·L^{-1} of BA, 0.1 mg·L^{-1} of IAA, and 2.0 mg·L^{-1} of 2,4-D, and MS2 medium was supplemented with 0.1 mg·L^{-1} of BA, 2.0 mg·L^{-1} of IAA, and 0.4 mg·L^{-1} of 2,4-D. MS, Murashige and Skoog; BA, 6-benzyladenine; IAA, indole-3 acetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid.

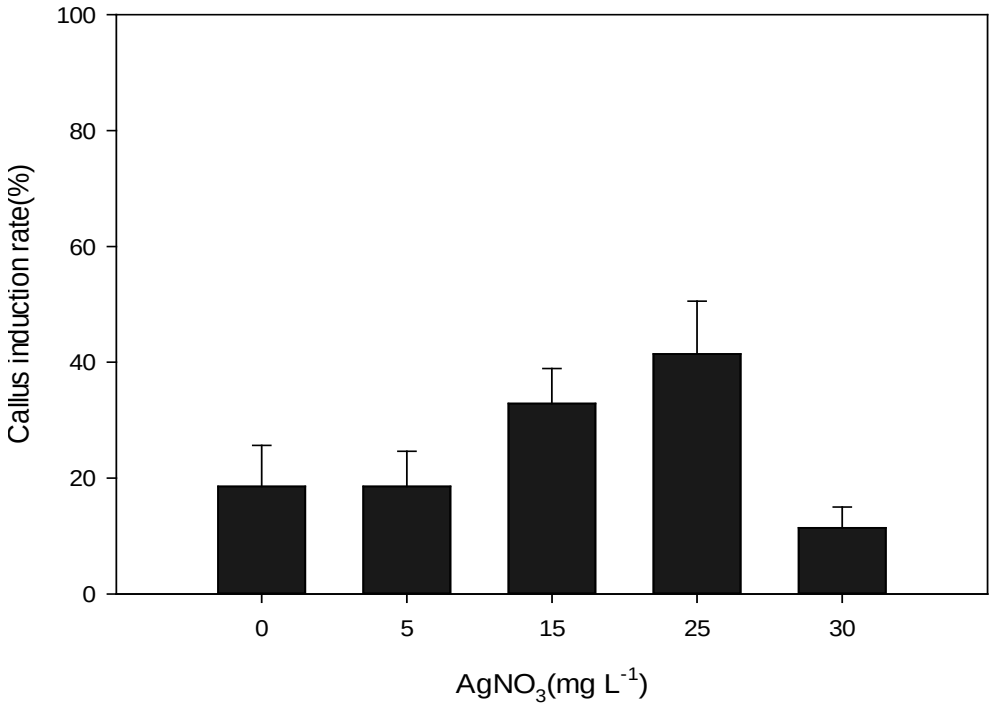

Figure 5. Effect of $AgNO_3$ concentration on anther-derived callus induction in Seolhyang strawberry anther culture.

of auxin and cytokinin are the same in *Muscari comosum* var. *Plumosum* (He et al., 2006). However, a culture medium with a higher concentration of 2,4-D than BA is more suitable for *Callerya speciosa* anther culture (family Fabaceae) (Huang et al., 2016). The callus induction rate with combined auxin and cytokinin treatment varies depending on plant varieties as well as the type and concentration of plant growth regulators.

Callus induction based on $AgNO_3$ and Fe-EDTA

On the basis of the $AgNO_3$ concentration, the callus induction rate was highest at 41.4% with 25 mg·L^{-1} of $AgNO_3$, whereas the callus induction rates in cultures with 5 and 15 mg·L^{-1} of $AgNO_3$ were 18.6 and 32.9%, respectively, confirming that the callus induction rate increases with the $AgNO_3$ concentration (Figure 5). However, the callus induction rate rapidly decreased to 11.43% with 30 mg·L^{-1} of $AgNO_3$. $AgNO_3$ inhibits cell aging by inhibiting ethylene generation in ethylene receptors. Therefore, $AgNO_3$ is reportedly effective for long-term callus cultures because it inhibits ethylene generated by aging and stress (Williams et al., 1990). Explants in culture media supplemented with a low concentration of $AgNO_3$ improved the embryogenic callus size and texture, whereas higher concentrations of $AgNO_3$ decreased the embryogenic callus induction rate. In addition, in *Solanum nigrum* (L.), $AgNO_3$ significantly induced more embryogenic calluses compared to $AgNO_3$-free culture media (Geetha et al., 2016). For strawberry callus culture, the results of this study confirmed that $AgNO_3$ is effective for callus maintenance and culture.

