(Research Article)

The effects of sucrose, activated charcoal, and coconut dust on *in vitro* corm induction of *Gladiolus hybrida* under aseptic technique

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Abstract

This study aimed to investigate the effect of sucrose concentrations: of 3%, 6%, and 9% together with or without two different supplementary matters: activated charcoal (0.01%) and coconut dust (10%) on *in vitro* growth and corm induction of *Gladiolus hybrida* when cultured for 5 weeks. The objective was to investigate the optimal culture media for corm production in long-term culture. The results showed that shoots grown on MS medium with the addition of 10% coconut dust at any sucrose concentrations had a high percentage of corm induction, ranging from 72 to 88. The treatment of 6% sucrose supplemented with 0.01% activated charcoal and 10% coconut dust yielded the highest corm induction percentage (88%) after being cultured for 5 months. Therefore, the optimum medium for corm induction of *Gladiolus hybrida* was MS medium supplemented with 6% sucrose, 0.01% activated charcoal, and 10% coconut dust.

Keywords: Sucrose, Activated charcoal, Coconut dust, *Gladiolus hybrida*

Introduction

Gladiolus (*Gladiolus hybrida*) is classified in the “Iridaceae” family, with colorful inflorescence such as white, pink, red, orange, yellow, and purple. Gladiolus is native to the subtropical region. The optimum temperature for gladiolus growth and development is in the range of 18 to 25 °C. The maximum temperature during cultivation was approximately 33 °C [1]. In Thailand, gladiolus can be cultivated in the northern and northeast provinces because of cool weather, such as in Chiang Mai, Chiang Rai, and Phetchabun. In tropical areas, the germination period to the flowering stage of gladiolus is 70 - 105 days, depending on variety, season, and environment. However, there are few reports of the gladiolus cultivars grown in Thailand [2]. Gladiolus is a newly introduced temperate plant, that has not yet been well cultured in the country and gladiolus’s corms need to be imported, causing high production costs. Therefore, growers tend to use the tissue culture technique for gladiolus production, because this technique can propagate large amounts of sub-corm and reduce costs. This technique can also maintain the genetic characteristics of the mother plant [3]. The medium composition is the most important factor for growth and corm production. Sucrose plays a critical role in corm or cormel formation in gladiolus using plant tissue culture [4]. Application of high sucrose

concentration (7%) produced various sizes of cormels, including large (2.8-3.2 mm), medium (2.1-2.6 mm), or small (0.8-1.2 mm) [5]. The optimized concentrations of sucrose used for *in vitro* corm, tube, or rhizome production had been investigated in many plant species with suitable sucrose ranging from 6% to 9% sucrose [5,6]. However, corm or cormel production typically depends on genotype or cultivars. Besides the concentration of sucrose, alternative supplementary or additives could affect *in vitro* micro corm production. Examples of additives used in plant tissue culture are activated charcoal and coconut dust. Using coconut dust as a matrix in MS liquid medium supplemented with 176 mM (6.024%) sucrose and 0.002 mM (3.7×10-3 mg/l) NAA can stimulate many micro corms [7]. As previously reported, an efficient protocol was developed for mass-scale *in vitro* corm production of Gladiolus (cv. 'White Friendship') using liquid culture (supplemented with 0.5 mg/l NAA and 6% sucrose) and coconut dust as a matrix [7]. Therefore, the addition of coconut dust in the gladiolus medium can improve plant growth, especially corm induction [7], while the addition of activated charcoal (AC) in the Aerides orchid cultivation can promote the growth of leaves and roots [8]. The role of activated charcoal in an improved protocol for *in vitro* rooting of *Acacia leucophloea* (Roxb.) Willd was also reported, and roots were regenerated on half-strength MS with 3% sucrose, 1 mg/l indole-3-butyric acid (IBA), and 200 mg/l activated charcoal [9]. *In vitro* rooting of gladiolus was much affected by auxin and activated charcoal presented in the medium [10]. The positive effect of activated charcoal might be related to ethylene adsorption or/and to the removal of undefined inhibitory substances from the culture medium. Therefore, the objectives of this study were to determine the effect of sugar concentrations, activated charcoal, and coconut dust on *in vitro* corm induction of gladiolus, and find the optimal culture media for long-term culture.

