Effect of plant growth regulators and culture conditions on *Acrostichum aureum* L. gametophytes biomass accumulation

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Abstract
Acrostichum aureum L. (A. aureum) is a prevalent species in mangrove areas, serving critical functions in erosion and landslide prevention. Numerous studies have reported on the applications and biological activities of this species such as antioxidant, antibacterial properties, anti-inflammatory and anti-tumor activity. However, A. aureum harvesting faces significant challenges due to the susceptibility of its biological activities by environmental conditions such as salinity or heavy metals.

This finding focuses on the effect of in vitro conditions and plant growth regulators to accumulate gametophyte biomass from A. aureum sterilized spores for bioactive compound research and applications. The gametophytes were collected from sterilized tubes, separated and placed into Petri dishes or Erlenmeyer flasks containing various factors such as macronutrient composition, light intensity, carbohydrate content, culture media physical states and plant growth regulators for 1 month, 2 months and 4 months. The results show that the most effective condition for gametophyte biomass accumulation was achieved in MS static liquid medium containing 20 g/L of glucose placed into Erlenmeyer flask under 600 lux light conditions with 0.252 ± 0.247 g of dry weight biomass after 2 months. This suggests an effective culture condition for biomass accumulation.

Keyword: *Acrostichum aureum*, spores' cultivation, gametophyte biomass accumulation, mangrove fern.

Introduction

Acrostichum aureum L., belonging to the family Pteridaceae, is a mangrove fern that contains numerous important bioactive compounds such as alkaloids, saponins, sterols, triterpenoids and flavonoids (including kaempferol and quercetin)[1,2,10]. These bioactive compounds hold significant potential for applications in pharmaceuticals and industry due to their anti-inflammatory, antibacterial and antioxidant properties. However, harvesting from natural habitats faces significant challenges, as the plant typically grows in environments contaminated with high salinity and heavy metals, which can adversely affect the quality of its biomass and biological activities[14]. Therefore, cultivating biomass under controlled conditions is essential to ensure the quality of raw materials for the effective extraction of these compounds.

To date, numerous studies have focused on the utilization of plant growth regulators and the adjustment of environmental parameters to affect the biomass of various fern species. Research has indicated that species belonging to the genera *Adiantum* sp., *Matteuccia struthiopteris, Pteridium aquilinum and Cyclosaorus contiguus* exhibit optimal growth in ½ strength MS medium, while *Cibotium barometz* and *Matteuccia struthiopteris* can thrive in ¼ strength MS medium[19]. Furthermore, the presence of sucrose, glucose, or fructose at a concentration of 0.1 g/L can stimulate the production of sporophytes or gametophytes from callus[3]. Similarly, sucrose can promote biomass accumulation when used at appropriate concentrations; however, when sucrose levels exceed 5-8%, it has been shown to negatively affect the development of *Ampelopteris prolifera*[12].

Regarding hormonal effects, NAA has been demonstrated to support shoot development in *Nephrolepis exaltata* and *N. bostoniensis*, while a combination of kinetin and 2,4-D has been shown to stimulate root formation in these species[11]. BA (0.01 mg/L) combined with IBA and NAA in a 2% sucrose medium has also been reported to promote morphological development in *Adiantum capillus-veneris*[17]. Bhatia et al[2] elucidated the gametophyte development of *A. aureum* from spores. This research shows the necessity of exploring the effects of growth conditions and plant growth regulators on the biomass of this fern species.

Material and Methods

Samples preparation: The leaves of *A. aureum* containing sporangia were harvested at brackish swamp area (Tien Giang province, south of Viet Nam). The parameters for nitrogen, phosphorus and potassium contents as well as pH were obtained from water samples at the plant growth site to construct the N:P:K composition of the modified MS medium (MS_m) with the remaining components being identical to the original MS medium. This MS_m medium was compared with MS medium[13] and C-Fern medium[9] (Table 1).

Leaf segments containing sporangia were cut into 10 cm^2 pieces and sterilized with 98% ethanol for 30 seconds and 5.25% sodium hypochlorite solution for 5 minutes. Samples were thoroughly rinsed with sterile distilled water for several times.

Table 1
Composition of the Culture Medium for Gametophyte Cultivation of *A. aureum* (mg)

Ingredients		MS_m	MS	C-Fern
Macro-nutrients	NH_4NO_3	20687	1650	125
	KNO_3	9111	1900	-
	$CaCl_2.{}_2H_2O$	440	440	26
	KH_2PO_4	876	170	500
	$MgSO_4.7H_2O$	370	370	120
Micro-nutrients	$FeSO_4.7H_2O$	27.5		
	Na_2EDTA	37.25		
	$MnSO_4.4H_2O$	22.3		
	$ZnSO_4.7H_2O$	8.6		
	H_3BO_3	6.2		
	KI	0.83		
	$Na_2MoO_4.2H_2O$	0.25		
	$CuSO_4.5H_2O$	0.025		
	$CoCl_2.6H_2O$	0.025		
Vitamin	Myo-inositol	100		
	Nicotinic acid	0.5		
	Pyridoxine.HCl	0.5		
	Thiamine.HCl	0.1		
	Glycine	2		