In this study, it was also observed that 25 mg·L^{-1} of Fe-EDTA resulted in the highest callus induction rate at 28.6%. With 0, 50, 75 and 100 mg·L^{-1} of Fe-EDTA, the callus induction rate was 16.1, 21.4, 15.8 and 18.6%, respectively. A Fe-EDTA concentration of >50 mg·L^{-1} showed no effect, because the callus induction rate was similar to cultures without Fe-EDTA treatment (Figure 6). In orchid cultures, Fe-EDTA is effective in callus induction and protocorm-like body formation (Silva et al., 2006). As a source of iron, Fe-EDTA is used in tissue culture of many plants because it prevents a decrease in IAA activity by photochemical reactions (Hangarter and Stasinopoulos, 1991). In this study, we found that adding an appropriate concentration of Fe-EDTA to MS medium can effectively increase callus induction.

Conclusion

Supplementing BA (0.4 mg·L^{-1}), IAA (0.1 mg·L^{-1}), 2,4-D (2.0 mg·L^{-1}), myo-inositol (100.0 mg·L^{-1}), $AgNO_3$ (25.0 mg·L^{-1}), and Fe-EDTA (50.0 mg·L^{-1}) in B5 can help in anther-derived callus induction in Seolhyang strawberries. This study identified the optimal conditions for haploid breeding of Seolhyang strawberries, which can also be

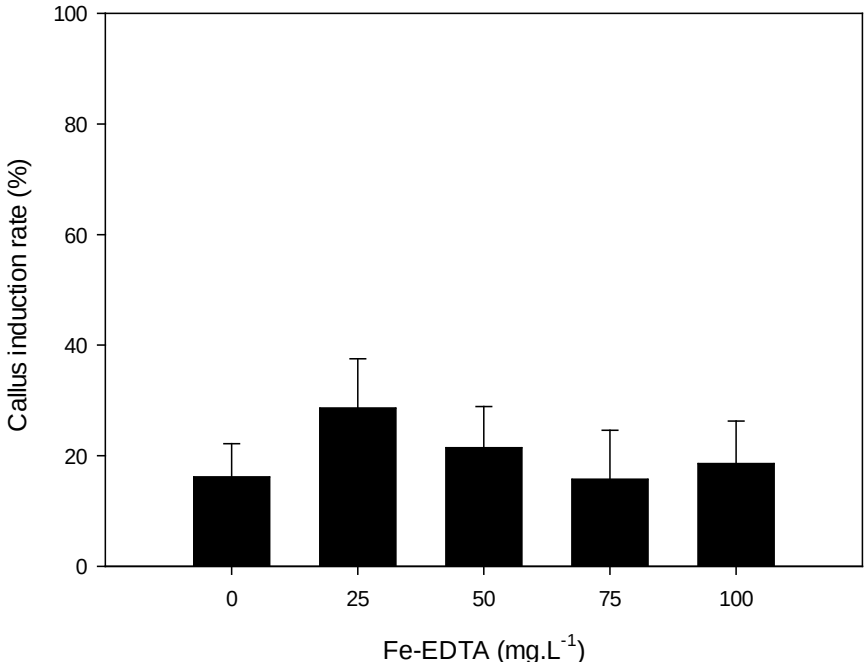

Figure 6. Effect of Fe-EDTA concentration on anther-derived callus induction in Seolhyang strawberry anther culture. Fe-EDTA, ferric ethylenediaminetetraacetic acid.

applicable for the cultivation of other strawberry cultivars.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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