Materials and Methods

Plant materials and establishment of shoot cultures

The aseptic gladiolus shoots, 1 cm long, were cultured for shoot multiplication in MS medium containing 1 mg/l 6-Benzylaminopurine (BAP) concentration, in aseptic conditions at a temperature of 25±2°C. The tissue cultures were exposed to the light intensity of 2000 – 3,000 lux for 16 hours a day. The medium was changed, and shoots were transferred to a new medium every 4 weeks until sufficient shoots were obtained for experiments.

Experiments for *in vitro* corm induction

Five pieces of new shoots, 1 cm long, were cultured in 12 treatments of medium shown in Table 1. This experiment was carried out in a completely randomized design (CRD) with five replications (five shoots per replication). These formulas were designed for testing the effect of sucrose concentrations (3%, 6%, and 9%), adding activated charcoal (0.01%), and coconut dust (10%). All plant stocks were cultured in the previously described conditions for five weeks.

For validating the optimal medium, the three most effective treatments were selected (3% sucrose + 0.01% activated charcoal + 10% coconut dust, 6% sucrose + 0.01% activated charcoal + 10% coconut dust, and 6 % sucrose). In this experiment, shoots were cultured in the previously described condition for five

months. Finally, corms derived from these three treatments were grown in small pots for the transplanting stage under greenhouse conditions, and germination was observed.

Growth observation and data collection

Leaf lengths were measured from the base of the petiole to the tip of a leaf. The percentage of corm induction was calculated according to the number of shoots that become corms. Root numbers were evaluated by counting and root lengths were measured from the base of the root to the root tip. Growth appearance was observed and photographed. ANOVA and means were separated using Duncan's Multiple Range Test (DMRT) in SPSS software.

Three different media were selected and repeated for validation. The first formulation was 3 % sucrose supplemented with 0.01% activated charcoal and 10% coconut dust. The second medium was 6% sucrose supplemented with 0.01% activated charcoal and 10% coconut dust. The last formula was basal medium with 6% sucrose without additives (activated charcoal and coconut dust). Plants were grown and cultured for five months. Corm formation: size, color (scored by grading 1= white, 2= green, and 3= brown), and fresh weight of corms were evaluated.

Table 1 Basal MS medium with different concentrations of sucrose supplemented with activated charcoal and coconut dust.

Treatments	Sucrose concentration (%)	Activated charcoal (AC) (%)	Coconut dust matrix (%)
1	3	0	0
2	3	0.01	0
3	6	0	0
4	6	0.01	0
5	9	0	0
6	9	0.01	0
7	3	0	10
8	3	0.01	10
9	6	0	10
10	6	0.01	10
11	9	0	10
12	9	0.01	10

Results

The statistical analysis revealed that the adjustment of sucrose concentration with the addition of coconut dust and activated charcoal had significant differences in leaf length, corm induction, root length, and root number. For leaf length, gladiolus shoots grown on MS basal medium using 3 % sucrose and supplemented with 0.01% activated charcoal and 10% coconut dust had the highest leaf length at 16.71 cm. Shoots grown on MS basal medium using 3% sucrose and free activated charcoal supplemented either with coconut dust or without coconut dust had high leaf lengths at 15.98 and 15.92 cm, respectively. While shoots grown on MS using 9% sucrose and 0.01% activated charcoal supplemented either with coconut dust or without coconut dust had the lowest leaf lengths at 7.84 and 7.64 cm, respectively (Fig. 1A).

For corm induction, the media adding 10% coconut dust in every sucrose concentration, either with or without activated charcoal, produced the highest corm induction in the range of 72 - 88%. The result showed that shoots grown on MS using 6% sucrose and 0.01% activated charcoal without coconut dust had the lowest corm induction at 29% (Fig. 1B). For root length, the *in vitro* gladiolus shoots cultured on MS medium in addition to 6% sucrose supplemented with 0.01% activated charcoal and 10% coconut dust produced the highest root length at 5.63 cm. Shoots cultured on MS medium having 6% sucrose without additives (activated charcoal and coconut dust) produced the second-best root length at 4.22 cm. Nevertheless, Shoots cultured on MS medium having 3% and 9% sucrose supplemented with only 0.01% activated charcoal (without coconut dust) had the lowest root length at 1.95 and 2.04 cm, respectively (Fig. 2A).