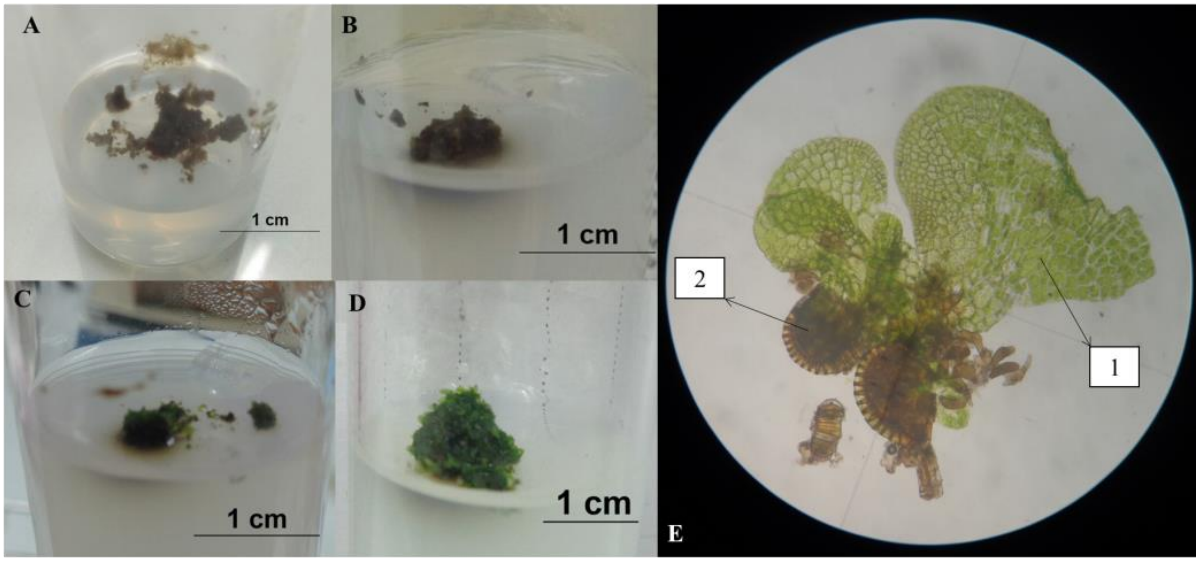

Figure 1: The development from *A. aureum* spores to gametophytes post-sterilization (A) after sterilization, (B) 7 – 10 days, (C) 10 – 15 days, (D) 20 days and (E) (1) The gametophytes and (2) sporangia under microscope.

Spores were scraped from the leaf and placed onto the surface of sterilized tubes containing modified MS medium with haft - strength macro mineral (½ MS_m) (agar 10 g/L, pH 6.5 ± 0.1, sucrose 10 g/L) (Fig. 1A). The spores were inoculated at 20 - 25°C under continuous light with light intensity of 2500 lux. The spore clusters began to swell within 7 days (Fig. 1B), germinated (Fig. 1C) and developed into large clusters after 20 days (Fig. 1D). Nine gametophytes isolated from multi-spore clusters were placed into each Petri dish/Erlenmeyer flask and cultured at 20 - 25°C under continuous light.

Effects of various basic culture conditions

Macronutrients: The gametophytes were cultured in the various media including MS_m, MS_m ½, MS_m ¼, C-Fern, C-Fern ½, C-Fern ¼ and MS medium ("½" denotes macronutrients half-strength and "¼" denotes macronutrients quarter-strength). The macronutrient parameters are based on the MS medium, MS_m medium and C-Fern medium separately. The micronutrients of the medium were retained according to MS medium composition.

Light Intensity: Different light intensity (2500 lux, 1250 lux, 600 lux and 0 lux) effects were investigated in MS_m ½ medium (sucrose 10 g/L) under fluorescent continuous light.

Carbohydrates: Two carbohydrates sources, sucrose and glucose with different concentrations (0 g/L, 10 g/L, 20 g/L, 30 g/L) were used in MS_m ½ medium separately and

compared with original carbohydrates source (sucrose 10 g/L).

The culture media physical states: The gametophytes were cultivated in Erlenmeyer flask with solid culture medium (20 ml solid MS_m ½ medium in Erlenmeyer), semi-solid culture medium (cotton wool 8 x 8 x 0.5 cm and 20 ml MS_m ½ medium), static liquid culture medium (20 ml MS_m ½ medium) and agitated liquid (20 ml liquid MS_m ½ medium at 100 rpm) and were compared to solid culture medium within Petri dish.

Effects of plant growth regulators

- **Auxin:** NAA (Naphthalene acetic acid) and 2,4-D (2,4-Dichlorophenoxyacetic acid) were used to investigate the effects of auxin at concentrations of 0.5, 1, 2 and 3 mg/L.
- **Cytokinin:** BA (Benzyladenine) is used at concentrations of 0.5, 1, 2 and 3 mg/L, AS (Adenine sulfate) at concentrations of 10, 20, 30 and 40 mg/L and kinetin at concentrations of 0.2, 0.5, 1 and 2 mg/L to investigate the effects of cytokinin.

Effect of optimal combined conditions: Based on the effects of culture conditions, the optimal settings were combined for gametophyte biomass accumulation. The optimal combined culture (Z_1) and the plant growth regulator culture (Z_1 + optimal plant growth regulator concentration - Z_2) were compared with the control culture condition.

Data collection: Observations and results were recorded as dry biomass weight (g), number of gametophytes in each container and diameter of each cluster (cm) after 1 month, 2 months and 4 months. Control samples were grown in ½ MS_m static liquid medium with 10 g/L sucrose under continuous light (2500 lux, 20 - 25^{o}C).

- **Dry Biomass Weight (g):** The biomass was dried at 50°C until a constant weight.
- **Number of Gametophytes**: The gametophytes separated from the clusters exhibit a coherent structure, with asymmetrical heart-shaped or round thallus forms, accompanied by rhizoids (Fig. 1E).
- **Gametophyte Cluster Diameter (cm):** The diameter of each gametophyte cluster was defined at its widest point in centimeters (cm).

Data analysis: The experiments were repeated at least 3 times. Values are presented as mean ± standard deviation. The data were statistically analyzed using ANOVA and Duncan's multiple range test at $p < 0.05$, with SPSS 20.0 software. The gametophytes cluster diameters were analyzed using ImageJ data analysis.