For roots number, shoots cultured on MS medium having 6% and 9% sucrose without charcoal and coconut dust, and shoots cultured on MS medium having 6% sucrose supplemented with 0.01% charcoal and 10% coconut dust had the highest roots number at 5.76, 5.24 and 5.48 roots, respectively (Fig. 2B). According to root length and root number, the data showed that MS in addition of 6% sucrose supplemented with 0.01% activated charcoal and 10% coconut dust enhanced rooting in gladiolus culture. The *in vitro* growth appearance of gladiolus plants in each medium was shown in Fig. 3. However, for five-week culture *in vitro* corms were not completely developed. They required more time to grow and store food.

During the validation, gladiolus shoots were cultured in three different formulations for five months and they produced great corms. According to ANOVA, the result exhibited that shoots cultured on MS medium having 6% sucrose supplemented with 0.01% activated charcoal and 10% coconut dust had the highest corm size and color scores as 10.06 and 2.87 respectively (Fig. 4A and 4B). For fresh weight per corm, both corms derived from shoots grown on MS medium having 6% sucrose supplemented with 0.01% activated charcoal and 10% coconut dust, and these shoots grown on MS medium having 6% sucrose without additives were heavier than corms derived from shoots cultured on MS basal medium using 3% sucrose and supplemented with 0.01% activated charcoal and 10% coconut dust (Fig. 4C). Morphology as corm size and color of corms obtained were heterogenous for all treatment. Round and brown corms were observed on MS medium having 6% sucrose supplemented with 0.01% activated charcoal and 10% coconut dust (Fig. 5). Further investigation on corm production showed the changes in the growth appearance of gladiolus shoots after being cultured for 5 months (Fig. 6). Finally, *in vitro* corms were successfully germinated in growing media using 100% peat moss in two-inch diameter pots and the first germination was found within four weeks (Fig. 7).

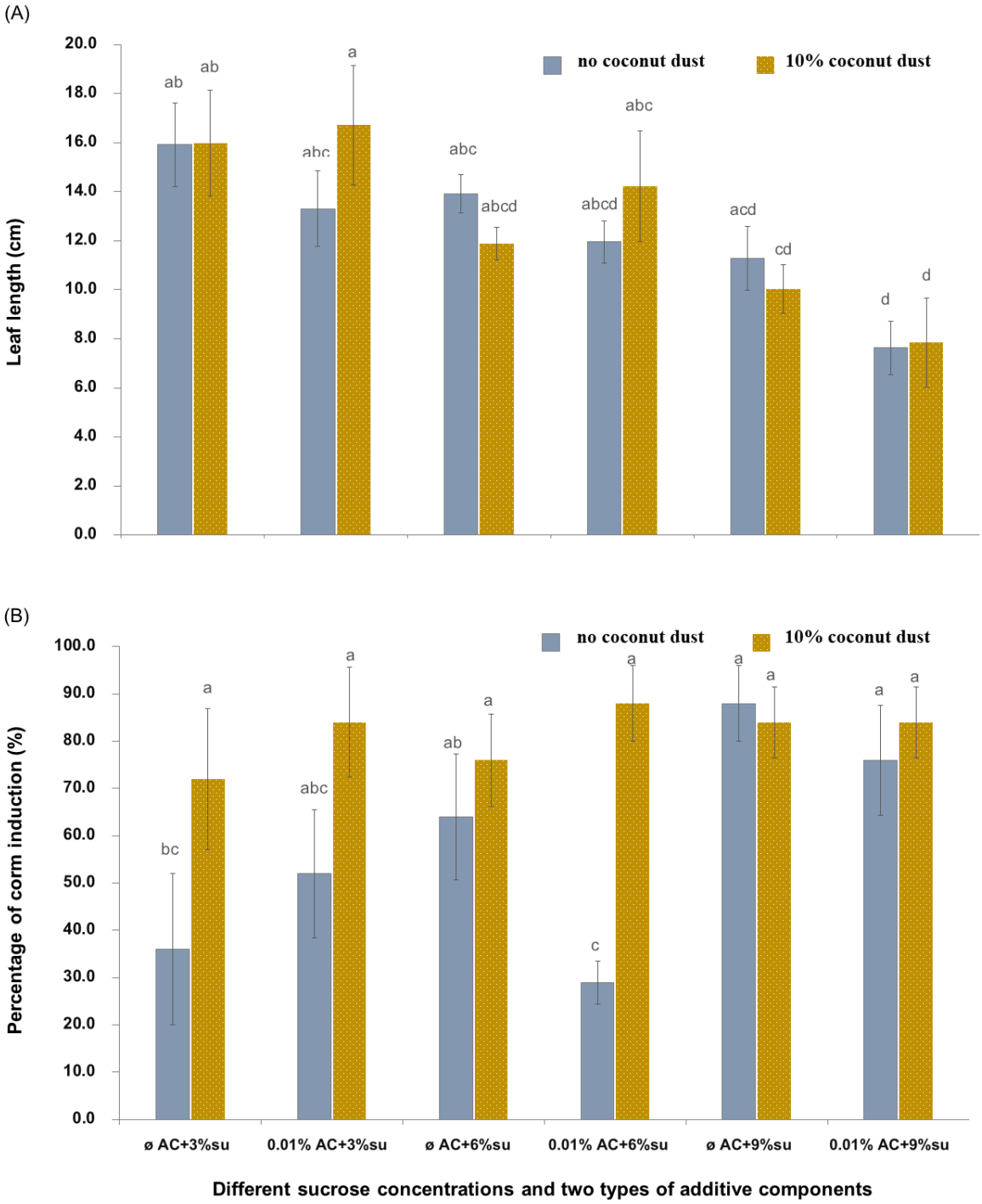

Fig. 1 Effect of different concentrations of sucrose supplemented with activated charcoal (AC) and coconut dust on (A) leaf length (cm) and (B) percentage of corm induction (%) after cultured for 5 weeks. Data represented as mean±SE (n=5)