Results

Effect of macronutrient content: Results in fig. 2 show that after 2 months of culture, the macronutrient content of MS medium yielded the highest biomass, with a dry weight of 0.258 ± 0.039 g ($p < 0.05$), the diameter of clusters was 0.898 ± 0.093 cm and had average of 32.400 ± 8.922 ($p < 0.05$) gametophytes, after 4 months dry weight biomass and diameter of clusters decreased significantly ($p < 0.05$). Control sample has 0.053 ± 0.016 g of dry biomass, the number of gametophytes was 14.900 ± 2.685 and the cluster size was 0.965 ± 0.208 cm after 4 months. C-Fern medium was unsuitable for gametophyte development, leading to wilting and death after 1 month of culture.

Effect of light: Results in fig. 3ABC indicated that *A. aureum* gametophytes developed better under the 600 lux treatment, yielding a dry weight of 0.054 ± 0.020 g, diameter of cluster was 1.728 ± 0.453 cm ($p < 0.05$), the maximum of gametophytes were samples under 600 lux at 2 months cultivation (21.500 ± 11.208, $p < 0.05$) and slightly decreased after 4 months. Those under light conditions show no morphological changes (Fig. 3 DEF). The gametophytes under 0 lux culture condition transform into morphological differences: elongated, slender shapes and tend to extend outward in search of light sources (Fig. 3G).

Effect of carbohydrate source: Fig. 4 shows the result of gametophytes accumulation after 4 months of cultivation under different carbohydrate treatments. The higher biomass of *A. aureum* gametophytes was observed in the 20 g/L glucose treatment, with a dry weight of 0.168 ± 0.080 g ($p < 0.05$), an average of 20.500 ± 5.836 gametophytes ($p < 0.05$) and a cluster diameter reached 1.316 ± 0.136 cm ($p < 0.05$). The biomass declined when the concentration of glucose increased to 30 g/L.

Effect of culture media physical states: Fig. 5 shows that the gametophytes of *A. aureum* grew better when cultured in Erlenmeyer flasks compared to Petri dishes, with dry weight biomass 0.237 ± 0.104 g ($p < 0.05$), 1.614 ± 0.446 cm diameters of gametophyte clusters ($p < 0.05$) and an average of 49.500 ± 39.598 gametophytes ($p < 0.05$) in the same media physical states (solid medium). The static liquid medium using Erlenmeyer flasks produced significant higher biomass (dry weight biomass: 0.413 ± 0.083 g, $p < 0.05$; diameter of clusters: 2.396 ± 0.707 cm, $p < 0.05$); number of gametophytes: 93.100 ± 60.307, $p < 0.05$) compared to both solid and agitated liquid.

Effect of growth regulator supplementation: The addition of BA (1 mg/L), NAA (0.5 mg/L), AS (40 mg/L) and kinetin (0.2 mg/L) slightly increased dry weight biomass, diameter clusters and number of gametophytes. 2,4-D treated sample wilted and died within the first month of cultivation. In all tested plant growth regulators, BA 1 mg/L obtained significantly higher in number of gametophytes after 2 months and 4 months (48.300 ± 21.975 gametophytes, $p < 0.05$) (Fig. 6) but not in biomass accumulation.

Combination treatments: To achieve better biomass yield under standard initial conditions, the combined culture conditions (Z_1) included the MS mineral medium

supplemented with 20 g/L glucose, cultured under a light intensity of 600 lux set in Erlenmeyer flasks. Z_2 was supplied with 1 mg/L BA. Their results were compared with *A. aureum* growth in static liquid cultures with normal condition.

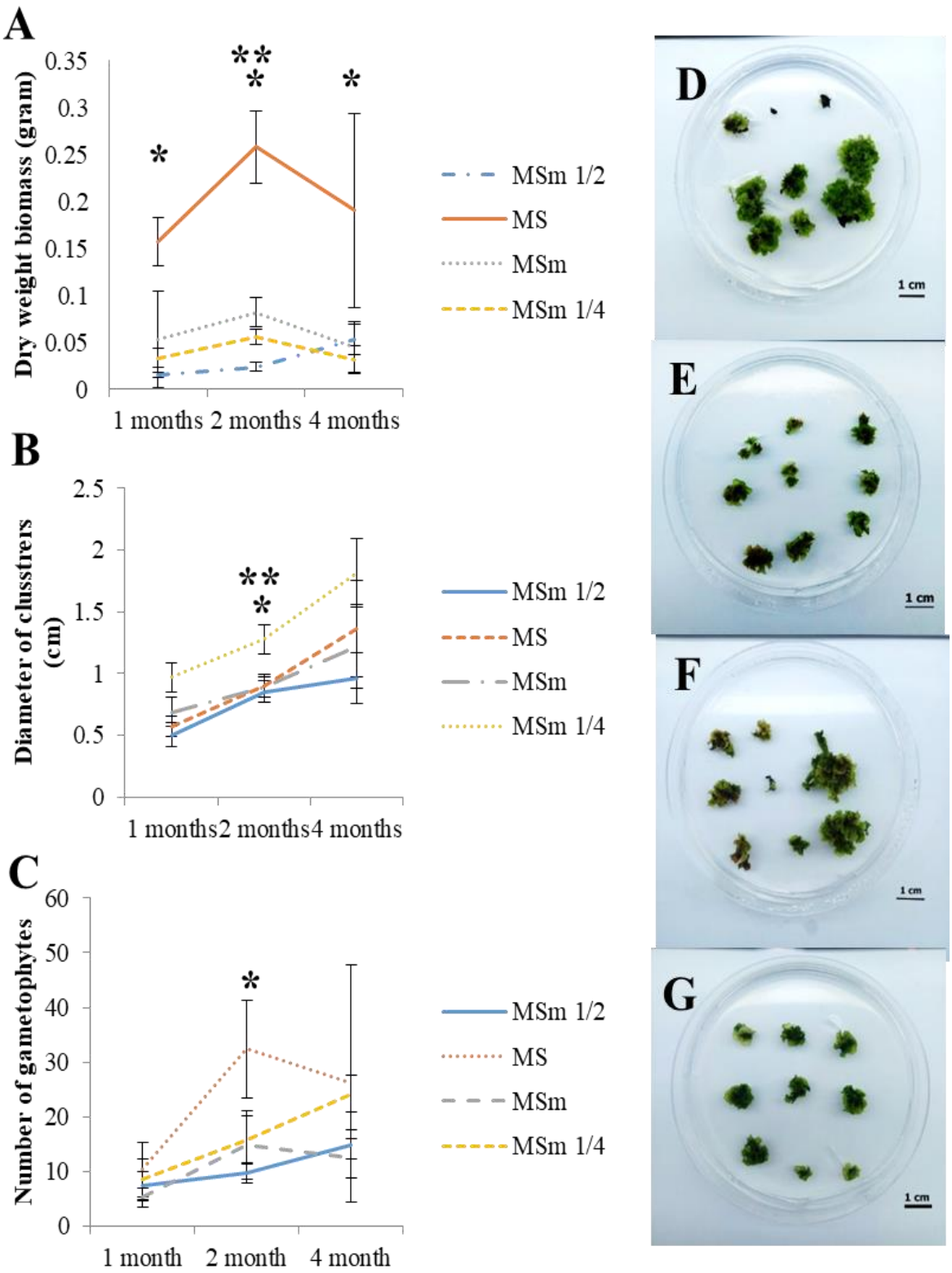

Figure 2: (A) *A. aureum* dry weight biomass, (B) clusters size and (C) number of gametophytes (D) accumulation on various macro-mineral medium: MS, (E) MS_m, (F) MS_m ½, (G) MS_m ¼, in 4 months. Error bar indicates standard deviation, (*) indicates the significant difference between MS macro-mineral medium and the others of the group, (**) indicates the significant difference of MS macro-mineral medium between 2 months and 4 months ($p < 0.05$).

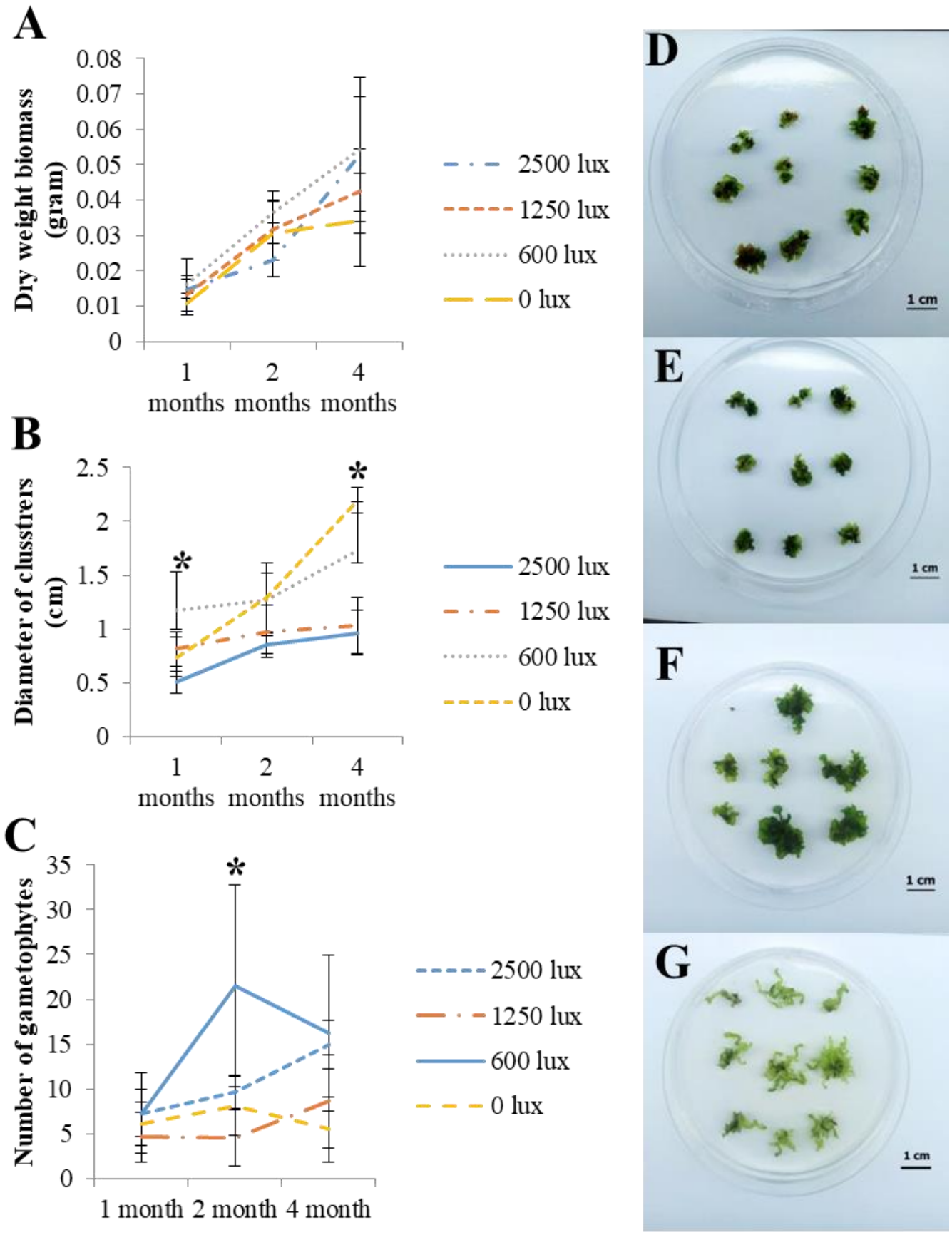

Figure 3: (A) *A. aureum* dry weight biomass, (B) clusters size and (C) number of gametophytes accumulation on various light intensity: (D) 2500 lux, (E) 1250 lux, (F) 600 lux, (G) 0 lux. Error bar indicates standard deviation, (*) indicates the significant difference between samples in 600 lux and the o others of the group ($p < 0.05$).