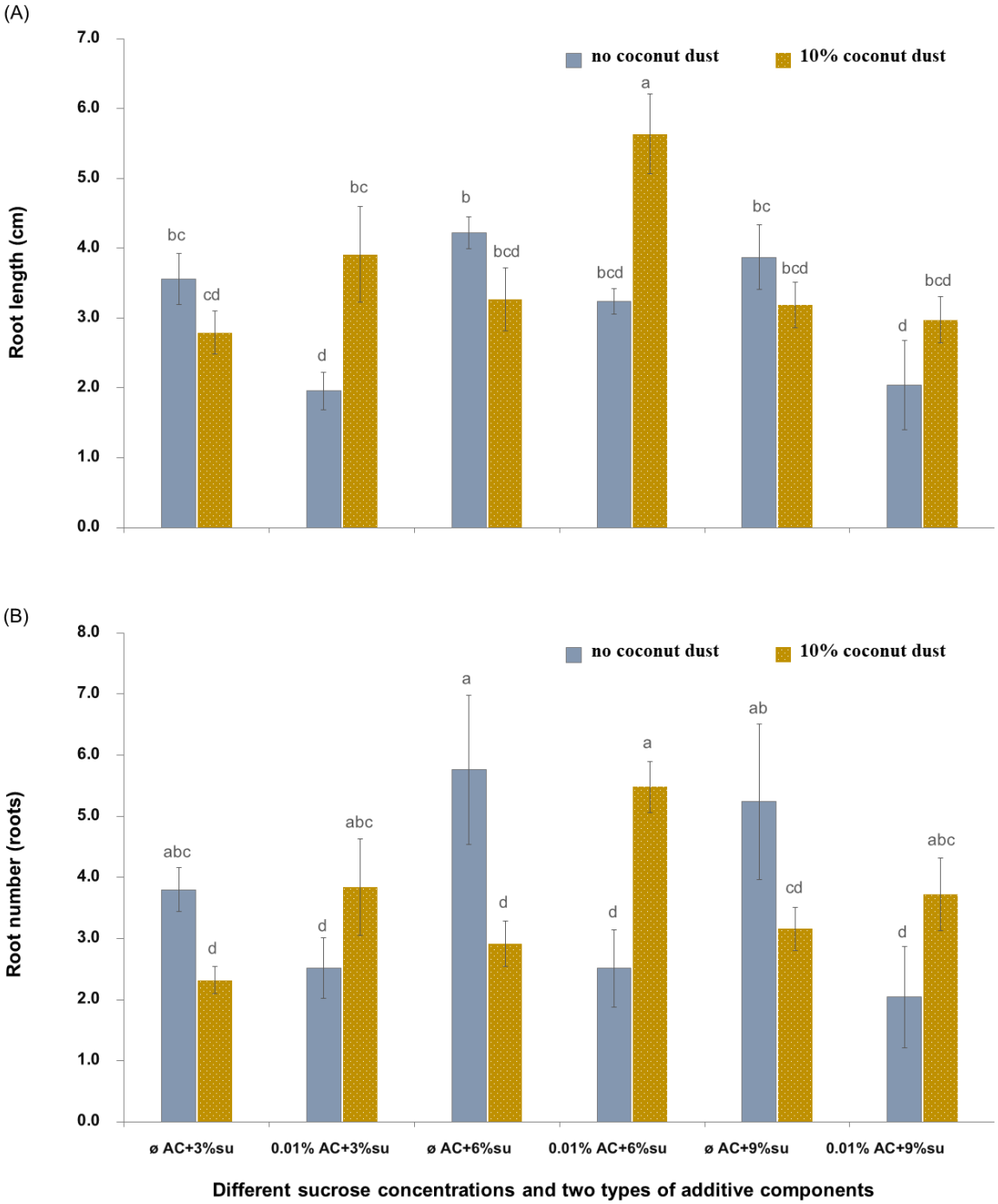

Fig. 2 Effect of different concentrations of sucrose supplemented with activated charcoal (AC) and coconut dust on (A) root length (cm) and (B) root number (%) after cultured for 5 weeks. Data represented as mean±SE (n=5)

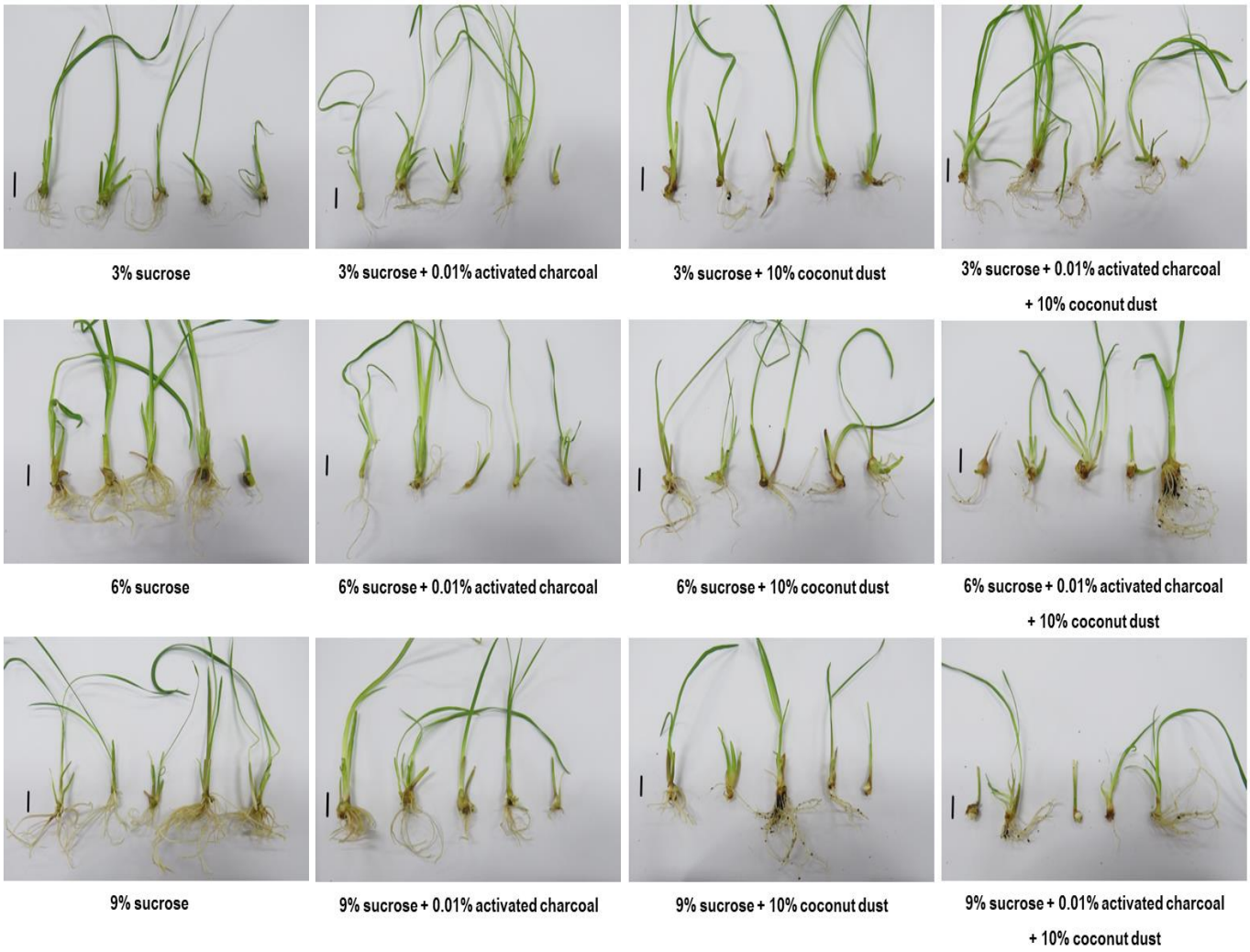

Fig. 3 Growth appearance of gladiolus shoots after being cultured in different medium conditions for 5 weeks. (Bar I = 1 cm)