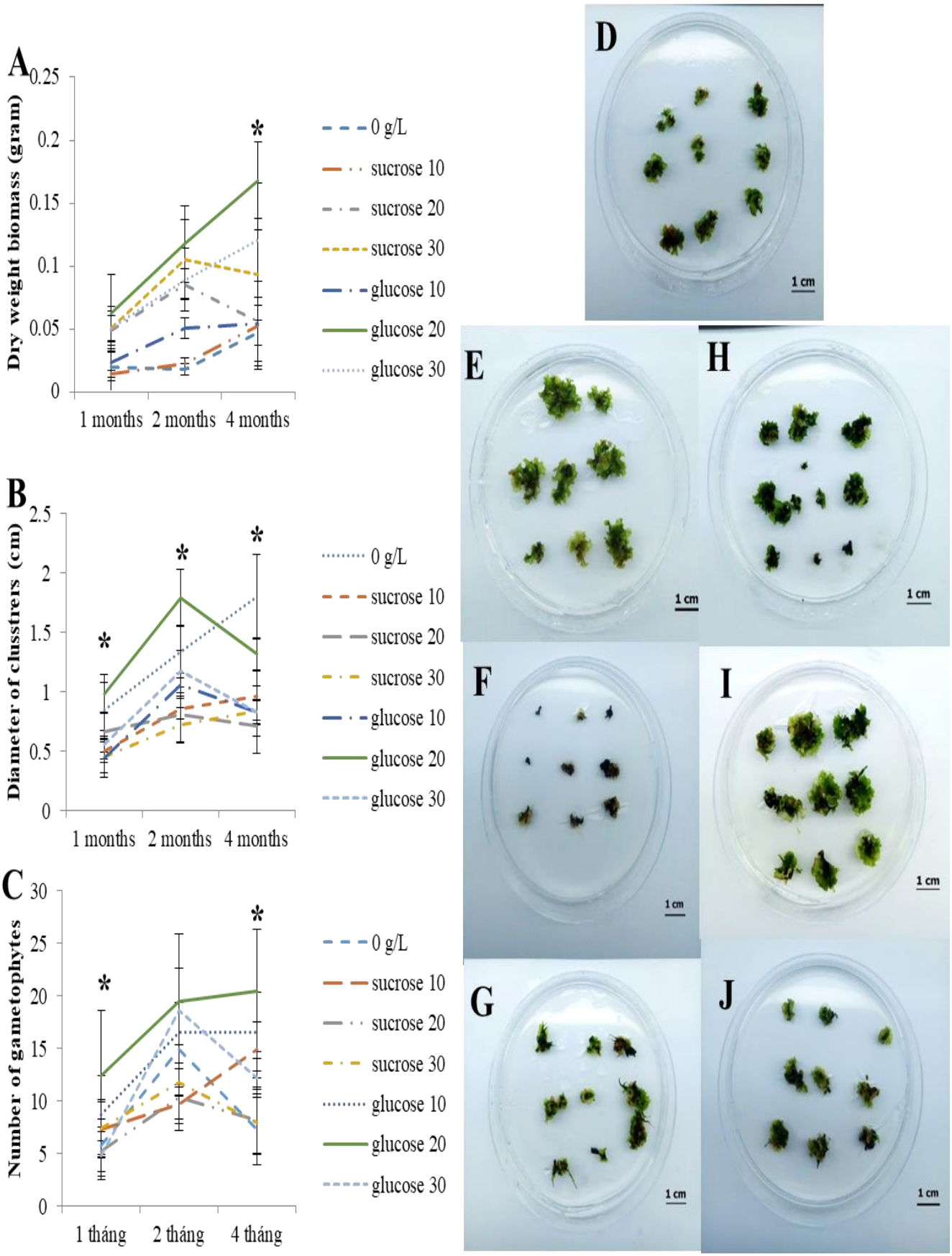

Figure 4: (A) *A. aureum* dry weight biomass, (B) clusters size and (C) number of gametophytes (D) accumulation on various sources of carbohydrates: 0 g/L, (E) sucrose 10 g/L, (F) sucrose 20 g/L, (G) sucrose 30 g/L, (H) glucose 10 g/L, glucose 20 g/L, glucose 30 g/L. Error bar indicates standard deviation, (*) indicates the significant difference between samples in glucose 20 g/L and the others of the group ($p < 0.05$).

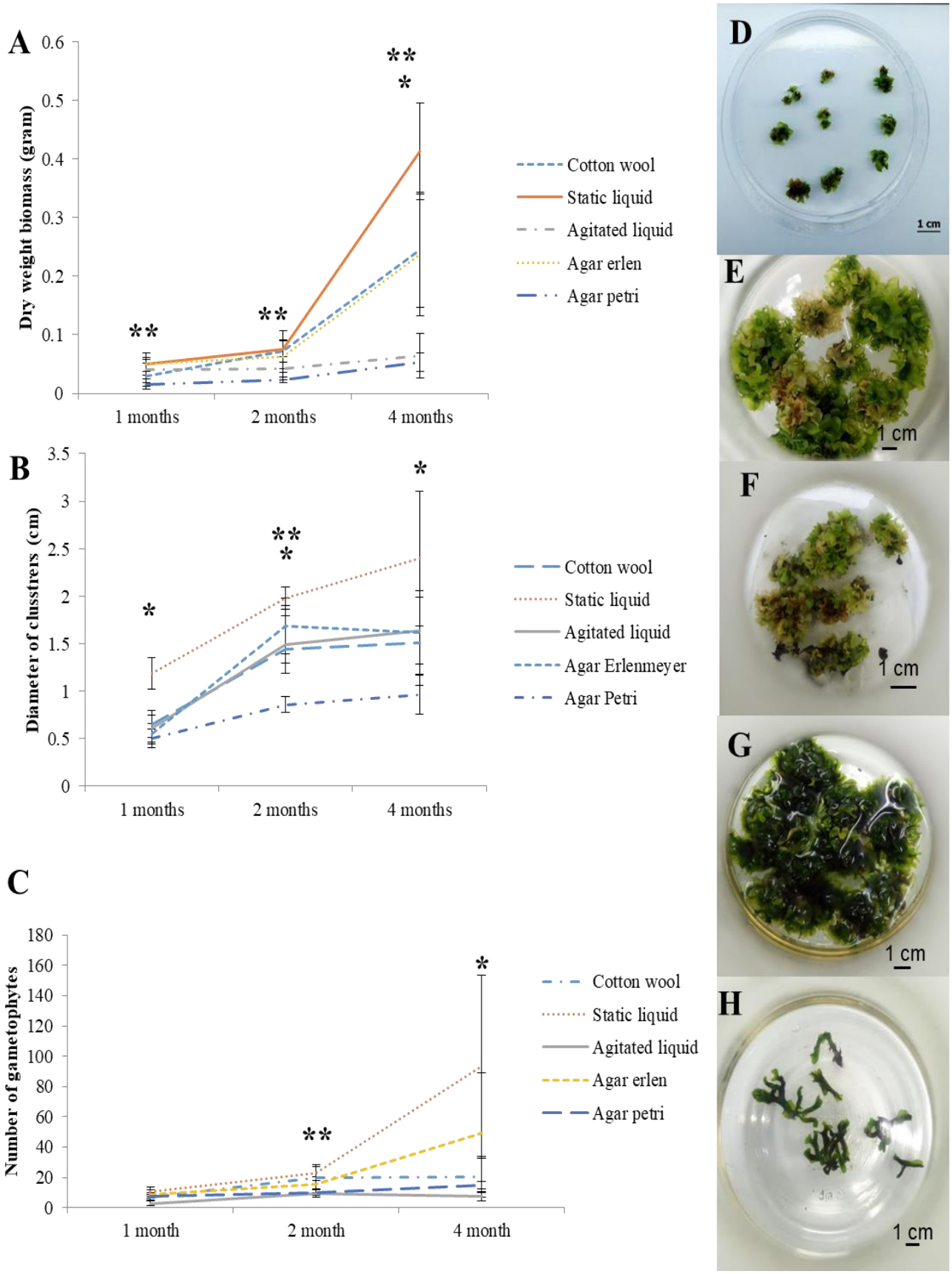

Figure 5: (A) *A. aureum* dry weight biomass, (B) clusters size and (C) number of gametophytes (D) accumulation on various media physical states: agar Petri, (E) agar Elenmeyer, (F) cotton wool, (G) static liquid, (H) agitated liquid. Error bar indicates standard deviation, (*) indicates the significant difference between static liquid and the others of the group, (**) indicates the significant difference between agar Erlenmeyer and agar Petri ($p < 0.05$).