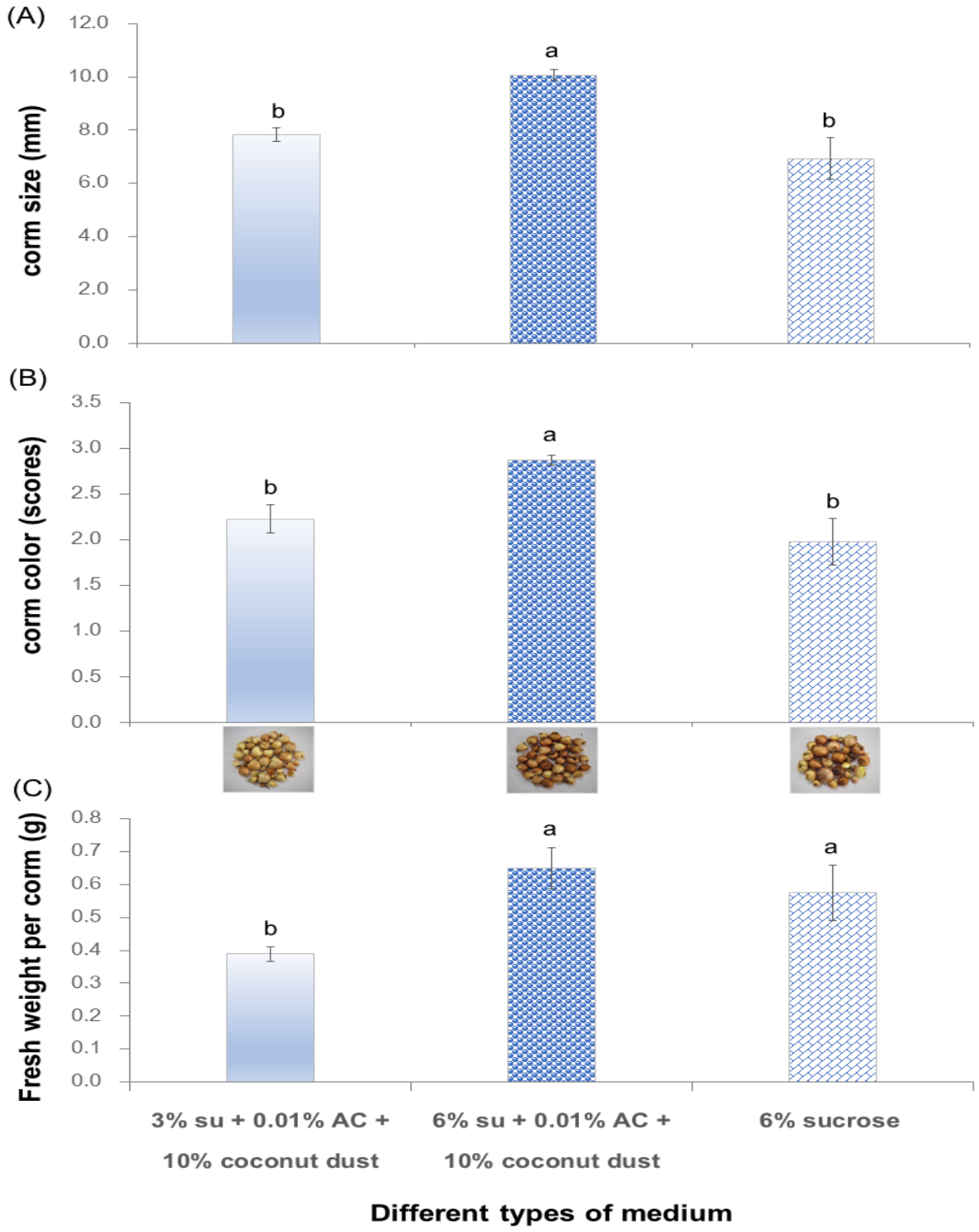

Fig. 4 Validation test of three different media for (A) corm size (mm), (B) corm color (scores), and (C) fresh weight per corm (g) after cultured for 5 months.