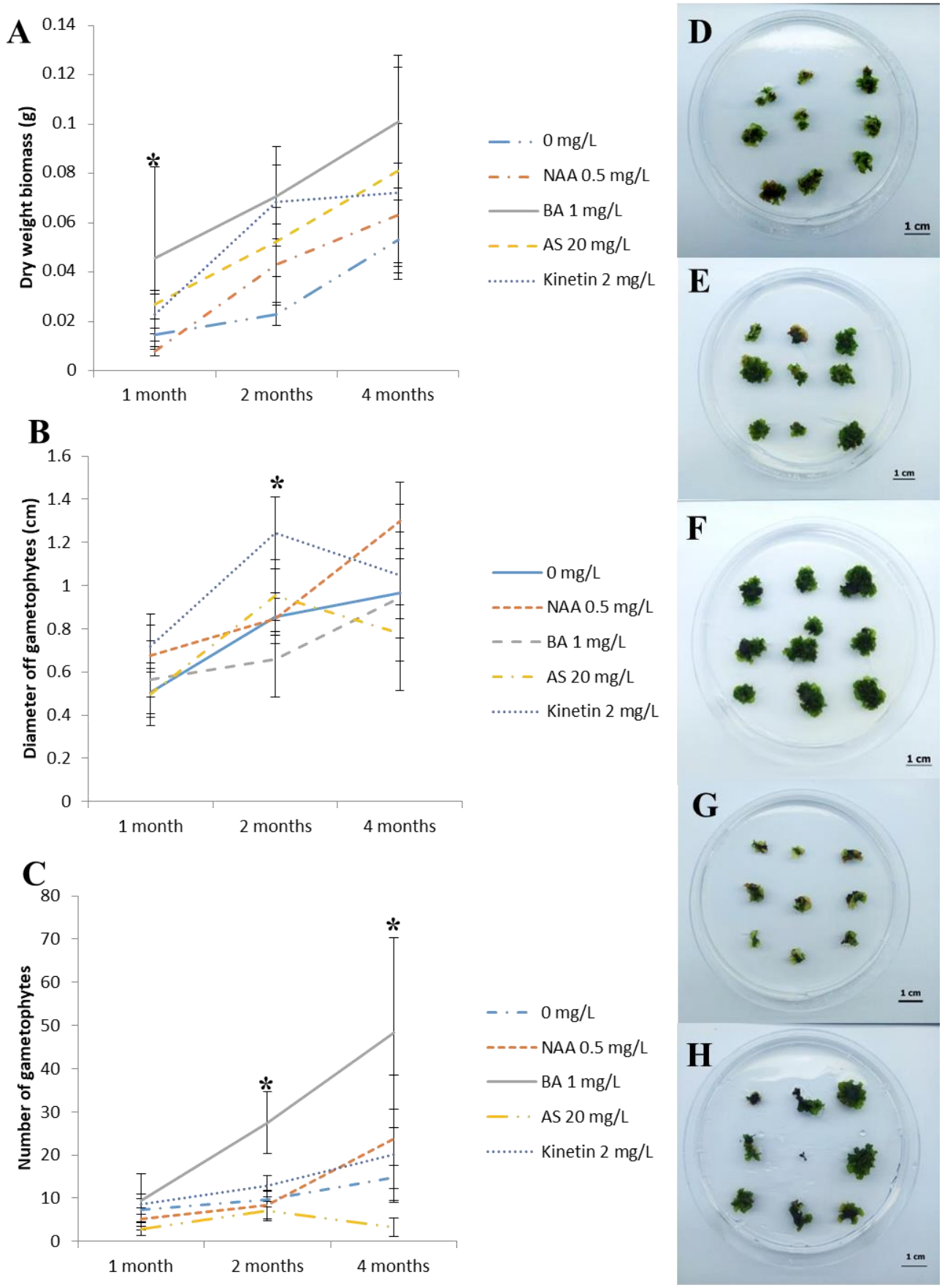

Figure 6: (A) *A. aureum* dry weight biomass, (B) clusters size and (C) number of gametophytes (D) accumulation on NAA 0.5 mg/L, (E) BA 1 mg/L, (F) AS 20 mg/L, (G) kinetin 2 mg/L. Error bar indicates standard deviation, (*) indicates the significant difference between BA 1 mg/L and the others of the group ($p < 0.05$).

The results (Fig. 7) show that the addition of BA into Z_1 did not increase the number of gametophytes or biomass. After 4 months of culture, Z_1 had a significant statistical increase when comparing to Z_2 in dry weight biomass (Z_1: 0.339 ± 0.068 g, Z_2: 0.090 ± 0.026 g), diameter of cluster (Z_1: 2.885 ± 0.462 cm, Z_2: 1.333 ± 0.142 cm, $p < 0.05$), number of gametophytes (Z_1: 51.100 ± 19.273, Z_2: 15.300 ± 6.395). Z_1 shows quicker dry weight biomass and diameter of clusters accumulation after 2 months of culture compared to static liquid in normal condition dry biomass weight (Z_1: 0.252 ± 0.247 g), (static liquid: 0,075 ± 0,032 g, $p < 0.05$), diameter of cluster (Z_1: 3.217 ± 0.714 cm, Static liquid: 1,975 ± 0,117 cm), number of gametophytes (Z_1: 39.600 ± 8.220, static liquid: 23.000 ± 5.270).