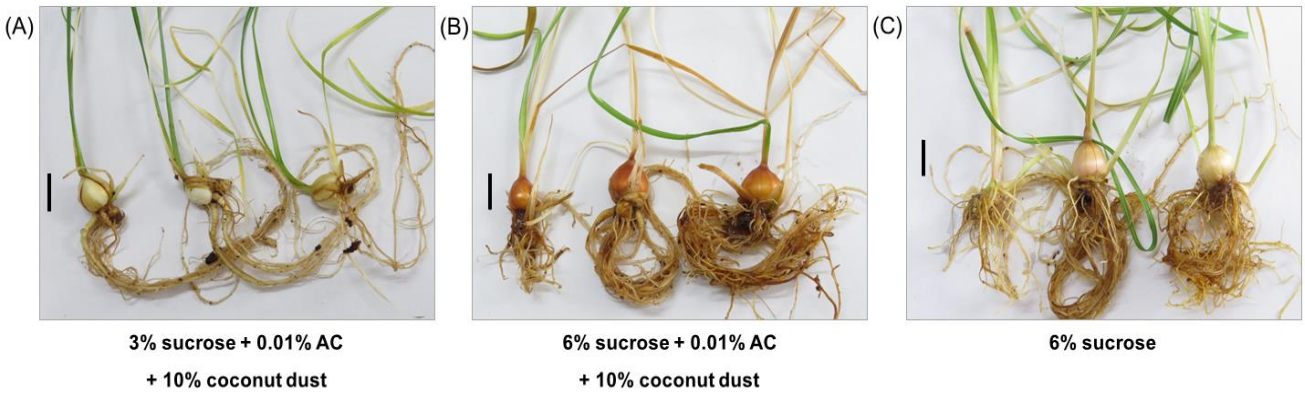

Fig. 5 Growth appearance of gladiolus shoots after cultured in different medium conditions (A) 3% sucrose + 0.01% AC + 10% coconut dust, (B) 6% sucrose + 0.01% AC + 10% coconut dust, and (C) 6% sucrose for 5 months. (Bar I = 1 cm).

Fig. 6 Changes in growth appearance of gladiolus shoots after cultured for 5 months. (A-B) initial stage, (C-D) enlargement stage, and (E-F) maturation stage.

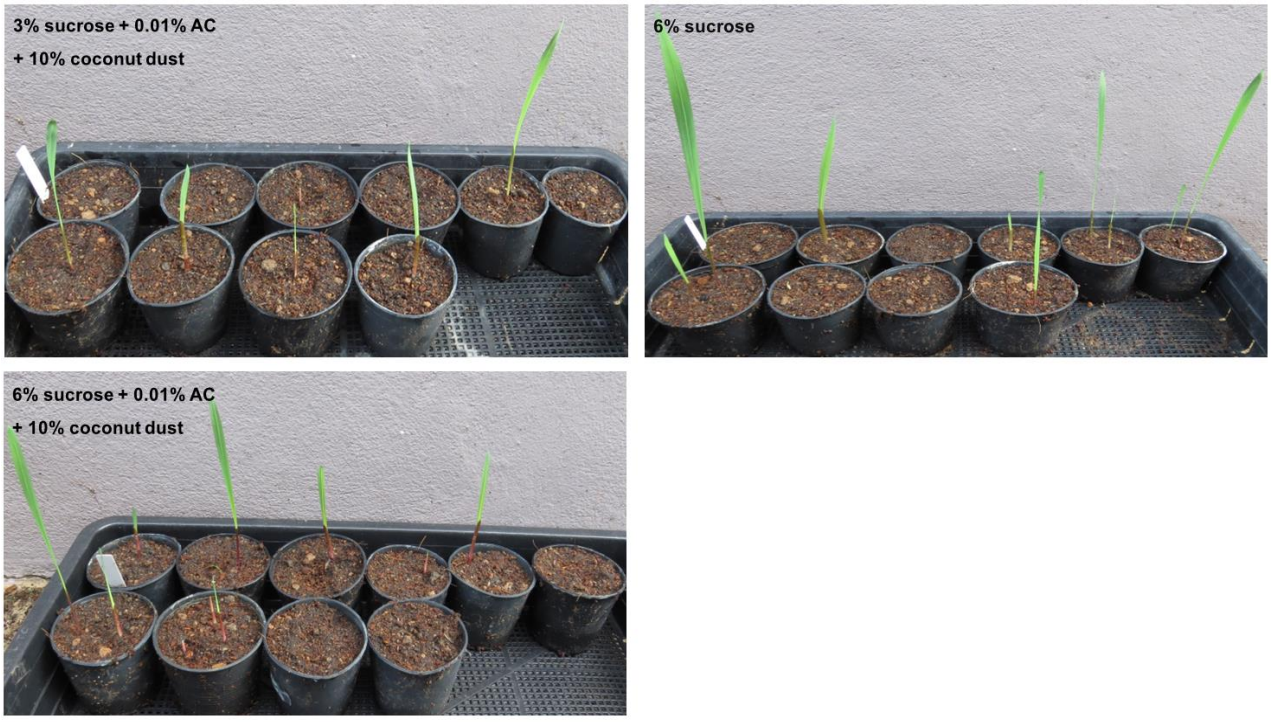

Fig. 7 Germination of *in vitro* gladiolus corms derived from different medium conditions after growing for 4 weeks.

Discussion and Conclusion

This study explored the best corm induction of Gladiolus using tissue culture technique. The media were supplemented with coconut dust and sucrose at varied concentrations, either with or without activated charcoal and coconut dust. We found that the higher sucrose concentrations tended to produce higher corm induction. In agreement with the previous study, the different sucrose concentrations at 88 mmol (3%) and 176 mmol (60 g/l or 6%), supplemented either with or without coconut dust can produce corm with the suitable 60 g/l (6%) sucrose supplemented with coconut dust that produced the best corm induction [4]. In addition, Jaimakaew et al. [11] studied the effect of sucrose concentration on the rhizome formation of the ginger King Yai cultivar and used different sucrose concentrations: 3, 6, 8, and 10% [11]. It was found that 6% of sucrose concentration, had the highest plant height at 11.8 cm and artificial stem diameter at 2.1 – 24 cm, while 10% sucrose, had the lowest plant height of 4.5 cm [11]. Changing sucrose concentrations affected stored parts of *in vitro* plants. Furthermore, the range of 3-8% sucrose produced 8.5 - 10.1 leaves, which was higher than 10% sucrose which produced only 3 leaves. This was because sucrose promotes the formation of tubers, in which carbohydrates are accumulated. Wahocho et al. [12] reviewed that sucrose is a critical factor for *in vitro* cormel formation in Gladiolus [12]. Micro rhizomes derived from regenerated *in vitro* plantlets were then successfully induced in MS medium using 6% sucrose showing an increase in micro rhizome biomass and the number of micro rhizomes of *Kaempferia parviflora* Wall. Ex Baker [13]. According to studying morphophysiological responses of *Billbergia zebrina* Lindl. as the effects of sugars, Santos et al. (2020) found that the types and concentrations of fructose, glucose, or sucrose employed in the culture medium did not affect the photosynthetic performance but the higher concentration above 3% sugars modified anatomical characters [14]. Therefore, the addition of high sucrose concentration affects the formation of tubers. The data corresponded to the result of this study which showed that 60 g/l of sucrose supplemented with 0.11 g/l of activated charcoal and 100 g/l of coconut dust, produced the highest corm induction at 88% . Nevertheless, the additives (activated charcoal and coconut dust) applied in this study might directly influence root induction. Bousselmame, Kenny, and Chlyah [15] reported that the addition of activated charcoal increased root induction and secondary roots in *Argania spinosa* L. cultured under an aseptic technique [15]. Although in this study we did not find any significant effect of activated charcoal, it had an important effect on morphogenesis. The activated charcoal may be mainly a response to the capability of irreversible adsorption when inhibitory compounds in the culture medium have been generated and this additive could substantially decrease the accumulation of toxic metabolites [16]. A positive effect of AC on the shoot and root formation has been found in *Paphiopedilum insigne* because the medium supplemented with 2 g/l of AC provided the highest multiplication rate (5.6 shoots per explant) and rooting frequency [17].

In summary, sucrose and coconut dust had effects on *in vitro* corm induction of gladiolus under aseptic conditions. The MS medium added with 60 g/l sucrose concentration and supplemented with 0.11 g/l of activated charcoal and 100 g/l of coconut dust was suitable for gladiolus cultivation and resulted in the best corm induction.

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