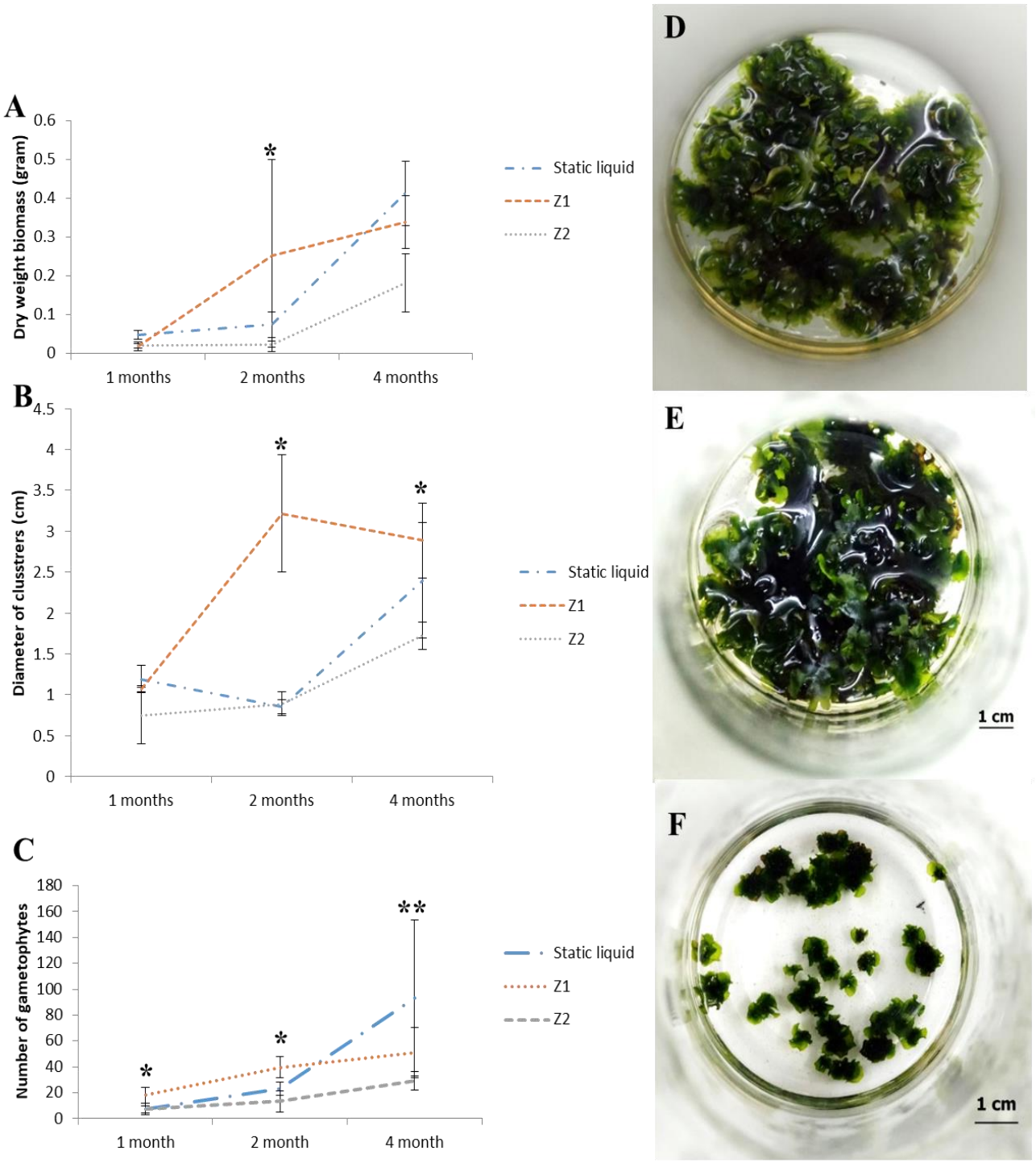

Figure 7: (A) *A. aureum* dry weight biomass, (B) clusters size and (C) number of gametophytes (D) accumulation on static liquid, (E) combination of culture conditions – Z1 and (F) the plant growth regulator culture – Z2. Error bar indicates standard deviation, (*) indicates the significant difference between Z_1 and the others of the group, (**) indicates the significant difference between Z_1 and static liquid ($p < 0.05$)

Discussion

Pteridaceae are extensively studied for their biochemical properties; however, research on biomass production is limited, focusing primarily on morphogenesis from spores or tissue culture from parts like shoots and leaves[9]. The development from *A. aureum* spore to a mature gametophyte takes approximately 20 days, which aligns with the findings on gametophyte development by Bharita et al[2]. The images of the gametophyte captured in fig. 1e resemble the morphological characteristics described in the literature with a complete shape, dark green color, heart-like form and adventitious roots; the gametophyte exhibits an asymmetrical distribution. For *in vitro* culture of this family, MS and C-Fern medium have been used in number of studies[6,16,19,25]. In this study, *A. aureum* showed superior growth in the standard MS medium compared to MS_m and C-Fern. This suggests that MS offers a balanced N:P:K ratio which is ideal for *A. aureum* gametophyte development. This supports MS as the optimal medium, consistent with findings in related fern studies.

A. aureum, being a fern, predominantly inhabits shaded areas[4] and thus is adapted to low-light conditions. Species such as *Onoclea sensibilis*[8] and *Osmunda regalis*[6] thrive in bright light conditions. However, *Onoclea sensibilis* also grows and develops better in complete darkness[12], while spores of species like *Lycopodium clavatum* and *Ophioglossum crotalophoroides* can germinate without light[22]. In this study, the gametophytes of *A. aureum* exhibited the highest biomass under 600 lux, which represents low-light conditions. Conversely, the gametophytes exhibited morphological changes at 0 lux, highlighting the important role of light in the morphological development of *A. aureum* gametophytes.

Various types of sugars are added to *in vitro* culture media and each fern species adapts to different types and concentrations of sugars depending on the developmental stage. Previous studies have shown that *Adiantum reniforme* var. *sinense* grows well with 15 g/L sucrose, while higher sucrose concentrations may inhibit growth[24]. Conversely, the addition of sucrose can increase spore germination rates in several fern species[18]. Meanwhile, *Pteridium aquilinum* var. *latiusculum* exhibits optimal callus growth in a medium containing 2% glucose[20]. In this study, *A. aureum* reached optimal biomass with 20 g/L glucose, which differs from previous findings and may be due to species-specific responses or unique ecological adaptations[21].

The culture media physical states have the great impact on the biomass of *A. aureum* gametophytes. Static liquid medium was found to be the most suitable for gametophyte development. This is likely because *A. aureum* is a species that thrives in wetland and mangrove areas where stagnant water is present, making it more compatible with liquid substrates that provide stable cultivation conditions. It also depends on their species adaption, for example, liquid medium is more suitable for the early development of gametophytes of epiphytic fern *Platycerium bifurcatum*[5]. Conversely, *Pteridium aquilinum* spores germinate better in solid medium[21]. The interesting finding in this study shows that biomass accumulation in agar culture within Erlenmeyer flasks exhibited significantly higher than those within Petri dishes. This indicates that the cultivation space plays a significant role in the biomass and the number of gametophytes produced.

The combined culture condition (Z_1) shows the potential for short-term biomass accumulation. After 2 months, Z_1 showed a statistically significant increase compared to Z_2: dry weight biomass which was 3.364 times higher. Cluster diameter was 3.760 times greater and the number of gametophytes was 1.722 times higher. However, extending the cultivation duration and culture space limitation may alter the effects of Z_1, potentially leading to growth cessation or biomass stress. In contrast, the static liquid medium of controlled condition appears more suitable for gametophytes number accumulation in long-term cultivation with number of gametophytes: 93.100 ± 60.307 in static liquid, 51.100 ± 19.273 in Z_1 ($p < 0.05$).

Numerous studies have investigated the effects of plant growth regulators on the development of Pteridaceae species. In this study, auxin (NAA and 2,4-D) and cytokinin (BA and kinetin) have been selected base on their previous results on Pteridaceae[7,11,15,23,24]. However, the addition of selected plant growth regulator is not particularly suitable for biomass development in this species. BA at 1 mg/L, which showed potential in number of gametophytes accumulation, also did not enhance the biomass, diameter clusters or number of gametophytes in combined condition after 4 months (compared to no plant growth regulator combine condition).

This study has investigated methods to effectively enhance the biomass of *A. aureum*. However, several culture factors such as pH, periodic light sources, different light colors such as red light, far-red light, blue light and other plant growth regulators (IBA, GA, ethylene…) are yet to be explored. Moreover, the increase in the size of each gametophyte is dependent on the cultivation space and expanding this space may enhance the biomass achieved.

Conclusion

Based on the findings, a static liquid MS medium enriched with 20 g/L glucose under 600 lux (continuous light, pH 6.5, 20 – 25°C) has been identified as the effective condition for *A. aureum* gametophytes biomass accumulation (mean dry weight 0.252 ± 0.247 g) within 2 months. These culture conditions can be utilized for biomass production in a short term.

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(Received 09th December 2024, accepted 11th January 2025)

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